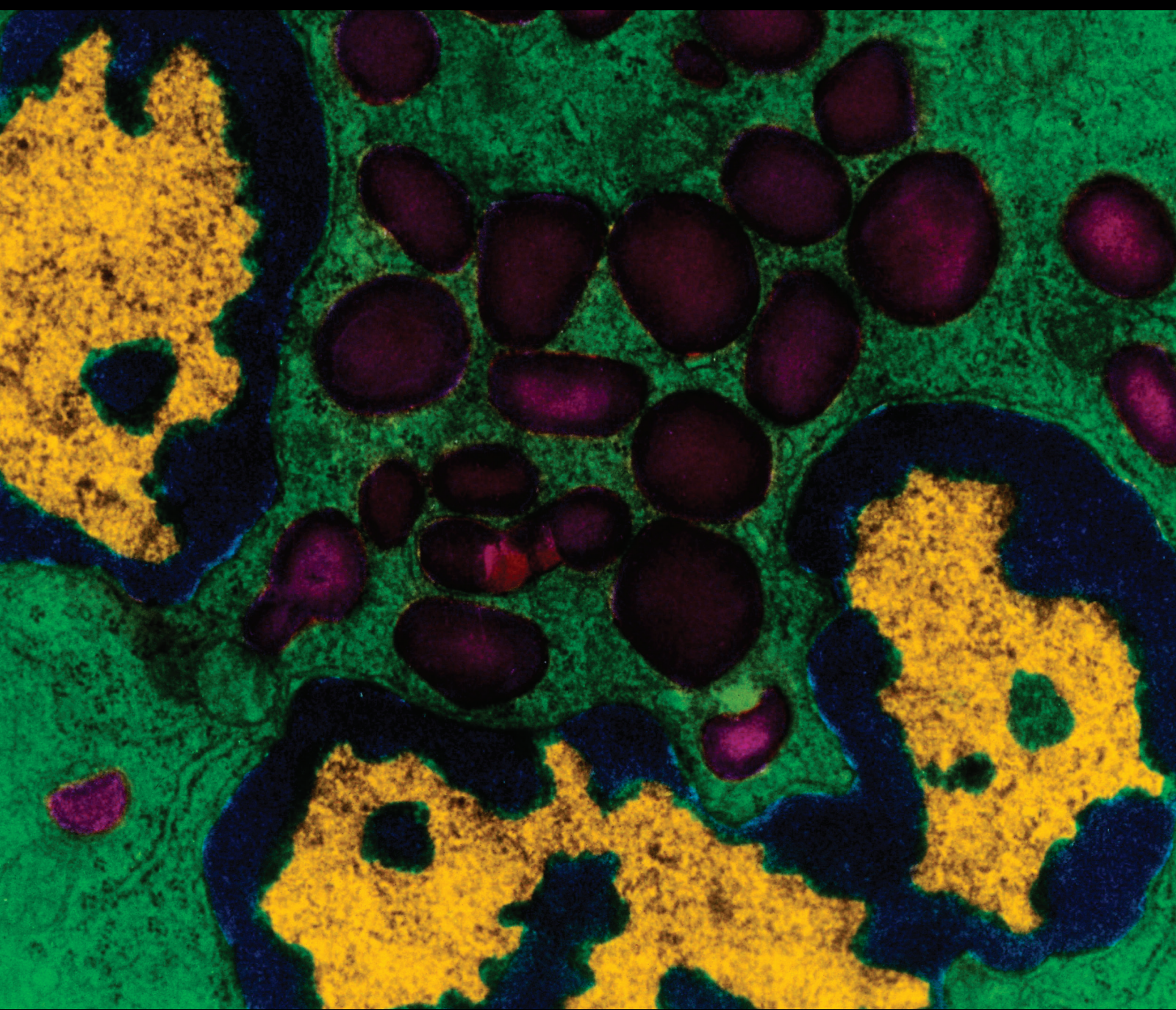
Mediators of Inflammation

Inflammatory and Fibrotic Mediators in Renal Diseases

Lead Guest Editor: Sandra Rayego-Mateos
Guest Editors: Marta Ruiz-Ortega and Roel Goldschmeding



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Editorial Board

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Mediators of Inflammation
Volume 2019, Article ID 7025251, 2 pages
https://doi.org/10.1155/2019/7025251

Editorial

Inflammatory and Fibrotic Mediators in Renal Diseases

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Received 29 November 2018; Accepted 29 November 2018; Published 10 February 2019

Chronic kidney disease (CKD) is a progressive disease that represents a public health problem in our modern society affecting 5-7% of the world's population. Recent clinical studies have described that CKD is an independent risk factor for cardiovascular disease. CKD patients have a 10 to 100 times higher risk than healthy people of developing cardiovascular diseases, including hypertension, diabetes, and hyperlipidemias, all of them associated with high morbidity and mortality. CKD is characterized by a progressive destruction of the renal parenchyma, sustained inflammation, and a functionality loss of the nephron that leads to end-stage renal disease (ESRD). The dysfunction of the nephron triggers cellular and molecular processes that seek to promote the compensatory growth of the nephron. In certain cases, this compensatory mechanism becomes pathological, contributing to renal damage progression leading to ESRD. Nowadays, one of the biggest problems in nephrology is the increased number of patients who progress up to ESRD. These patients require, in 100% of cases, renal replacement therapy such as dialysis (hemodialysis or peritoneal dialysis) or kidney transplantation. Importantly, 50% of patients who undergo dialysis, cannot stand more than three years with these replacement therapies, hence the importance of developing new strategies that could restore kidney function or, at least, delay disease progression.

This special issue contains 6 papers, including original research articles, clinical research articles, and a review, ranging from detailed studies of the key molecular mechanism associated to inflammation and fibrosis in renal diseases.

In the clinical field, there is a need for more studies that can deepen in the identification of new biomarkers for early detection and prevention of kidney disease progression. The manuscript entitled "Circulating $CD14^+CD163^+CD206^+$ M2 Monocytes Are Increased in Patients with Early Stage of Idiopathic Membranous Nephropathy" by J. Hou et al. studied the changes in peripheral blood monocytes and their clinical significance in patients with an early stage of idiopathic membranous nephropathy (IMN). Approximately 30–40% of patients with this common type of nephrotic syndrome progress to CKD. Recent studies have shown the importance of the differentiation of macrophages in the pathogenesis of chronic inflammatory process, including CKD. Monocytes are the progenitors of dendritic cells or macrophages in the tissues. The ratio of M1/M2 monocytes/macrophages is known to be modified in several kidney diseases but this has not been studied in detail for IMN. M1 monocytes release proinflammatory cytokines such as IL-12 (Th1 immune response), while M2 monocytes secrete anti-inflammatory mediators such as IL-10 (Th2 immune response) and have been suggested to exert profibrotic actions. In this manuscript, the authors show that CD14 +CD163+CD206+ M2 monocytes are relevant for the pathological process in incipient disease and are correlated with the severity of the disease. The authors also propose that $IL\text{-}10^+$ M2 cells should be a useful marker for evaluating the severity of incipient IMN. Cell-based therapy related to M2 macrophages is an active field of research. The phenotype and function of macrophages at the different CKD stages are not well defined yet, since injection of different types of macrophages (bone marrow-derived, splenocyte-derived, or cell line-derived macrophages) in different models of experimental renal damage has revealed

opposite results. Therefore, future studies are needed to define possible macrophage-based therapies.

The study by A. Żyłka et al. entitled "Markers of Glomerular and Tubular Damage in the Early Stage of Kidney Disease in Type 2 Diabetic Patients" analyzed several markers of glomerular and tubular damage in patients with type 2 diabetes with these inclusion parameters: eGFR > 60 ml/min/1.73 m2 and uACR < 300 mg/g, and at different stages of CKD (G1/G2, A1/A2). In this study, the authors identified serum cystatin C and urine IgG, transferrin, and NGAL as the best indicators of glomerular damage, whereas urine NGAL, KIM-1, or uromodulin could be indicators of tubular damage. The urinary markers are associated with increase of albuminuria, while both serum and urine NGAL were significantly associated with eGFR decline. Although these findings still need to be confirmed in a large number of patients, these data indicates that combination of biomarkers in serum and urine could be useful for the clinical management of diabetic patients.

Cardiovascular and heart damage is the most frequent consequence of CKD. Serum levels of sST2 (soluble suppression of tumorigenicity 2), a novel biomarker of fibrosis and cardiac remodeling in heart failure patients, are independent of eGFR and age. Previous studies described that the BCN Bio-HF score, an algorithm derived from a real-life cohort, based on soluble ST2 in addition to other predictive serum biomarkers (NT-proBNP and hs-cTnT) and clinical variables/treatments (beta-blockers ARBs/ACEI, statins, and furosemide), could be a useful tool in CV risk stratification of nondialyzed CKD patients. M. Plawecki et al. in their manuscript entitled "sST2 as a New Biomarker of Chronic Kidney Disease-Induced Cardiac Remodeling: Impact on Risk Prediction" evaluate the predictive potential of serum sST2 in CKD patients and show that serum sST2 alone is not a good predictive biomarker of major adverse coronary events or death in CKD patients.

Y. Xu et al. in their manuscript entitled "Apoptosis-Associated Speck-Like Protein Containing a CARD Deletion Ameliorates Unilateral Ureteral Obstruction Induced Renal Fibrosis and Endoplasmic Reticulum Stress in Mice" showed the key role of the apoptosis-associated speck-like protein containing a CARD (ASC), a component of the inflammasome, in the progression of interstitial fibrosis in a model of experimental renal damage. The genetic deletion of ASC in mice submitted to unilateral ureter obstruction significantly reduced the inflammatory cell infiltration in the kidney. The ASC knockout mice showed a diminution in apoptosis (lower number of TUNEL-positive cells, decreased levels of Bax and caspase 3, and increased levels of Bcl-2) and in fibrosis assessed by the expression of ECM proteins, such as fibronectin and collagen I. Additionally, they demonstrated improvement of endoplasmic reticulum stress as evidenced by the reduced BIP, p-eIF2α/eIF2α, ATF4, and CHOP protein levels in the obstructed ASC-deleted mice compared to obstructed WT mice. These results demonstrated the key role of the ASC inflammasome component in the regulation of the inflammatory and fibrotic processes in experimental renal damage.

Another study submitted to this special issue by K. Ramani et al. is entitled "IL-17 Receptor Signaling Negatively Regulates the Development of Tubulointerstitial Fibrosis in the Kidney." In this study, the authors showed the crucial role of the IL-17A signaling pathway in the modulation of renal fibrosis induced by comparing fibrosis upon ureteral obstruction in the Il17ra genetic deleted as compared to wild-type mice. Interestingly, this study demonstrated the novel relationship between the antifibrotic kallikrein-kinin system (KKS) and the IL-17A signaling pathway in the kidney. Interestingly, they found that treatment of the more fibrosis-prone Il17ra-/- mice with bradykinin, the major end-product of KKS activation, confers protection against fibrosis by upregulating the expression of matrix degrading enzymes such as the metalloproteinase MMP2. Moreover, the group identified the bradykinin receptors Bdkrb2 and Bdkrb1 as downstream mediators of IL-17-KKS-axis and administration of a bradykinin receptor 1 or 2 agonist in vivo showed a clear diminution of renal fibrosis.

Finally, the review article from S. *Rayego-Mateos* et al. entitled "Role of Epidermal Growth Factor Receptor (EGFR) and Its Ligands in Kidney Inflammation and Damage" describes the key role of the EGFR signaling pathway in the pathogenesis of inflammatory renal damage, with special attention to the role of some EGFR ligands in this process such as TGF-A and HBEGF that play a crucial role in EGFR transactivation through the modulation of key factors of renal damage and inflammation such as Ang II, aldosterone, and TWEAK. The authors specially analyzed the role of CTGF/CCN2, one recently described EGFR ligand with a strong impact in inflammatory and profibrotic processes in kidney disease.

In summary, this special issue will help to understand the inflammatory and profibrotic mechanisms that regulate key processes in the renal pathology and the new therapeutic strategies that are developed for the treatment and prevention of CKD.

Conflicts of Interest

The guest editors declare that there is no conflict of interest regarding the publication of this special issue.

Acknowledgments

This work was supported by the Instituto de Salud Carlos III and Fondos FEDER European Union (PI014/00041, PI17/00119), Red de Investigación Renal (REDinREN; RD16/0009), Comunidad de Madrid (B2017/BMD-3751 NOVELREN-CM), and Sociedad Española de Nefrología. The "Juan de la Cierva de Formacion" training program of the Ministerio de Economía, Industria y Competitividad, Gobierno de España, supported the salary of SR-M (FJCI-2016-29050).

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 8739473, 22 pages
https://doi.org/10.1155/2018/8739473

Review Article

Role of Epidermal Growth Factor Receptor (EGFR) and Its Ligands in Kidney Inflammation and Damage

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Received 31 May 2018; Revised 29 October 2018; Accepted 7 November 2018; Published 23 December 2018

Academic Editor: Martha Lappas

Chronic kidney disease (CKD) is characterized by persistent inflammation and progressive fibrosis, ultimately leading to end-stage renal disease. Although many studies have investigated the factors involved in the progressive deterioration of renal function, current therapeutic strategies only delay disease progression, leaving an unmet need for effective therapeutic interventions that target the cause behind the inflammatory process and could slow down or reverse the development and progression of CKD. Epidermal growth factor receptor (EGFR) (ERBB1), a membrane tyrosine kinase receptor expressed in the kidney, is activated after renal damage, and preclinical studies have evidenced its potential as a therapeutic target in CKD therapy. To date, seven official EGFR ligands have been described, including epidermal growth factor (EGF) (canonical ligand), transforming growth factor-α, heparin-binding epidermal growth factor, amphiregulin, betacellulin, epiregulin, and epigen. Recently, the connective tissue growth factor (CTGF/CCN2) has been described as a novel EGFR ligand. The direct activation of EGFR by its ligands can exert different cellular responses, depending on the specific ligand, tissue, and pathological condition. Among all EGFR ligands, CTGF/CCN2 is of special relevance in CKD. This growth factor, by binding to EGFR and downstream signaling pathway activation, regulates renal inflammation, cell growth, and fibrosis. EGFR can also be "transactivated" by extracellular stimuli, including several key factors involved in renal disease, such as angiotensin II, transforming growth factor beta (TGFB), and other cytokines, including members of the tumor necrosis factor superfamily, showing another important mechanism involved in renal pathology. The aim of this review is to summarize the contribution of EGFR pathway activation in experimental kidney damage, with special attention to the regulation of the inflammatory response and the role of some EGFR ligands in this process. Better insights in EGFR signaling in renal disease could improve our current knowledge of renal pathology contributing to therapeutic strategies for CKD development and progression.

1. Introduction

Chronic kidney disease (CKD) is a devastating progressive disease that affects 5–7% of the world's population. CKD has become a public health priority due to the increasing incidence of type 2 diabetes mellitus, hypertension, obesity, and aging [1]. CKD is characterized by diverse insults that trigger persistent inflammation, development of fibrosis, and loss of renal function ultimately leading to end-stage renal disease. Nowadays, the therapeutic protocols applied for the treatment against CKD have limited effectiveness underscoring the importance of the development of new molecular diagnostic approaches and therapeutic targets to either prevent or delay the progression of renal diseases.

Many preclinical studies have shown that the epidermal growth factor receptor (EGFR) can be a potential therapeutic target for renal diseases, as we will review here. Activation of the EGFR signaling pathway is linked to the regulation of

several cellular responses, including cell proliferation, inflammatory processes, and extracellular matrix regulation—all of them being involved in the onset and progression of renal damage. Nowadays, there are seven official EGFR ligands, including the following: EGF (canonical ligand), transforming growth factor-α (TGFA), heparin-binding EGF-like growth factor (HBEGF), amphiregulin (AREG), betacellulin (BTC), epiregulin (EPR), and epigen (EPGN) [2–7]. Recently, the connective tissue growth factor (CTGF/CCN2) has been described as a novel EGFR ligand [7]. Among all EGFR ligands, CTGF has been considered as a therapeutic target and a potential biomarker of human renal diseases [8–15]. The aim of this review is to summarize the contribution of EGFR pathway activation in experimental kidney damage, with special attention to the regulation of the inflammatory response and the role of some EGFR ligands in this process.

1.1. The EGFR Activation Pathways. The binding of neurotransmitters, hormones, or growth factors (ligands) to their membrane receptors produces biochemical changes inside the cell, which lead to a specific response to the initial stimulus. There are different groups of membrane receptors, all defined by their signal transduction mechanisms; these include ionotropic receptors, G protein-coupled receptors (GPCRs), and receptors with tyrosine kinase (RTK) activity. The EGFR (also known as HER1; ERBB1) is a transmembrane glycoprotein of 1186 aa (180 KDa) that belongs to the ERBB family of tyrosine kinase receptors, which is composed of members such as HER2/neu (ERBB2), HER3 (ERBB3), and HER4 (ERBB4). EGFR comprises a cysteine-rich extracellular domain (responsible for ligand binding), a transmembrane domain, and an intracellular domain with tyrosine kinase regions (activation domain) [16]. In most cases, EGFR is activated either directly or indirectly, by EGFR transactivation.

The first step of direct EGFR activation begins with the binding of specific ligands to the receptor. The seven official EGFR ligands have been extensively studied and share a common structure involved in EGF binding [17, 18], but information about the novel described ligands, such as CTGF, is scarce. EGFR ligands activate this pathway in different ways: (1) direct activation by soluble ligands, (2) the juxtacrine mode, when the ligand is anchored to the cell membrane, (3) autocrine signaling, in which EGFR activation occurs in the same cell, (4) the paracrine form if acting on a neighbouring cell [19], and (5) the extracrine form, which combines features of autocrine, paracrine, and juxtacrine signaling as well as possibly endocrine signaling, since EGFR and AREG can be detected in human plasma exosomes [20] (Figure 1).

All EGFR ligands can be found as soluble proteins, but some of them are also present as biologically active precursors anchored to the plasma membrane, including HBEGF, TGFA, AR, and BTC. The release of EGFR ligands from the cellular membrane is an important point in the EGFR transactivation process [21–25]. Interestingly, EGFR transactivation can be prompted by physiological and nonphysiological stimuli. The physiological stimuli capable of bringing about this effect include chemokines, adhesion molecules, and growth factors that require previous interaction with its specific receptors (GPCRs or not). EGFR transactivation by nonphysiological processes such as hyperosmolarity, oxidative stress, mechanical stress, ultraviolet light, and γ radiation is mediated by the inactivation of certain phosphatases that antagonize the intrinsic kinase activity of the receptor, thus allowing EGFR autophosphorylation [26].

The affinity of EGFR for its ligands depends on the tissue and pathological condition. Most of the studies have been done comparing the seven official EGFR ligands [17, 18]. These ligands display different ligand-biding affinities at around 3 orders of magnitude [17, 18]. Moreover, depending on the specific ligand that binds to EGFR, different cellular responses can be activated. Structural studies have described how EGFR activation occurs but ligand-related activation is less understood [18]. After EGFR ligand interaction, the receptor undergoes a conformational change leading to the formation of homo- or heterodimers. Then, the intracellular domain is activated in its tyrosine residues by phosphorylation, promoting the autophosphorylation of these same residues in their homologue. Phosphorylated residues in turn serve as a binding site for certain molecules that have domains of SRC homology; this interaction leads to different signaling cascades [27]. Earlier studies described that the different intracellular signaling triggered after EGFR activation depends on the phosphorylation of certain residues in the intracellular domain of the receptor. In SAA cells (NIH3T3 fibroblasts that overexpress human EGFR) stimulated with EGF, treatment with a phosphopeptide that blocks the autophosphorylation site Tyr1068 of EGFR induced a significant inhibition of EGFR/Grb2 interaction and RAS/MAP kinase activation [28]. EGFR activation translates signals to the nucleus, modulating the activity of transcription factors such as c-JUN, c-FOS, c-MYC, and NFKB and regulating gene transcription [16]. In bronchial epithelium, EGFR activation is linked to phosphorylation of the tyrosine residue (Tyr1173) associated with the activation of JNK, AP1, p38 MAPK, and NFKB pathways [29]. On the other hand, SRC kinase protein can act as cotransducer of EGFR signals and SRC-dependent EGFR activation mediated by EGFR phosphorylation of Tyr 845 and Tyr 1101 [30, 31].

EGFR transactivation (indirect activation) is triggered when different molecules bind to their specific receptors. A variety of these molecules have been identified and can be broadly categorized into the following groups: G protein-coupled receptors (e.g., Ang II and ET-1), cytokines (e.g., TNFA, TWEAK, and other TNF receptor family proteins), growth factors (e.g., TGFB and other proteins, including TPA and LPA), integrins, ion channels, and other physical stimuli [32–38]. Many of these molecules that transactivate EGFR are very relevant in renal damage, including Ang II, TGFB, and TNF receptor family proteins, showing the importance of EGFR transactivation in renal pathology. After the specific binding of these molecules to their own receptors, several second messengers, including intracellular Ca^{2+}, reactive oxygen species (ROS), and protein kinases, such as PKC, can be released [39–41]. These intracellular signals trigger a signaling cascade, leading to the activation of metalloproteases/disintegrins from the family of ADAMs.

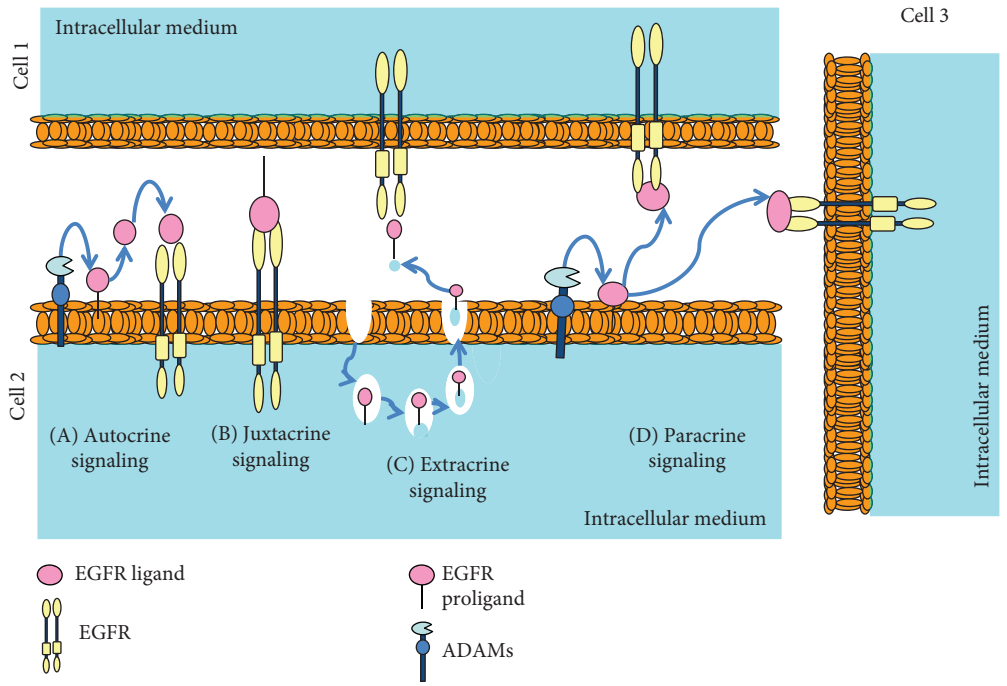

Figure 1: Types of signaling via epidermal growth factor receptor (EGFR) ligands: (A) the autocrine form if EGFR activation occurs in the same cell; (B) the juxtacrine form, when the ligand is anchored to the cell membrane; (C) the extracrine form, which combines features of autocrine, paracrine, and juxtacrine signaling as well as possibly endocrine signaling; and (D) the paracrine form if acting in a neighbouring cell. Adapted from Singh et al. 2016.

In general, the EGFR ligands involved in EGFR transactivation are inactive transmembrane precursors located in the cellular membrane which need to undergo proteolytic processing and be released as soluble ligands into the extracellular medium in order to bind to EGFR [42], being the most studied EGF, TGFA, and HBEGF. This proteolytic processing is carried out by ADAMs. Therefore, EGFR transactivation via ADAMs leads to the release of EGFR from the cellular membrane and subsequent binding to EGFR and pathway activation. Alternatively, in some cases, EGFR transactivation can occur independently of MMPs/ADAMs and is mediated by intracellular protein kinases, as in the case of Src kinase [43–46] (Figure 2).

1.2. ADAMs: Key Proteins in EGFR Transactivation. ADAMs are a family of 23 glycoproteins expressed as transmembrane surface proteins consisting of an extracellular metalloproteinase domain followed by a disintegrin-like domain, a cysteine-rich domain, a transmembrane domain, and finally a cytoplasmic tail. Their proteolytic activity is mediated by the zinc-dependent metalloproteinase domain, with the other domains contributing to substrate recognition and regulation. These domains give them its characteristics of adhesion molecules and proteases [47]. The first ADAMs described were involved in reproductive functions, mainly spermatogenesis and the attachment of sperm to the ovule (ADAM1 and ADAM2). However, only 12 of the human ADAMs (ADAM8, 9, 10, 12, 15, 17, 19, 20, 21, 28, 30, and 33) contain a functional catalytic consensus sequence (HEXGEHXXGXXH). The physiological function of the proteinase-inactive ADAMs (ADAM2, 7, 11, 18, 22, 23, 29, and 32) remains largely unknown, although some members of this group play important roles in development and function as adhesion molecules rather than proteinases [48, 49]. Numerous transmembrane proteins have been identified as targets of ADAM-mediated proteolysis [50]. Some of these substrates can be cleaved by different ADAMs, while others appear to be specific to an individual ADAM.

Recently, the roles of different ADAMs such as ADAM9, 10, 12, 15, 17, and 19 in the release and/or activation of cell surface proteins have been described. Among those proteins cleaved by ADAMs are several EGFR ligands, such as EGF, TGFA, and HBEGF [47, 51]. Depending on the tissue, different ADAMs may be involved in the release of the EGFR ligands. For example, kidney angiotensin II- (Ang II-) induced transactivation of EGFR is mediated by ADAM17 and by the release of TGFA [52], while in the heart, ADAM12 regulates this process through the release of HBEGF [53].

These glycoproteins are synthesized in the Golgi apparatus; under the action of the furin protease, they undergo a conformational change that induces activation. In their active form, they are transported to the plasma membrane where they exert their sheddase activity on the inactive precursors of the EGFR ligands. Functional upregulation of ADAM activity is generally observed in association with cytosolic Ca^{2+} elevation, purinergic receptor agonists, or membrane-perturbating agents. In some cases, such as ADAM17, sheddase activity is amplified by other signaling pathways, including activation of protein kinase C (PKC) and receptor tyrosine kinases. Furthermore, certain physicochemical properties of the lipid bilayer also govern the action of ADAM proteases [54].

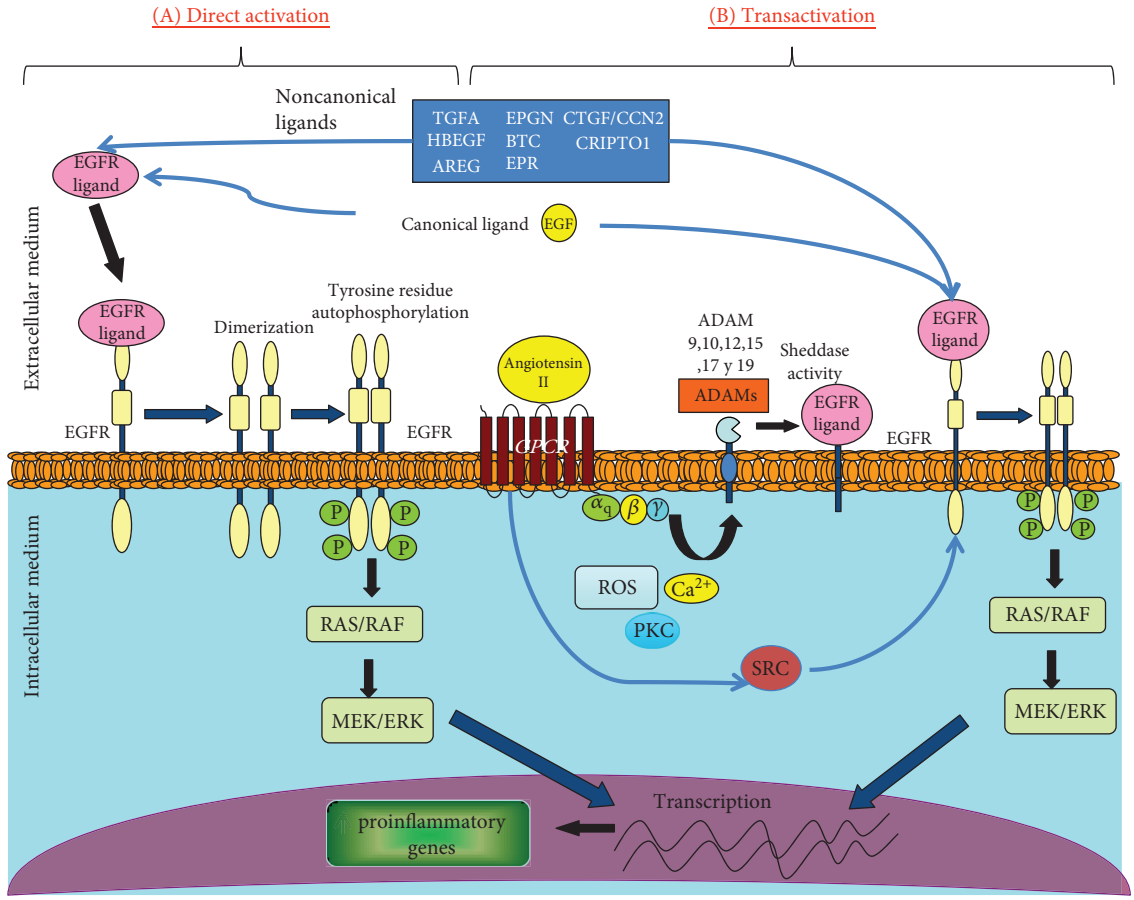

Figure 2: Different EGFR signaling systems in the inflammatory process: (A) direct ligand-receptor activation: the first step for the direct activation of EGFR begins with the binding of the ligand to the receptor. In general, EGFR ligands are located as inactive transmembrane precursors, which, in order to bind to their receptor, need to undergo proteolytic processing and be released as soluble ligands into the extracellular medium. This proteolytic processing is carried out by metalloproteases/disintegrins of the ADAM family. (B) Indirect ligand-receptor activation/transactivation: this process is triggered by the binding of molecules such as Ang II, thrombin, and ET1 to their specific receptor. After this binding, the release of second messengers is induced, such as intracellular $Ca2^{+}$, ROS, and certain protein kinases such as PKC, which induces activation of metalloproteases/disintegrins of the family of ADAMs. After EGFR ligand interaction, the receptor undergoes a conformational change inducing the formation of homo- or heterodimers. Then, the intracellular domain is activated in its tyrosine residues by phosphorylation, promoting the autophosphorylation of these same residues in their homologue. Phosphorylated residues in turn serve as a binding site for certain intracellular kinases that are capable of activating EGFR independently to MMPs, as in the case of the SRC kinase.

Once these precursors are proteolytically processed, they are released into the extracellular environment and can interact with EGFR and activate this signaling pathway [42]. Therefore, modulation of ADAM activity is of paramount importance to EGFR activation. Indeed, several strategies for ADAM inhibition are being considered as pharmacological targets. Thus, therapeutic strategies have focused on the inhibition, induction, and activation of ADAM expression as well as the use of small molecules blocking the active site and events blocking the domain for substrate recognition [55].

1.3. EGFR in Pathological Processes. Malfunctioning of EGFR, or its related pathways, has been shown to be relevant to the pathogenesis of several malignancies, playing a role in the development of ovarian, breast, and colorectal cancers as well as non-small cell lung and head and neck carcinomas [56]. Moreover, a strong correlation between EGFR expression and prognosis has been found in ovarian, head and neck, bladder, and esophageal cancers [57]. Thus, anti-EGFR therapy could have a place in the treatment of some of these tumors; in fact, the use of erlotinib or cetuximab may be employed as second-line therapy after failure of mono- or polychemotherapy in squamous cell lung carcinoma [58]. Recent studies suggest that EGFR activation can be involved in inflammatory diseases. Overexpression of EGFR may be related to a number of skin disorders, such as psoriasis or atopic dermatitis [59]. Apart from these spontaneously occurring diseases, it has been described that inhibition of EGFR by either monoclonal antibodies or small-molecule EGFR tyrosine kinase inhibitors brings about a monomorphic acneiform reaction, which, interestingly, is directly correlated with overall survival [60]. EGFR blockade is being explored as a new treatment for the disease [61], and future studies on inflammation are needed.

1.4. EGFR in the Kidney in Normal and Pathological Conditions. EGFR is expressed in the kidney, specifically localized in the glomerulus and the tubulointerstitial compartment [62]. This receptor plays a key role in renal electrolyte homeostasis [42]. However, its role in renal pathology is somewhat contradictory since both beneficial and deleterious actions have been found. Interestingly, upregulation of the EGFR pathway (including several of its ligands, as TGFA, HBEGF, and CTGF) has been described in human and experimental chronic renal pathologies, including glomerulonephritis, diabetic nephropathy, transplant rejection, and polycystic kidney disease [63–65], as well as in experimental models of acute kidney injury (AKI), such as ischemia/reperfusion or folic acid administration [66–68]. Intensive research in many experimental studies has shown that EGFR blockade exerts beneficial effects in progressive kidney disease, mainly ameliorating fibrosis [69, 70]. However, EGFR inhibition in AKI models exerts opposite results, presenting deleterious effects. Studies in models of ischemic injury using waved-2 mice (an EGFR mutation that induces a reduction in receptor tyrosine kinase activity) and wild-type mice treated with the EGFR kinase inhibitor erlotinib showed significantly decreased renal function in the depleted/treated mice [71, 72]. On the contrary, activation of the EGFR pathway can accelerate renal recovery in the early AKI phase by means of a mechanism that involves induction of renal tubular cell regeneration and protection of these cells from apoptosis, as demonstrated by the *in vivo* administration of several ligands (EGF, HBEGF) in experimental acute ischemic injury [69, 73–78]. These data were confirmed *in vitro* in cultured proximal tubular epithelial cells, in which activation of EGFR by the ligands EGF [76], HBEGF [46], and EPR [78, 79] induced cell proliferation and migration, supporting their protective role in renal repair after AKI. Thus far, there are no studies of CTGF modulation in experimental AKI.

Interestingly, the consequences of EGF signaling activation depend on species. In models of hydronephrosis, EGF administration causes cell death in mice, while it induces cell survival in rats [80]. Moreover, in a rat model of cisplatin-induced nephrotoxicity, the EGFR inhibitor erlotinib induced renoprotective properties by modulation of apoptosis and proliferation of tubular cells [81].

1.5. Official and Novel Described EGFR Ligands and Signaling in Renal Physiopathology. Most EGFR ligands have a similar globular structure, with a common fold defined by six conserved cysteine residues that form three disulphide bonds, termed EGF motifs, through which they interact with EGFR [82–86]. In contrast, CTGF does not possess an EGF motif and interacts with EGFR through its C-terminal domain [7] and future studies are needed to compare its structural binding to EGFR with other ligands. Several EGFR ligands contain amino-terminal heparin-binding domains (HBEGF domains), including HBEGF, AR, and CTGF [87, 88]. Contradictory results have been also reported in diabetic nephropathy. For instance, high glucose induces transactivation of EGFR and profibrotic responses in mesangial cells by increasing HBEGF release [89, 90]. However, Dey et al. [91] reported a beneficial effect of ADAM-mediated EGFR transactivation by bradykinin, leading to a decrease of podocyte permeability. EGFR ligands have generally been classified based on their interaction with EGFR and can be categorized into two classes: high affinity or low affinity. The high-affinity ligands are EGF, TGFA, HBEGF, and BTC, which bind to EGFR and have a dissociation constant (Kd) of between 1 and 100 nM, while the low-affinity ligands, AR, EPR, and EPGN, have a Kd greater than 100 nM [17]. CTGF has a Kd of 126 nM, which places it within the group of ligands of lower affinity [7].

1.6. EGF (Canonical Ligand). EGF is the canonical EGFR ligand and the one with the highest EGFR affinity described to date. EGF was identified from submaxillary gland extracts during nerve growth factor studies [92]. The kidney is the key source for EGF production, and several studies have identified elevated urine EGF levels as an independent risk factor for CKD progression [93–97].

EGF is produced in the kidney as a membrane-anchored prepro-EGF located in the apical membrane of epithelial cells [93, 98, 99]. Earlier studies conducted in rats showed positive EGF immunostaining, mostly in the proximal tubules, as well as increased *EGF* mRNA levels in the thick ascending limb of Henle's loop and distal convoluted tubules [100, 101]. EGF regulates physiological processes in the kidney. Several studies have shown the important role of EGF on sodium and magnesium transport through the regulation of epithelial ion channels such as epithelial sodium channel (ENaC) or $Na^+/K^+/2Cl^-$ cotransporter (NKCC1), among others [102–105]. Many studies have described the importance of EGF in polycystic kidney diseases [106–108] as well as the role of EGF in cell proliferation, including in tubular epithelial cells [109, 110]. Exogenous EGF administration has been found to reduce tissue injury caused by ischemia/reperfusion injury (IRI) showing beneficial antiapoptotic and antioxidant effects [111–113]. However, *in vitro* studies described prooxidative effects. In mammalian cells, EGF enhances the production of intracellular reactive oxygen species by dual oxidase 1 [114] and these oxidative species, which are induced by EGF, modulate ADAMs (positive regulator of EGFR signaling) or protein tyrosine phosphatases (negative regulator of EGFR signaling) [115–118]. AKI is a multifactorial pathology characterized by renal tubular damage, inflammation, and, frequently, a transient decrease in renal function. After the initial injury, there is a recovery phase, associated with proximal tubular cell proliferation and migration (as part of a regeneration process). But if this regenerative process fails to resolve, it may lead to fibrosis and loss of renal function [42, 119, 120]. Several experimental studies have shown the involvement of EGF in renal regeneration after IRI damage [121–123], but studies on EGF signaling in renal inflammation are scarce.

1.7. Transforming Growth Factor-α (TGFA). TGFA is one of the most widely studied EGFR ligands. TGFA is expressed in normal adult human kidney [121], and TGFA protein is detected in the urine of healthy human subjects [122]. Several experimental models of renal damage have described upregulation of TGFA. In an experimental model of renal mass

reduction, elevated TGFA protein levels were markedly increased after nephron reduction and prior to the development of renal lesions [123]. Experimental studies have demonstrated the involvement of TGFA in renal fibrosis, as in the model of Ang II-induced renal damage [52]. Moreover, elevated mRNA expression levels of *TGFA* were found in inflammation-induced renal damage triggered by systemic administration of TWEAK. In addition, treatment with a pharmacological inhibitor of ADAM17 (WTACE2) diminished renal inflammation associated with downregulation of TGFA and inhibition of EGFR pathway activation [38]. In *in vitro* studies in cultured tubular epithelial cells, stimulation with recombinant TGFA upregulated proinflammatory gene expression [38]. In these cells, upregulation of proinflammatory cytokines and chemokines induced by stimulation with aldosterone and TWEAK was blocked by a TGFA-neutralizing antibody [124]. Additionally, in Xenopus 2F3 cells, chronic treatment with TGFA over 24 hours inhibits the epithelial sodium channel ENaC by decreasing the number of channels in the membrane through the modulation of MAPK1/2 pathways but acute treatment with TGFA for 1 hour activates ENaC via PI-3 kinase [125]. In models of middle cerebral artery occlusion in mice and rats, TGFA treatment significantly reduced infarct size, suggesting that TGFA can induce angiogenesis, neurogenesis, and neuroprotection after stroke [126, 127].

Several studies have investigated the effect of TGFA loss on different pathological conditions. Research using a model of acute intestinal mucositis in *TGFA* knockout mice described that the lack of TGFA in intestinal epithelial cells resulted in higher apoptosis and lower proliferation [128]. In a model of bleomycin-induced lung injury, *TGFA*-null mice exhibited diminished pulmonary inflammation and fibrosis compared to wild-type mice [129]. Another study about peripheral nerve injury showed that *TGFA* knockout mice have no effect in regeneration process, probably due to compensatory expression mechanisms of other EGFR ligands [130]. These studies demonstrated that EGF-related growth factors could present specific and unique functions in certain tissues and cells.

1.8. Heparin-Binding Epidermal Growth Factor (HBEGF). HB-EGF is a 22 kDa protein originally identified in macrophage-like U-937 cells [131]. HBEGF is synthesized as a transmembrane precursor protein (pro-HBEGF) that can be cleaved by metalloproteinases such as ADAM17 to release a mature soluble HB-EGF (sHBEGF), a different form that is more functionally active *in vivo* than the precursor protein [82, 132]. The soluble form of HBEGF is capable of linking to heparan sulfate proteoglycans present in the cell surface, favouring local expression and accumulation of growth factors [133]. HBEGF participates in several physiological and pathological events, including wound healing [134], atherosclerosis [135], and tumor progression [136].

Some studies have analysed the role of HBEGF in renal pathology. Studies carried out on *HBEGF*-deficient mice showed the involvement of this ligand in podocyte damage in progressive glomerulonephritis. The loss of *HBEGF* was associated with lower renal inflammatory infiltration and decreased albuminuria levels prior to the appearance of renal cell proliferation [137]. Several studies using mice with specific *HBEGF* deletion in endothelium demonstrated attenuated renal damage in streptozotocin- (STZ-) induced diabetic renal injury [138] and in response to Ang II infusion [139]. In these conditional knockout mice, the inflammation in the perivascular area or renal interstitium (tested by F4/80- and CD3-positive stained cells) and the renal fibrosis caused by Ang II were significantly reduced compared to those in control mice [139]. Pharmacological blockade of ADAM17 by WTACE2 in a model of renal injury induced by TWEAK administration reduced *HBEGF* renal mRNA expression levels associated with lower inflammatory cell infiltration [38]. *In vitro* studies have clearly demonstrated that HBEGF regulates cell proliferation, including in glomerular epithelial cells [69, 140], and also regulates proinflammatory gene expression, as observed in cultured tubular epithelial cells [38]. In inner medullary collecting duct cells, the sustained exposure to sHBEGF induces the transition from an epithelial to a mesenchymal phenotype by upregulating the E-cadherin transcriptional repressor SNAIL2, thus contributing to renal fibrosis [141].

1.9. Amphiregulin (AREG). AREG is constitutively expressed in different cell types during development and homeostasis [142] and participates in several physiological processes, including the regulation of pulmonary morphogenesis [143] and the proliferation of keratinocytes [144]. Although AREG was originally described as an epithelial cell-derived factor, multiple studies have shown that it can also be expressed by activated immune cells in different inflammatory processes. Emerging evidence shows that AREG plays a critical role in restoring tissue integrity after infection or inflammation [145–148] and induces tolerance by promoting the restoration of tissue integrity after damage associated with acute or chronic inflammation [149, 150]. The immune system plays an important role in the EGFR signaling pathway, thus contributing to the progression of the inflammatory process. Cells such as basophils express high amounts of AREG after exposure to IL3 [151] and other types of immune cells such as neutrophils, CD8 T cells, and T regulatory lymphocytes [149]. Interestingly, AREG is expressed only in proinflammatory-type M1 macrophages [152]. Inflammation, ischemia, and hypoxia induce AREG expression in the brain. Under these situations, glial cells show upregulation of AREG, which protects against neuronal cell death. In neuro-2a cells, administration of AREG inhibits endoplasmic reticulum stress and cell death [153].

There are few studies about the role of AREG in kidney damage. In autosomal dominant polycystic kidney disease, the use of anti-AREG antibodies and inhibitors of activator protein-1 (AP1) can reduce cell proliferation in cystic cells by reducing AREG expression and EGFR activity [154]. In a model of streptozotocin-induced diabetes, the genetic blockade of *EGFR* or pharmacological inhibition using erlotinib showed a downregulation of phospho-AKT, CTGF, and AREG expression compared to that in diabetic mice [155]. However, there are no studies assessing the role of AREG gene deletion of direct modulation in experimental renal disease.

1.10. Betacellulin (BTC). BTC was first described in 1993 by Shing and et al. as a mytogen from pancreatic B cell tumors [5]. BTC was detected in the normal kidney at low levels; its location in the nephron remains unclear [156]. BTC could have different roles depending on the organ where it is expressed. A murine model of *BTC* overexpression demonstrated a reduction in body weight in *BTC* transgenic animals, which was accompanied by a reduction in kidney and pancreas weight, whereas the lungs of these animals were overgrown and their hearts had the same weight as those in controls [157]. Regarding inflammation, few studies have evaluated the role of BTC in this process, and to our knowledge, there is none in the kidney. One study demonstrated that BTC upregulates COX-2 expression in human granulose cells [158]. With regard to particular diseases, elevated BTC levels have been described in rheumatoid arthritis patients [159] and other authors observed that *BTC* overexpression protects against acute pancreatitis by activating stress-activated protein kinase [160].

1.11. Epiregulin (EPR). EPR was originally purified from conditioned medium of the fibroblast-derived tumor cell line NIH3T3/T7 [6] and is another example of the less widely studied EGF ligands. Higher EPR concentrations were found in patients with inflammatory diseases [161], including patients suffering from rheumatoid arthritis [159]. In this pathology, EPR inhibition suppresses the development of experimental autoimmune arthritis [161]. EPR regulates several immune-related processes. EPR is involved in peptidoglycan-mediated proinflammatory cytokine production in antigen-presenting cells and in innate immunity [162]. In a model of wound healing in corneal epithelial cells, *EPR* knockout mice presented an increased number of infiltrating cells in the wound area and this difference was related to the upregulation of several proinflammatory factors, including IL6, CXCL1, CXCL2, and CCL2 [163]. In the kidney, EPR promotes proliferation and migration of renal proximal tubular cells [78]. In 2016, Boyles et al. [164] commented on unpublished data with regard to the potential beneficial effects of a specific EPR neutralization antibody in experimental diabetes. However, the direct role of EPR on kidney inflammation requires further study.

1.12. Epigen (EPGN). EPGN was first identified in 2001 by Lorna Strachan. It consists of 152 amino acids and a transmembrane domain. EPGN is present in many tissues such as the testes, heart, and liver and is characterized as a low-affinity ligand [165, 166]. Several studies have shown that EPGN participates in cell proliferation. EPGN is a mitogen for HaCaT cells [165]. In epithelial cells, EPGN stimulates phosphorylation of c-ERBB1 and MAP kinase proteins [165]. The EPGN transgenic overexpression during embryonic development induces sebaceous gland hyperplasia [167], and the activation of NRF2 causes sebaceous gland enlargement in an EPGN-dependent manner [168]. *EPGN*-null mice exhibit peripheral demyelinating neuropathy that induces muscular dystrophy [169]. Data on inflammation, however, is scarce. One study described the involvement of EPGN in the inflammatory process in the skin via ERK pathway activation [170]. Other studies reported that recombinant EPGN is unable to activate ERBB2 in the presence of other ERBBs. Additionally, soluble EPGN has more mitogenic activity than EGF, although its binding affinity is lower [171].

1.13. The Two Unofficial/Novel Ligands

1.13.1. Teratocarcinoma-Derived Growth Factor 1 (TDGF1; CRIPTO1; CR-1). CRIPTO1 is another molecule that binds to EGFR but differs to official EGFR ligands because it does not possess an EGF-like motif. In addition to binding to EGFR, CRIPTO1 also acts as a coreceptor for the TGFB subfamily. CRIPTO1 is critically important in early embryogenesis, maintenance of stem cells, and the progression of some types of cancer [172]. There are few studies on the role of this EGFR ligand in pathophysiological processes. A cancer study reported that CRIPTO1 was expressed in a certain type of non-small cell lung cancer that causes intrinsic resistance to specific inhibitors of EGFR tyrosine kinase activity and participates in EMT [173]. A recent study suggests a potential role of this EGFR ligand in cardiac repair. In human cardiac ventricular fibroblasts, CRIPTO1 production was increased in response to reparative factors, such as NRG1B, and the blockade of PI3K, ERBB2, and ERBB3 by pharmacological inhibitors or neutralizing antibodies significantly reduced CRIPTO1 levels [174]. Currently there are no studies about the role of CRIPTO1 in inflammatory processes or in kidney diseases.

1.13.2. Connective Tissue Growth Factor (CCN2/CTGF): A Newly Described EGFR Ligand. CTGF (also known as CCN2) is a cysteine-enriched secretable matricellular protein with a molecular weight of 38 kDa. CTGF was identified in the conditioned medium of endothelial cells of the umbilical cord vein [175]. This protein has a modular structure made up of a secretory peptide at the N-terminal end followed by 4 functional modules [176]: (1) the insulin-like growth factor- (IGF-) binding domain, which stimulates the production of matrix proteins in renal cells [177–179]; (2) the von Willebrand factor type C domain, which is rich in cysteines and participates in protein oligomerization and synthesis. In Xenopus cells, CTGF binds directly to TGFB through this domain and promotes binding to its receptor, leading to the activation of the Smad response promoter [180]; (3) the thrombospondin-1 domain, which is involved in the union of soluble macromolecules or matrix proteins and participates in the union of CTGF to VEGF [181, 182]; and (4) the C-terminal domain, a dimerization domain involved in binding to the cell surface, possesses mitogenic activity for fibroblasts, and is responsible for the interaction with fibronectin [183]. This domain contains heparin-binding EGF sites [87, 88] and a region with a cysteine knot motif that resembles PDGF, TGFB, and NGF [184]. Finally, the N-terminal domain contains putative binding sites for IGF and TGFB.

Between module 2 and module 3, there is a hinge region that can be processed by multiple proteases, including the MMPs 1, 2, 3, 7, 9, and 13, generating two protein portions

(one with the N-terminal domain and the other with the C-terminal domain), both with biological activity. This region can also be proteolyzed by elastase and plasmin, which can cleave the individual modules to produce four fragments [185]. In addition, it has been observed *in vitro* that MMP2 processes CTGF, thereby generating the C-terminal fragment of 10–12 kDa. In biological fluids and in the medium of cells in culture, the presence of CTGF has been described in its different forms: complete molecule CTGF, C-terminal fragment, and the N-terminal fragment [186, 187]. However, the *in vivo* biological effects of CTGF and its fragments have not been investigated in depth.

1.14. Role of CTGF in Pathological Processes: A Key Mediator in Renal Inflammation. CTGF plays an important role in multiple cellular processes such as development, differentiation, cell proliferation, extracellular matrix (ECM) remodelling, and angiogenesis [8]. According to the cell type, a large variety of factors and molecules are involved in the induction and regulation of CTGF expression, including GPCR agonists, such as Ang II; growth factors such as TGFB, BMP, VEGF, IGF, GMCSF, and IL4; high concentrations of glucose; AGEs; hypoxia; mechanical stress; and oxidative stress [188–196].

CTGF is a developmental gene that is not expressed in adult tissues. However, under pathological conditions, CTGF could be induced in several diseases such as scleroderma, pulmonary fibrosis, and hepatic fibrosis [178, 196, 197] and in a multitude of renal diseases, including diabetic nephropathy [10, 198, 199]. In several independent studies, CTGF has been proposed as a biomarker for human diabetic nephropathy and other forms of CKD [8, 10–12] and also for cardiac dysfunction in patients exhibiting myocardial fibrosis and chronic heart failure [13]. Plasma CTGF levels predict end-stage renal disease and mortality in diabetic nephropathy [12]. Moreover, urine CTGF can also be used as predictor/biomarker of CKD, including diabetic nephropathy [9, 10, 14]. Patients with reduced right ventricular function had higher plasma CTGF levels than those with normal or mildly reduced right ventricular function [15].

Initial studies demonstrated the role of CTGF as a mediator of the profibrotic action of TGFB [200] and other factors involved in renal damage, such as Ang II [201] or endothelin-1 [202]. Additionally, experimental studies showed that blockade of endogenous CTGF using different approaches, including antisense oligonucleotides or gene silencing, demonstrated beneficial effects in fibrotic-related diseases, including experimental lung, liver, and vascular damage, as well as models of chronic renal damage, including diabetic nephropathy [200, 201, 203–205]. Interestingly, CTGF exerts opposite effects in other pathologies in much the same way as other EGFR ligands. CTGF overexpression conferred cardioprotection in Ang II-infused mice and in ischemia-reperfusion injury [206, 207]. However, the role of CTGF in AKI has not been investigated in depth. A recent study by our group described the beneficial effects of *CTGF* gene deletion, reducing proliferation, the induction of the G2M phase of cellular cycle, and fibrosis in the kidney using a model of CTGF injection over 10 days [70]. Near total inhibition of *CTGF* below baseline levels reduced tubulointerstitial fibrosis in different models of renal damage, such as folic acid administration or obstructive nephropathy [70, 208].

Many reports suggest that CTGF can also be considered a cytokine involved in the regulation of immune and inflammatory responses. CTGF can activate several cells of the immune system. CTGF is a chemotactic factor for immune cells, including mononuclear cells [209], and induces cell adhesion and migration [210]. In human CD4 lymphocytes, CTGF, in combination with IL16, contributes to Th17 differentiation [211]. Interestingly, monocyte-derived macrophages do not produce CTGF on stimulation with TGFB, lipopolysaccharide, but CTGF is taken up by macrophages *in vitro* [209]. In an early study, our group demonstrated that *in vivo* administration of the C-terminal CTGF fragment induced an acute renal inflammatory response, characterized by infiltration of inflammatory cells in the kidney (lymphocytes and macrophages) and activation of the NFKB and subsequent induction of proinflammatory factors such as CCL2, CCL5, and IL6 [212]. In later studies, we found that CTGF induces a sustained renal inflammatory response linked to activation of the Th17 response, characterized by the presence of interstitial infiltration of Th17 ($IL17A^+/CD4^+$) cells and upregulation of Th17-related factors (STAT3 and RORγt) [211]. Recent evidence suggests that CTGF can also regulate inflammation in other pathological conditions. Studies performed with conditional *CTGF* knockout mice have found lower macrophage accumulation and downregulation of proinflammatory factors in peritoneal-induced damage [213]. In experimental models of alcohol-induced inflammatory process in the pancreas, overexpression of *CTGF* in mice plays a novel role, regulating inflammation by increasing infiltration of macrophages and neutrophils and increasing inflammatory mediators such as IL1B or CCL3 [214]. In another study in a model of skin fibrosis induced by Ang II, pharmacological blockade with a neutralizing antibody against CTGF mitigated the inflammation and fibrotic process in the dermis and diminished the number of cells expressing PDGFRB, procollagen, αSMA, pSMAD2, CD45, and FSP1 [215].

Recent studies have analysed the relationship between CTGF and other signaling pathways. Several have demonstrated the crucial role played by integrins, proteoglycans heparan sulfate, and low-density protein receptors in CTGF cellular responses [216]. The existence of CTGF binding sites in the cell membrane was suggested in 1998 in chondrocyte studies [217]. In 2005, a potential CTGF receptor was described in mesangial cells, the receptor tyrosine kinase of nerve growth factor (TRKA), a member of the TRK membrane receptor family (TRKA, TRKB, and TRKC) [218]. Some studies have confirmed that CTGF also activates TRKA in murine cardiomyocytes and tubuloepithelial cells [7, 219]. In 2013, we described that CTGF can bind to EGFR through its C-terminal module and via a process modulated by $\alpha V\beta 3$ integrin [7]. The CTGF-EGFR interaction activates this signaling pathway linked to the modulation of proinflammatory factors and the recruitment of lymphocytes and macrophages in the kidney [7]. At the vascular level, we also observed that

CTGF-EGFR activation is linked to the oxidative process, endothelial dysfunction, and vascular inflammation through the NOX1 and NFKB pathways [220]. These results demonstrate that the activation of the EGFR pathway by the new ligand CTGF regulates inflammatory processes (Figure 3) and identify EGFR as a potential therapeutic target for the treatment of chronic kidney disease and vascular diseases closely linked with kidney damage.

In summary, EGFR activation has dual effects in AKI or CKD, ameliorating renal damage in experimental AKI by activating the regenerative process that occurs following acute renal damage through the induction of proliferation and migration of tubular cells. In contrast, EGFR activation exerted deleterious effects on CKD by activation of a fibrotic-related process, as observed in long-term models of renal damage [42, 70, 119, 120] (Figure 3).

1.15. Role of ERBB Crosstalks in Renal Inflammation. Previous studies described the possible crosstalk between EGFR and other receptors. The most obvious EGFR crosstalk is related to its heterodimerization with other members of the ERBB family (ERBB2, ERBB3, and ERBB4), whose function is amplifying and diversifying the signals [221–223].

Depending of the type of dimerization of ERBB1 (with itself or with other ERBB receptors as ERBB2), the signaling is very different [224, 225]. Only ERBB1/EGFR, ERBB3, and ERBB4 are able to bind ligands, an obligatory process for activation of the tyrosine kinase domain and intracellular signaling [226, 227]. ERBB2 has no known ligands, and it acts as a signal transducer in the recruitment of other components of the heteromeric complexes like ERBB1, ERBB3, or ERBB4 [228, 229]. ERBB3 has no kinase activity, and the biological relevance of the complex formation with other ERBB receptors (ERBB2-ERBB3 and ERBB2-ERBB4) has been analysed in studies with *ERBB2*,*3*,*4*-null mice [230–233].

As described above, EGFR transactivation can be induced by several GPCRs, different cytokines, integrins, and diverse tyrosine kinase receptors (TKRs) [36, 234, 235]. Moreover, it is possibly a crosstalk induced by ligand-independent EGFR transactivation, which consists of physical interactions between EGFR and other receptors such as platelet-derived growth factor receptor (PDGFR) [236] or IGF1R [237] and c-MET [238].

Several studies developed in monocytes have described a crosstalk between EGFR and GPCRs, linked to the regulation of inflammatory responses. The pharmacological blockade of EGFR or N-formyl-l-methionyl-l-leucyl-phenylalanine (fMLP) receptor, with AG1478 or cyclosporine H, respectively, decreased oxidative stress, CD11b upregulation, and EGFR/fMLP phosphorylation induced by their respective ligands. This crosstalk is SRC and ERK dependent [239]. In cervical cancer, GPCR TF-PAR2 (tissue factor protease-activated receptor 2) transactivates EGFR and mediates resistance to cisplatin, decreasing cisplatin-induced apoptosis [240]. Another study in monocytes has described a crosstalk between EGFR and TRKA in response to the stimulation of GPCRs [239], which further confirms the interaction between these two receptors. TRKA is a member of the TRK membrane receptor family (TRKA, TRKB, and TRKC). These receptors interact with neurotrophins and form homo/heterodimers with the low-affinity neurotrophin receptor, p75NTR [241].

There are few studies about EGFR crosstalks in renal inflammation. Most of them are related to EGFR transactivation. Lautrette et al. [52] demonstrated a possible crosstalk between EGFR and Ang II receptors in the kidney. In this in vivo study, the authors showed that Ang II is capable of transactivating EGFR and overexpression of a dominant negative isoform of EGFR prevents the frequency and severity of renal lesions in Ang II mice as well as interstitial cell infiltration.

In rat renal fibroblasts, palmitic acid (PA) activated the EGFR signaling pathway through TLR4/c-Src signaling. Previous studies described that fatty acids directly activate Toll-like receptor 4 (TLR4) and this process can induce c-SRC kinase activation [242]. In NRK-52E cells, the pharmacological blockade of EGFR or c-SRC previous to PA stimulation suppressed EGFR activation and its downstream signaling pathways ERK and AKT, closely related with renal inflammation and oxidative stress. The genetic silencing of *TLR4* through siRNA transfection blocked the PA-induced phosphorylation of EGFR, c-SRC, ERK, and AKT [243].

A study carried out in hepatocyte growth factor (HGF) transgenic mice demonstrated that the HGF/c-MET system significantly reduced LPS-induced renal and vascular injuries by the diminution of inflammation and ROS production, though EGFR ubiquitin degradation [244].

In vitro studies have described an EGFR crosstalk with aldosterone receptors. In cultured tubular epithelial cells, aldosterone caused EGFR transactivation, by a process mediated by ADAM17 and release via TGFB, and upregulated proinflammatory genes, via ERK and STAT1 activation [124]. In mesangial cells, aldosterone also activates EGFR linked to ROS production, ERK signaling, and modulation of cell growth [245].

A study developed in renal cells using gene silencing and pharmacological inhibitors of EGFR and TRKA demonstrated a clear crosstalk between EGFR and TRKA in response to stimulation with CTGF [7]. The analysis of the phospho-proteomic profiles of TRKA and EGFR shows a considerable similarity in the signaling originated by these RTKs [246]. EGFR crosstalk with TNF-related proteins is also involved in renal inflammation. TWEAK, a TNF member, is a cytokine that engages its receptor Fn14 to activate ADAM17, which releases the mature ligands HBEGF and TGFA that, in turn, transactivate EGFR. In cultured tubular epithelial cells, Fn14 gene silencing inhibited TWEAK-induced EGFR phosphorylation. Conversely, EGFR inhibition blocked TWEAK-induced responses, including activation of the ERK kinase pathway and upregulation expression of proinflammatory factors [38]. Moreover, pharmacological EGFR blockade inhibited TWEAK-induced renal inflammation. In vitro studies in renal cells have described that CTGF is involved in TGFB-induced EGFR transactivation [7]. However, the functional consequence of this EGFR/TRII crosstalk has not been investigated.

In conclusion, there are some possible crosstalks between ERBB1 and other receptors and their different interaction

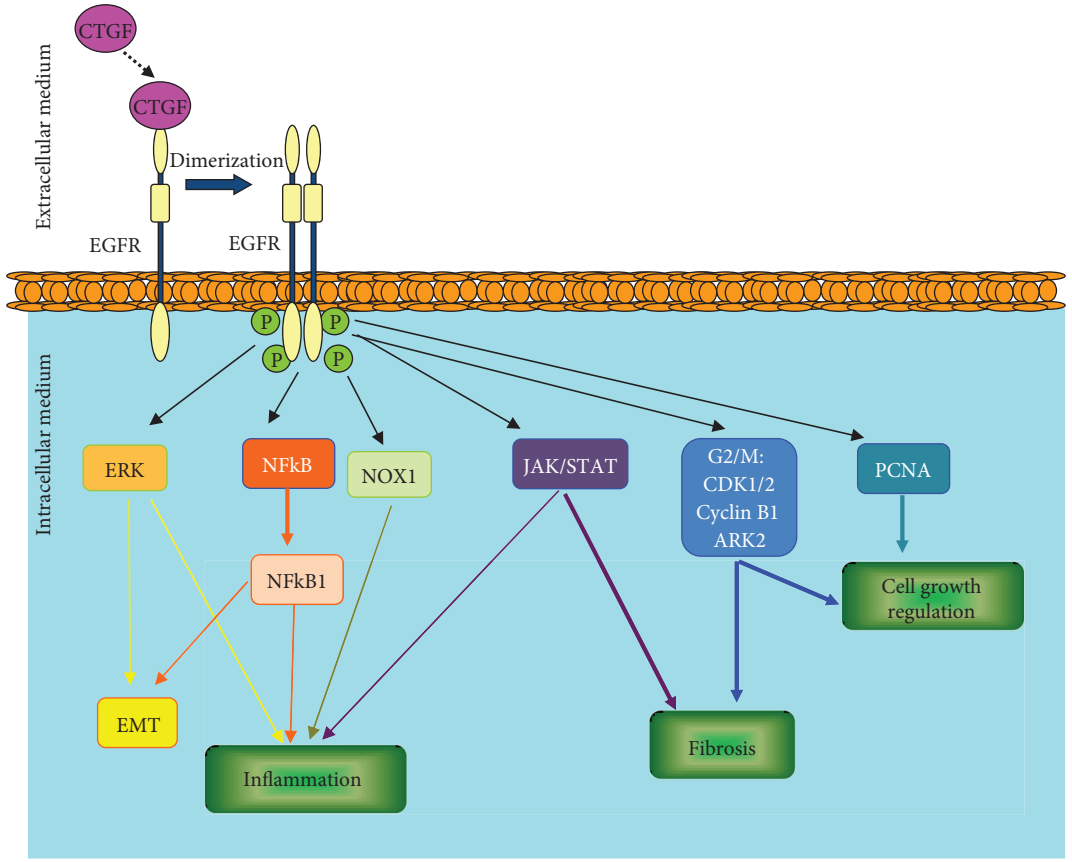

Figure 3: Different signaling pathways related to EGFR activation induced by CTGF/EGFR interaction. CTGF binds to EGFR through its C-terminal module. This interaction activates the EGFR signaling pathway linked to the modulation of different pathways closely related with cell growth, oxidative process, inflammation, EMT, and fibrosis in renal damage.

will trigger diverse signaling pathways, as well as amplify and diversify the intracellular signals related to inflammation and other key processes in kidney diseases. However, more studies in this topic are needed.

1.16. Therapy Targeting the ERBB1 Receptor in Kidney Disease. Several experimental studies have suggested that blocking EGFR could be an important tool to treat kidney diseases [42], especially by regulating inflammation, cell proliferation, and fibrosis [7, 38, 69]. Studies conducted in an autosomal recessive polycystic kidney model showed that treatment with an EGFR kinase inhibitor decreased the formation of cysts and improved renal function. In addition, similar results were observed using *WAVED-2* mutant mice (which present a point mutation in EGFR that reduces their 90% tyrosine kinase activity) [247]. In subsequent studies using the same mice model, it was observed that the beneficial effect of EGFR blockade was increased when is combined with the inhibition of ADAM17 [248]. Previous studies described that the EGFR blockade in different mice models of renal damage, induced by CCN2 and TWEAK injection, reduced the inflammatory infiltration of T lymphocytes and macrophages as well as the gene and protein expression of the proinflammatory mediators CCL2, CCL5, or IL6 [38]. In vitro studies developed in tubuloepithelial cells and an experimental model of renal inflammation in mice induced by TWEAK injection showed that the pharmacological blockade of ADAM17 with TAPI-2 and WTACE-2, respectively, inhibited the upregulation of proinflammatory mediators at gene and protein levels. Studies performed in models of renal mass reduction (subtotal nephrectomy) and prolonged ischemia showed that the truncated expression of a dominant negative of EGFR in proximal tubular cells decreased the infiltration of mononuclear cells, the accumulation of interstitial collagen, and renal tubular proliferation [249]. In diabetic rats, treatment with EGFR kinase inhibitors decreased the proliferation of tubuloepithelial cells, in addition to increase glomerular size [77]. In experimental models of hypertension induced by several factors (Ang II, leptin, monocrotaline, or ET1), EGFR blockade by different approaches including antisense oligonucleotides for EGFR, inhibitors of the EGFR kinase, and mutated *WAVED-2* mice reduced the characteristic effects of tissue damage observed in these models [250–253]. Systemic administration of Ang II induces severe fibrotic lesions in the kidney. However, the infusion of this peptide in mice that express a dominant negative form of the renal tubular-specific EGFR, protected them against the lesions produced by Ang II. In addition, it has been observed that in knockout mice for *TGFA* and

in mice treated with a specific inhibitor of ADAM17, renal fibrosis induced by Ang II decreases [52].

The origin of the study of the EGFR signaling pathway was in tumor pathology [254, 255]. In various types of cancer, including tumors of the head, neck, lung, and breast and colorectal tumors, the ErbB family of receptors (EGFR/HER1/ERBB1, HER2/neu/ERBB2, HER3/ERBB3, and HER4/ERBB4) is unregulated, producing an inappropriate cell stimulation [254, 256]. In malignant tumors, it has been described that HER2 and EGFR/HER1 are overexpressed and it has been established that overexpression of EGFR correlates with a worse clinical prognosis [257]. In the specific case of lung cancer, EGFR overexpression has been described in 90% of tumors. Several mechanisms can trigger an aberrant expression of EGFR, including in particular the overexpression of the protein, its gene amplification, appearance of mutations, the overexpression of EGFR ligands, and finally the loss of the regulatory mechanisms of these processes [256]. It should be noted that mutations in this receptor are one of the indicators that correlate best with the efficacy of EGFR inhibitors [256]. Specifically, a marked beneficial effect and greater response to treatment with these EGFR inhibitors have been observed in patients with mutations in exon 19 (codons 746–750) and exon 21 (substitution of leucine by arginine in codon 858 (L858R)) of EGFR compared to patients without these mutations [258].

The EGFR signaling pathway plays a crucial role in tumor processes since it modulates the cell cycle, inhibits apoptosis, induces angiogenesis, and promotes the motility of cancer cells and metastasis [259]. The first therapeutic approaches against EGFR began with the development of reversible pharmacological inhibitors of EGFR such as gefitinib (competes with ATP to bind the intracellular tyrosine kinase domain of EGFR, preventing its phosphorylation). Subsequently, erlotinib was designed, which presented a better pharmacokinetic and toxicity profile [260]. However, the response to gefitinib and erlotinib did not improve survival and improvements were only observed in those patients with mutations in EGFR [261, 262]. Another newly developed inhibitor is afatinib, with dual specificity against EGFR/ERBB1 and HER2/ERBB2 [263]. Due to the involvement of these pathways in embryonic development and cell proliferation, most efforts so far are focused on anti-EGFR therapies. These therapies involve the use of tyrosine kinase inhibitors, which are small molecules that bind intracellularly and interfere with signaling of the receptor, and the use of monoclonal antibodies that block the extracellular domain of the EGFR kinase. Among the developed antibodies, cetuximab stands out, which improved the survival rates in patients with lung cancer and colorectal cancer in combination with chemotherapy [264, 265]. In a kidney cancer study, administration of a murine/human chimeric anti-EGFR antibody (C225) was shown to inhibit the growth of normal renal cell carcinoma explants in NUDE mice [266].

EGFR regulates vascular homeostasis and pathophysiology. Studies with spontaneously hypertensive rats showed that vascular smooth muscle cells expressed high levels of EGFR and increased proliferation [267]. In models of experimental hypertension, EGFR blockers reduced blood pressure elevation and improved vascular lesions [69, 250, 268–270]. In atherosclerosis, increased expression of EGFR and some of its ligands, such as HBEGF, were described in the different stages of the atherogenic process [135, 271–273]. EGFR activation has also been involved in the vascular complications of diabetes [274–276].

1.17. Future Perspectives. All these studies show the complexity of EGFR pathway activation and its involvement in the pathogenesis of kidney damage. The canonical EGFR ligand EGF participates in acute renal damage mainly regulating cell proliferation, and future studies focusing on its role in renal regeneration are important. The other official ligands, TGFA and HBEGF, have an important role in the process of EGFR transactivation, by modulating key factors of renal damage, including Ang II, aldosterone, and TWEAK, mainly by regulation of renal inflammation. The recently described EGFR ligand CTGF is a potential therapeutic target that exerts proinflammatory and fibrotic properties, although more research is needed to completely understand EGFR binding and its involvement in EGFR transactivation in vivo. These data suggest that inhibiting EGFR or some of its ligands is an interesting therapeutic strategy for CKD and future studies are warranted.

Abbreviations

AREG:	Amphiregulin
EPGN:	Epigen
aa:	Amino acids
ADAMs:	Membrane-anchored disintegrin metalloproteases
ADPKD:	Autosomal dominant polycystic kidney disease
AKI:	Acute kidney injury
AKT:	Protein kinase a
Ang II:	Angiotensin II
AP1:	Activator protein 1
BMP:	Bone morphogenetic protein
BTC:	Betacellulin
CCL3:	Chemokine (C-C motif) ligand 3
CCN2/CTGF:	Connective tissue growth factor
CKD:	Chronic kidney disease
COX2:	Cyclooxygenase 2
CYR61:	Protein rich in cysteines 61
EGF:	Epidermal growth factor
EGFR/c-ERBB1:	Epidermal growth factor receptor
EMT:	Epithelial mesenchymal transition
EPR:	Epiregulin
ERK:	Intracellular signal regulation kinase
ET1:	Endothelin-1
FGF:	Fibroblast growth factor
GMCSF:	Granulocyte macrophage colony-stimulating factor
GPCRs:	Receptors coupled to G proteins
HBEGF:	Heparin-binding epidermal growth factor
HER2/neu (ERBB2):	Receptor tyrosine-protein kinase erbB-2

HER3 (ERBB3): Receptor tyrosine-protein kinase erbB-3
HER4 (ERBB4): Receptor tyrosine-protein kinase erbB-4
HGF: Hepatocyte growth factor
IGF: Insulin-like growth factor
IL1B: Interleukin 1 beta
IL4: Interleukin 4
IL6: Interleukin 6
IRI: Ischemia/reperfusion injury
JNK: c-Jun N-terminal kinase
LPA: Lysophosphatidic acid
LPR: Low-density protein
MAPK: Protein kinases activated by mitogen
MCP1/CCL2: Monocyte chemoattractant protein-1
MMPs: Metalloproteinases
NFKB: Nuclear factor-κB
NGF: Nerve growth factor
NKCC1: $Na^+/K^+/2Cl^-$ cotransporter
PDGF: Platelet-derived growth factor
PDGFRB: Platelet-derived growth factor receptor beta
PA: Palmitic acid
PI3K: Phosphatidylinositol 3-kinase
PKC: Protein kinase C
PLC: Phospholipase C
RANTES/CCL5: Regulated on activation, normal T expressed, and secreted
RORγt: RAR-related orphan receptor gamma
ROS: Reactive oxygen species
RTKs: Receptors with tyrosine kinase activity
SMAD: Mothers against decapentaplegic homolog
SRC: Tyrosine-protein kinase
STAT3: Signal transducer and activator of transcription 3
STZ: Streptozotocin
TGFA: Transforming growth factor-α
TGFB: Transforming growth factor-β
Th17: T helper 17 cells
TLR4: Toll-like receptor 4
TNFA: Tumor necrosis factor-α
TPA: Tetradecanoyl-phorbol-13-acetate
TRKA: Receptor of nerve growth factor A
VEGF: Vascular endothelial growth factor
WTACE2: Pharmacological inhibitor of tumor necrosis factor-alpha-converting enzyme (TACE)
αSMA: α-Smooth muscle actin.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by the Instituto de Salud Carlos III and Fondos FEDER European Union (PI014/00041, PI17/00119), Red de Investigación Renal (REDinREN; RD16/0009), Comunidad de Madrid (B2017/BMD-3751 NOVELREN-CM), and Sociedad Española de Nefrología. The "Juan de la Cierva de Formacion" training program of the Ministerio de Economía, Industria y Competitividad, Gobierno de España supported the salary of SR-M (FJCI-2016-29050).

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 5103672, 14 pages
https://doi.org/10.1155/2018/5103672

Research Article

IL-17 Receptor Signaling Negatively Regulates the Development of Tubulointerstitial Fibrosis in the Kidney

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Received 29 May 2018; Revised 13 August 2018; Accepted 13 September 2018; Published 14 October 2018

Guest Editor: Sandra Rayego-Mateos

Chronic inflammation has an important role in the development and progression of most fibrotic diseases, for which no effective treatments exist. Tubulointerstitial fibrosis (TF) is characterized by irreversible deposition of fibrous tissue in chronic kidney diseases. Prolonged injurious stimuli and chronic inflammation regulate downstream events that lead to TF. In recent years, interleukin-17 (IL-17) has been strongly linked to organ fibrosis. However, the role of IL-17 receptor signaling in TF is an active area of debate. Using the unilateral ureteral obstruction (UUO) mouse model of TF, we show that IL-17 receptor A-deficient mice (*Il17ra*$^{-/-}$) exhibit increased TF in the obstructed kidney. Consequently, overexpression of IL-17 restored protection in mice with UUO. Reduced renal expression of matrix-degrading enzymes results in failure to degrade ECM proteins, thus contributing to the exaggerated TF phenotype in *Il17ra*$^{-/-}$ mice. We demonstrate that the antifibrotic kallikrein-kinin system (KKS) is activated in the obstructed kidney in an IL-17-dependent manner. Accordingly, *Il17ra*$^{-/-}$ mice receiving bradykinin, the major end-product of KKS activation, prevents TF development by upregulating the expression of matrix-degrading enzymes. Finally, we show that treatment with specific agonists for bradykinin receptor 1 or 2 confers renal protection against TF. Overall, our results highlight an intriguing link between IL-17 and activation of KKS in protection against TF, the common final outcome of chronic kidney conditions leading to devastating end-stage renal diseases.

1. Introduction

Fibrosis affects all vital organs and is responsible for a staggering 45% of deaths in the US [1]. Unresolved inflammation triggers downstream signaling events that lead to organ fibrosis [2, 3]. No better example of this grave clinical scenario exists than in renal tubulointerstitial fibrosis (TF) [4]. TF is characterized by irreversible deposition of fibrous tissue in the tubular space in patients with chronic kidney injury leading to end-stage renal diseases (ESRD) [5]. Every year, approximately half a million ESRD patients receive dialysis or transplantation (http://www.niddk.gov). Inadequate early diagnostic tools and lack of antifibrotic medications further aggravate the prognosis in these patients. Thus, the need for safe and effective drugs to prevent TF is desperately needed in the clinic. However, the development of antifibrotic therapeutic strategies is hindered by our lack of understanding of the profibrotic events in the kidney during chronic kidney diseases.

Renal inflammation confers an initial protective response to injury. However, unresolved inflammation can drive excessive deposition of extracellular matrix (ECM) protein in the tubular space [3, 5]. The profibrotic cascade from initial damage to fibrosis is regulated by cytokines that drive activation of renal tubular epithelial cells and myofibroblasts, macrophage infiltration, and production of TGFβ, kidney proteases, and growth factors. To date, the primary focus of the renal fibrosis field centers around understanding the origin of myofibroblasts and tissue remodeling processes. Far less effort has been dedicated to dissecting the role of cytokines in regulating profibrotic events. Yet, cytokines remain an appealing therapeutic opportunity, given the clinical success

of anticytokine biologics therapy. Few studies have implicated IL-4, IL-5, and IL-13 (Th2 cytokines) in fibrosis [6]. In contrast, under inflammatory settings where IFN-γ (Th1 cytokine) dominates, fibrosis is attenuated [6]. Thus, tight regulation of the balance between pro- and antifibrotic cytokines dictates the outcome of profibrotic events, a process that is poorly understood.

The homeostasis of ECM proteins is a highly regulated process, involving multiple proteases and their regulators. An uneven deposition of ECM components either due to increased synthesis or diminished degradation of ECM proteins eventually replaces the normal renal parenchyma with fibrous tissue [7]. Two protease systems, the plasminogen activation system and the matrix metalloproteinase (MMP) family, play a critical role in degrading ECM components in the fibrotic kidney [8]. Each of these proteolytic systems is regulated by their respective endogenous activators and inhibitors, confirming a delicate balance of the activity of these enzymes by both positive and negative regulation.

IL-17 family cytokines (IL17A-F) are produced by T-helper 17 (Th17) cells and other innate IL-17 producers, such as invariant NKT, $\gamma\delta$ T, natural Th17, and group III innate lymphoid cells [9, 10]. IL-17A (IL-17) bind to cognate receptors (IL-17RA/RC) of target cells and activate Act1, NF-κB, and C/EBPγ/δ and drive the expression of IL-17-responsive genes [10]. Numerous studies, including work from our lab, demonstrated a critical role for IL-17R signaling in renal inflammation following kidney injury [11–13]. In these settings, IL-17 induces the expression of cytokines and chemokines that facilitate the influx of innate effector cells in the kidney [11]. However, the role of IL-17 in TF development is highly debatable. Consistent with its proinflammatory function, one study has implicated IL-17 as a profibrotic cytokine in TF [14]. IL-17 produced by kidney infiltrating $\gamma\delta^+$ T and $CD4^+$ T cells were shown to mediate fibrosis via RANTES-driven influx of inflammatory cells. Contrary to this study, a recent report demonstrated a surprising antifibrotic role of IL-17 in the TF following ureteral obstruction [15]. Likewise, IL-17 prevents TF development in deoxycorticosterone acetate + angiotensin II-induced fibrosis in the kidney [16]. Nevertheless, the contribution of IL-17R signaling in the progression of TF is poorly understood.

Our published study showed that IL-17 activates the kallikrein-kinin system (KKS) through the induction of renal expression of kallikrein 1 (Klk1) during systemic fungal infection [17]. Klk1 is a serine protease that cleaves high and low molecular weight kininogens to generate kinins and bradykinin [18]. Bradykinin via bradykinin receptor b1 (Bdkrb1) and bradykinin receptor b2 (Bdkrb2) regulates blood pressure. Additionally, bradykinin receptor activation plays a key role in renal protection [19]. Mice lacking components of the KKS or humans with polymorphisms in KKS-related genes exhibit increased risks for chronic renal disorders [19–24]. Interestingly, mice deficient in Bdkrb1 or Bdkrb2 also show exaggerated TF following kidney injury [25, 26]. In this setting, Bdkrb1 or Bdkrb2 activation induces the expression of matrix-degrading enzymes that facilitate the removal of ECM proteins and favor healing in the injured kidney. Although activation of KKS is linked to many acute and chronic kidney diseases, regulation of KKS in the kidney particularly during TF remains remarkably understudied in comparison to other organs including skin, salivary glands, and liver.

Here, we show that IL-17 is upregulated and plays an unappreciated antifibrotic role in the kidney following UUO. Accordingly, mice deficient in *Il17ra* signaling show exaggerated fibrosis in the obstructed kidney and overexpression of IL-17 prevented TF development in WT mice. Failure to degrade ECM proteins due to reduced renal expression of matrix metalloproteinase-2 (MMP2) and tissue plasminogen activator (tPA) contribute to the exaggerated TF phenotype in $Il17ra^{-/-}$ mice. Mice lacking the IL-17 receptor A subunit ($Il17ra^{-/-}$) exhibited diminished Klk1 expression in the kidney. Treatment with bradykinin reduced TF development in $Il17ra^{-/-}$ mice via upregulating Mmp2 and tPA levels in the obstructed kidney. Finally, we show that both Bdkrb1 and Bdkrb2 are equally required for protection against TF. These data identify a previously unrecognized link between IL-17 and KKS-mediated renal protection against TF, which may provide the basis for clinical intervention in this disease.

2. Materials and Methods

2.1. Mice. C57BL/6 (WT) mice were obtained from Jackson Laboratory (Bar Harbor, ME). IL-17 receptor A-deficient ($Il17ra^{-/-}$) mice on the C57BL/6 background were generously provided by Amgen (San Francisco, CA) and bred in-house. 8- to 10-week-old male mice were used for all the experiments. All studies were carried out under approved protocols of the University of Pittsburgh Institutional Animal Care and Use Committee adhering to the guidelines in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

2.2. Unilateral Ureteral Obstruction Model of Kidney Fibrosis. Unilateral ureteral obstruction (UUO) was executed as described previously [27]. Briefly, under anesthesia, the left ureter was isolated and ligated 2–4 mm below its origin (UUO kidney). The right ureter is left unobstructed (non-UUO kidney). Seven days post-surgery, mice were sacrificed, and the UUO and contralateral non-UUO kidneys were harvested for further analysis.

2.3. Morphological and Histological Analysis for UUO. The UUO and non-UUO kidneys were fixed in formalin, dehydrated, and paraffin-embedded. Serial sections (4–5 μm) were stained with periodic acid-Schiff (PAS) (Sigma-Aldrich, St. Louis, MO). To visualize kidney fibrosis via detection of total collagen, serial kidney sections were stained with Masson's trichrome and Picrosirius red stains, as described before (Sigma-Aldrich, St. Louis, MO). Morphometric analysis of the tubular inflammation and interstitial fibrosis was evaluated as previously described [28].

2.4. Immunofluorescence Staining. Frozen sections (5 μm thickness) were fixed in acetone and blocked with 1% BSA in PBS. The staining for collagen I, collagen III, and αSMA was performed using FITC-conjugated mouse anti-collagen I,

anti-αSMA (SouthernBiotech, Birmingham, AL), and anti-collagen III antibodies (Novus Biologicals, Littleton, CO), respectively. Slides were mounted with Vectashield mounting medium with or without 4′,6′-diamidino-2-phenylindole (Vector Labs, Burlingame, CA) and visualized using an EVOS FL Auto microscope (Life Technologies, Carlsbad, CA).

2.5. Hydroxyproline Assay. Hydroxyproline is a component largely seen only in collagen. Measurement of hydroxyproline levels can therefore be used as an indicator of collagen content. 10 mg of kidney tissue was homogenized with 12 M HCl (Sigma-Aldrich, St. Louis, MO) at 120°C overnight. Hydroxyproline levels were quantified using a commercially available hydroxyproline assay kit (Chondrex, Redmond, WA) following the manufacturer's instructions. The samples were finally read at 560 nm. Values were represented as mg collagen/mg kidney tissue.

2.6. Isolation of RNA and Real-Time PCR. Total RNA was isolated from kidney tissue by using the RNeasy Micro Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). cDNA was synthesized using SuperScript III First-Strand (Invitrogen, Carlsbad, CA). Gene expression was measured by qPCR with PerfeCTa SYBR Green FastMix ROX (Quanta BioSciences, Gaithersburg, MD) on the 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Gene expression levels were then determined for target genes using commercially available QuantiTect primers (Qiagen, Valencia, CA) by real-time PCR. The expression of each gene was normalized to that of GAPDH.

2.7. Flow Cytometry Analysis of Single-Cell Suspension from the Kidney. Kidneys were perfused with 15 mL prewarmed PBS. The perfused kidneys were digested with collagenase B (0.230 U/mL; Roche Applied Science, Indianapolis, IN) in 10% FCS in RPMI for 30 min (37°C) with occasional shaking. RBC lysis was performed, and cells were resuspended in 10 mL RPMI media. The cells were slowly layered over 5 mL Lympholyte-M (Cedarlane, Burlington, Canada). The tubes were spun for 1200 rpm for 30 min at room temperature. The cell layer at the interface of the media and Lympholyte-M was collected and washed twice with PBS and used for flow cytometry staining. The single-cell suspensions from the kidney were surface-stained with fluorochrome-conjugated antibodies against Ly6G (clone: IA8; eBioscience, San Diego, CA, USA), Ly6C (clone: AL-21; eBioscience, San Diego, CA, USA), CD11b (clone: M1/70; eBioscience, San Diego, CA, USA), and F4/80 (clone: BM8; eBioscience, San Diego, CA, USA) followed by flow cytometry. The data were analyzed using FlowJo software (Tree Star, Ashland, OR).

2.8. Adenoviruses. Adenoviruses expressing IL-17A (Ad-IL-17) and control (Ad-ctrl) were kindly provided by Dr. J. Kolls (U. Pittsburgh). Mice were injected via the tail vein with 1×10^9 pfu of Ad-IL-17 or Ad-ctrl virus 72 hours prior to UUO surgery.

2.9. Oral Antibiotic Treatment. Mice were provided *ad libitum* autoclaved drinking water supplemented with ampicillin (0.5 mg/mL, Sigma-Aldrich, St. Louis, MO), metronidazole (0.5 mg/mL Sigma-Aldrich, St. Louis, MO), neomycin (0.5 mg/mL, Med-Pharmex, Pomona, CA), vancomycin (0.25 mg/mL, Nova Plus, New York, NY), and sucralose (4 mg/mL, Splenda, McNeil Nutritionals, LLC). Splenda was added to make the antibiotic cocktail more palatable. Control mice received autoclaved water with Splenda only. Antibiotic treatment was started 2 weeks prior to UUO surgery and continued for the duration of the experiment.

2.10. Bradykinin and Bradykinin Receptor Agonists. Mice were injected i.p. with 200 μL bradykinin (300 nmol/kg/day) (R&D Systems, Minneapolis, MN). Control mice received equal volumes of PBS. For experiments with selective agonists, mice received i.p. injection of Bdkrb1 (1 mg/kg/day) or Bdkrb2 (750 nmol/kg/day) selective agonists as indicated (R&D Systems, Minneapolis. MN). Control mice received equal volume of PBS.

2.11. Western Blots. Kidney tissues (10 mg) were homogenized in RIPA buffer (Thermo Scientific, Pittsburgh PA). Concentration of protein was quantitated by the BCA quantitation assay (Thermo Scientific, Pittsburgh, PA). Equal amounts of sample were subjected to electrophoresis and transferred to PVDF membranes (Millipore, Billerica, MA). After blocking with 5% milk in TBS, the blots were incubated with anti-mouse Klk1 (LifeSpan Biosciences, Seattle, WA) or anti-mouse beta-actin (Abcam, Cambridge, MA) overnight in 4°C. The blots were then washed and incubated for 1 hour at room temperature with individual secondary antibodies accordingly. Bands were detected using an enhanced chemiluminescence detection system (Thermo Scientific, Pittsburgh, PA) and developed with a FluorChem E imager (ProteinSimple, San Jose, CA).

2.12. Gelatin Zymography. Gelatin zymographic analysis of MMP2 and MMP9 proteolytic activity in kidney tissue homogenates was performed according to the method described previously [27]. Briefly, kidney homogenates were prepared essentially according to the methods described before [29]. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit. A constant amount of protein (30 μg) from the kidney tissue homogenates was loaded onto commercially 10% Zymogram (gelatin) gel (Life Technologies, Carlsbad, CA). After electrophoresis, proteinase activity was detected as unstained bands on a blue background representing areas of gelatin digestion, as per the manufacturer's protocol.

2.13. Statistical Analyses. Results are expressed as mean ± SD. Differences between groups were calculated for statistical significance using 2-tailed paired Student's t tests and ANOVA as appropriate. A p value less than 0.05 was considered significant.

3. Results

3.1. IL-17 and IL-17-Responsive Genes Are Upregulated in the Obstructed Kidney. To determine the expression of IL-17 family of cytokines in the obstructed kidney, WT mice were subjected to UUO and renal transcript expression of *Il17a*,

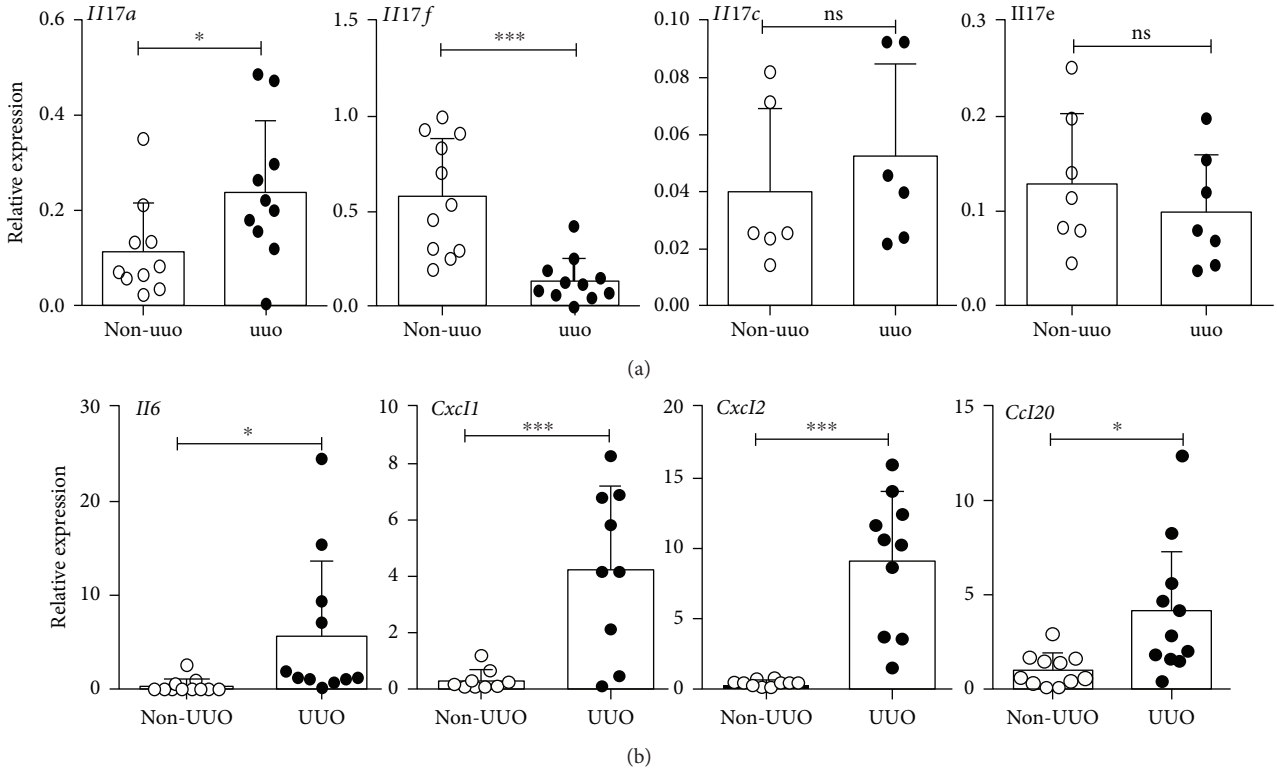


Figure 1: IL-17 and IL-17 target gene expressions are increased in the obstructed kidney of WT mice. WT mice were subjected to UUO ($n = 7$–11). Mice were sacrificed at day 7 post-surgery, and renal transcript expressions of (a) Il17a, Il17f, Il17c, and Il17e, and (b) IL-17-responsive genes (Il6, Ccl20, Cxcl1, and Cxcl2) in the UUO and non-UUO kidneys were evaluated by qPCR. In the scattered plots, each dot represents individual mouse and error bars indicate mean ± SD. The data are pooled from three independent experiments. p value ≤0.05 (*), ≤0.001 (***), and ≤0.0001 (****). ns: not significant.

Il17f, *Il17c*, and *Il17e* were evaluated by qPCR at day 7 post-surgery. Confirming previous reports, we observed a significant increase in *Il17a* mRNA expression in the UUO kidney compared to non-UUO (Figure 1(a)) [14]. While the *Il17f* mRNA level was reduced in the obstructed kidney, transcript expressions of *Il17c* and *Il17e* were comparable between the UUO and non-UUO kidneys.

We next assessed the functional consequence of IL-17A (IL-17) production in the UUO kidney by measuring the transcript expression of IL-17-responsive cytokines and chemokine genes. The mRNA levels of *Il6*, *Ccl20*, *Cxcl1*, and *Cxcl2* were significantly elevated in the UUO kidney compared to non-UUO at day 7 post-UUO surgery (Figure 1(b)). These results indicate that expressions of IL-17A (IL-17) and IL-17-responsive inflammatory genes are increased in the kidney following ureteral obstruction.

3.2. IL-17RA Signaling Is Critical for Protection against TF following UUO. To define the contribution of IL-17RA signaling in TF, WT and *Il17ra*$^{-/-}$ mice were evaluated for renal tissue damage and fibrosis at day 7 post-UUO surgery. Surprisingly, mice deficient in *Il17ra* signaling demonstrated significantly increased tissue damage and collagen deposition in the renal parenchyma, as evidenced by PAS (Figure 2(a)), Masson's trichome (Figure 2(b)), and Picrosirius red staining, respectively (Figure 2(c)). The non-UUO kidneys from WT and *Il17ra*$^{-/-}$ mice showed no fibrotic changes. In line with the histopathology data, we observed a significant increase in the total collagen content in the obstructed kidneys of *Il17ra*$^{-/-}$ mice than WT animals (Figure 2(d)). Moreover, to ensure that the observed phenotype in *Il17ra*$^{-/-}$ mice is not due to increased IL-17 signaling via IL-17RC, we measured *Il17rc* mRNA level in the obstructed and nonobstructed kidneys of WT and *Il17ra*$^{-/-}$ mice. As shown in Figure 2(e), *Il17rc* transcript level was comparable between the groups. These results highlight an unexpected antifibrotic role for IL-17RA signaling in the pathogenesis of TF.

Since IL-17RA serves as a receptor subunit for *Il17*, *Il17f*, *Il17c*, and *Il17e*, we next sought to verify the renal protective role for IL-17 in TF [10]. To that end, we infected WT mice either with an adenovirus overexpressing IL-17 (Ad-IL-17) or adeno-control vector (Ad-Ctrl). We have previously shown that Ad-IL-17 infection in mice resulted in 200-fold more serum IL-17 than the Ad-ctrl vector, which could be detected 6 days post-Ad-IL-17 injection (last time point analyzed) [17]. Additionally, the increase in IL-17 level was not associated with systemic inflammation, as serum TNFα and IL-1β levels were undetectable [17]. In comparison to mice injected with Ad-ctrl, IL-17 overexpression led to significantly reduced fibrotic changes and total collagen deposition in the obstructed kidney at 7 days post-UUO (Figures 3(a) and 3(b)). Overall, our results suggest that IL-17 prevents the deposition of collagen in the tubulointerstitial space following UUO.

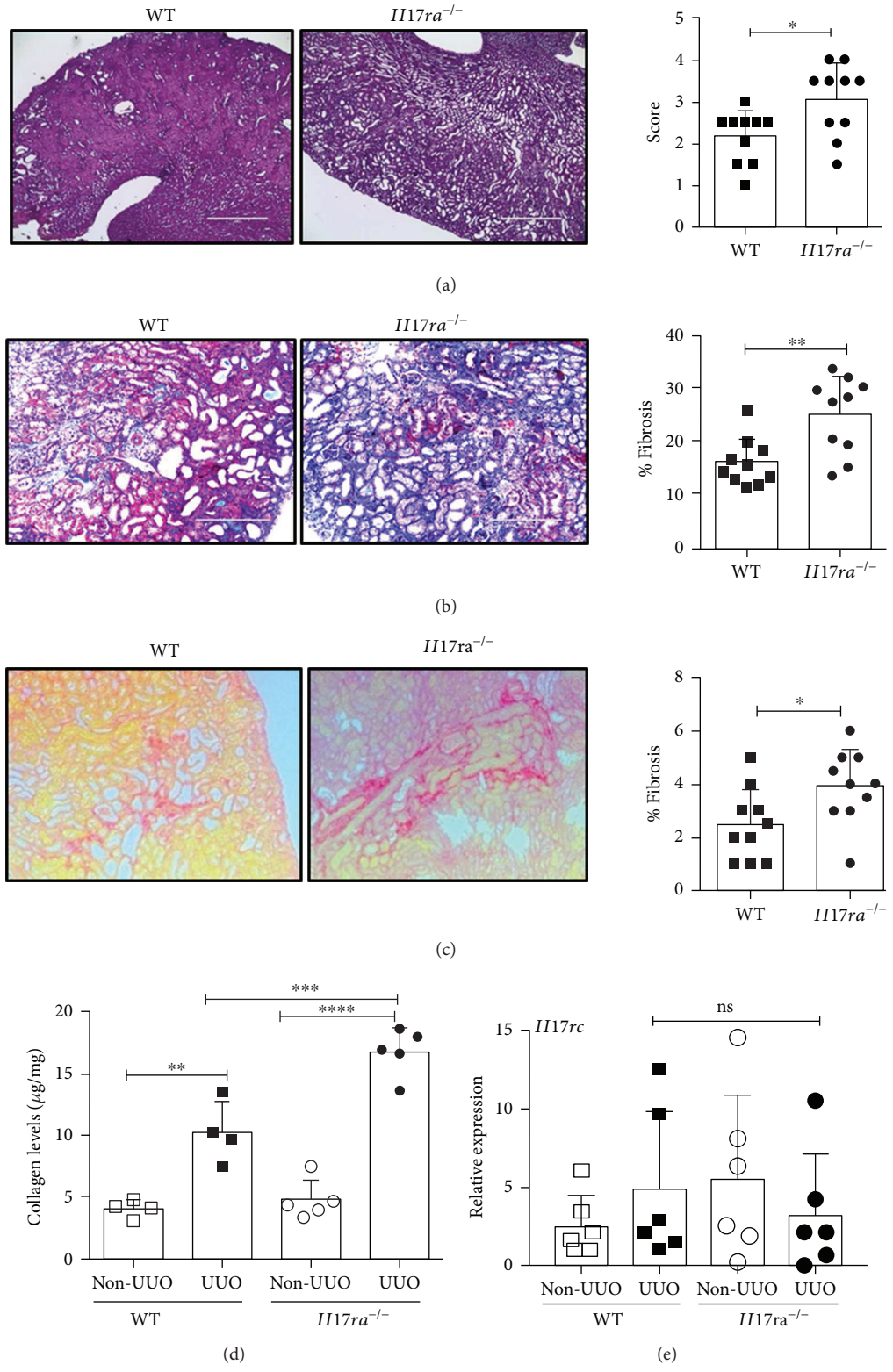


Figure 2: Exaggerated TF in Il17ra$^{-/-}$ mice following UUO. WT and Il17ra$^{-/-}$ mice ($n = 10$) were subjected to ureteral obstruction. At day 7 post-surgery, UUO kidneys were stained with (a) PAS, (b) Masson's trichrome, and (c) Picrosirius red stain to evaluate inflammatory changes and TF development. The inflammation score and percentage fibrosis were quantified blindly based on stained sections. Photomicrographs are representative of 2 independent experiments. Original magnification: 4x (a), 20x (b), and 10x (c). (e) The absolute collagen content in the UUO and non-UUO kidneys ($n = 4$–5) was quantified using hydroxyproline assay. (e) Kidneys were evaluated for Il17rc mRNA expression by qPCR. In the scattered plots, each dot represents individual mouse and error bars indicate mean ± SD. The data are pooled from two independent experiments. p value ≤0.05 (*), ≤0.01 (**), ≤0.001 (***), and ≤0.0001 (****).

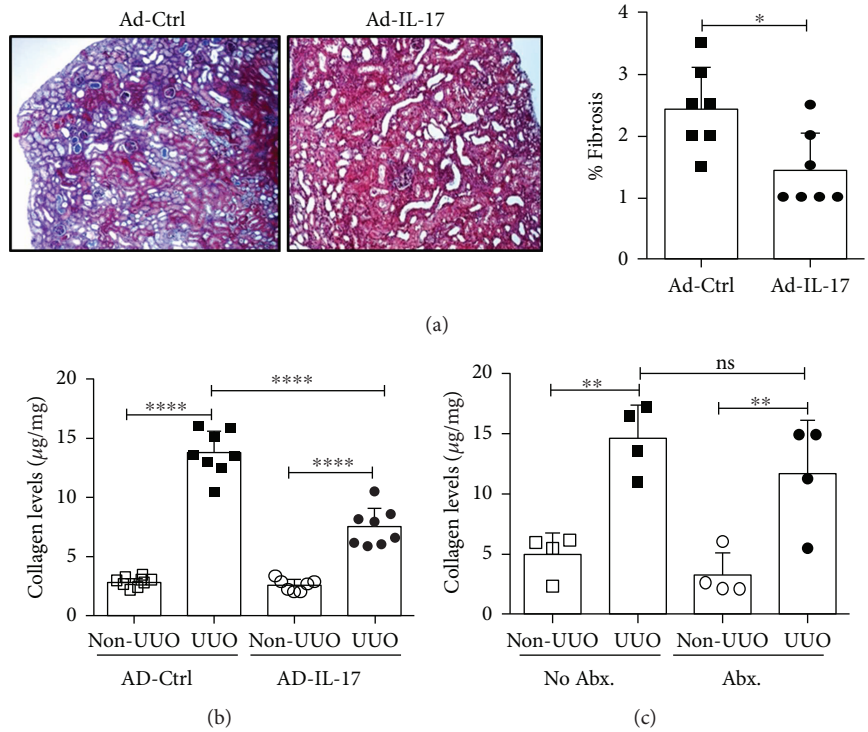


Figure 3: Overexpression of IL-17 reduced TF development in the WT mice. WT mice ($n = 7$–8) were either injected with adenovirus overexpressing IL-17 (Ad-IL-17) or control vector (Ad-ctrl) (10^9 pfu) 72 h prior to performing UUO surgery. The TF development was evaluated at day 7 post-surgery by (a) Masson's trichrome staining of serial sections from UUO kidneys. Photomicrographs are representative of 2 independent experiments. Original magnification: 10x. (b) The total collagen content in the kidneys was evaluated by hydroxyproline assay. (c) WT mice ($n = 4$) were treated with a cocktail of antibiotics (Abx) in drinking water starting day 7 (relative to UUO surgery) and then throughout the experiment. Control mice (no Abx treatment) received drinking water only. At day 7 post-surgery, total collagen content in the kidney was evaluated by hydroxyproline assay. In the scattered plots, each dot represents individual mouse and error bars indicate mean ± SD. The data are pooled from two independent experiments. p value ≤0.05 (*), ≤0.01 (**), and ≤0.0001 (****). ns: not significant.

The role for IL-17 in the TF development has been conflicting based on existing literature. A previous study by Peng et al. identified IL-17 as a profibrotic cytokine in the mouse model of UUO [14]. In stark contrast, a recent study has shown increased TF in the absence of IL-17 following ureteral obstruction [15]. The apparent discordance between these findings is presently unclear. It is possible that difference in the gut microbiome, shown to drive IL-17 response in the kidney diseases, may account for the seeming disagreement between the study by Peng et al. and our results [30]. To define the involvement of the gut microbiome in IL-17-driven TF, we depleted gut microbiota by treating WT mice with a cocktail of antibiotics before performing UUO surgery. Mice treated with antibiotics showed comparable level of total collagen content in the obstructed kidney than did untreated mice (Figure 3(d)). These results suggest that differences in the gut microbiome between mice may not account for the disagreement between the TF phenotypes observed by Peng et al. and our studies.

3.3. Increased Synthesis of ECM Proteins Does Not Contribute to the Exaggerated TF in Il17ra$^{-/-}$ Mice. Previous studies have shown that the balance between ECM proteins' synthesis and degradation determines the severity of ECM deposition and eventual development of TF [7]. To assess whether IL-17RA signaling negatively regulates the deposition of ECM proteins, we evaluated collagen I and collagen III deposition in the WT and *Il17ra*$^{-/-}$ kidney in mice with UUO. Indeed, immunofluorescence staining revealed increased deposition of collagen I and collagen III in the renal parenchyma of *Il17ra*$^{-/-}$ kidney following ureteral obstruction (Figure 4(a)).

We further determined whether exaggerated deposition of ECM proteins in the *Il17ra*$^{-/-}$ mice could be due to increased expression of ECM protein genes. We measured transcript levels of *Col1a1* and *Col3a1* in the WT and *Il17ra*$^{-/-}$ kidneys following ureteral obstruction. UUO significantly enhanced transcript expression of *Col1a1* and *Col3a1* compared to non-UUO kidney. However, mRNA levels of *Col1a1* and *Col3a1* were comparable between the obstructed kidneys of WT and *Il17ra*$^{-/-}$ mice at day 7 post-surgery (Figure 4(b)). Additionally, immunofluorescence staining and qPCR revealed similar number of ECM protein secreting α-smooth muscle actin (αSMA$^+$) myofibroblasts and *αSMA* mRNA expression between the groups, respectively (Figures 4(c) and 4(d)).

Following ureteral obstruction, macrophages infiltrate the kidney and facilitate TF by secreting profibrotic growth factors including TGFβ [7]. A recent study has demonstrated a critical role of hematopoietic cell-specific IL-17R signaling in regulating TF [31]. To assess the impact of

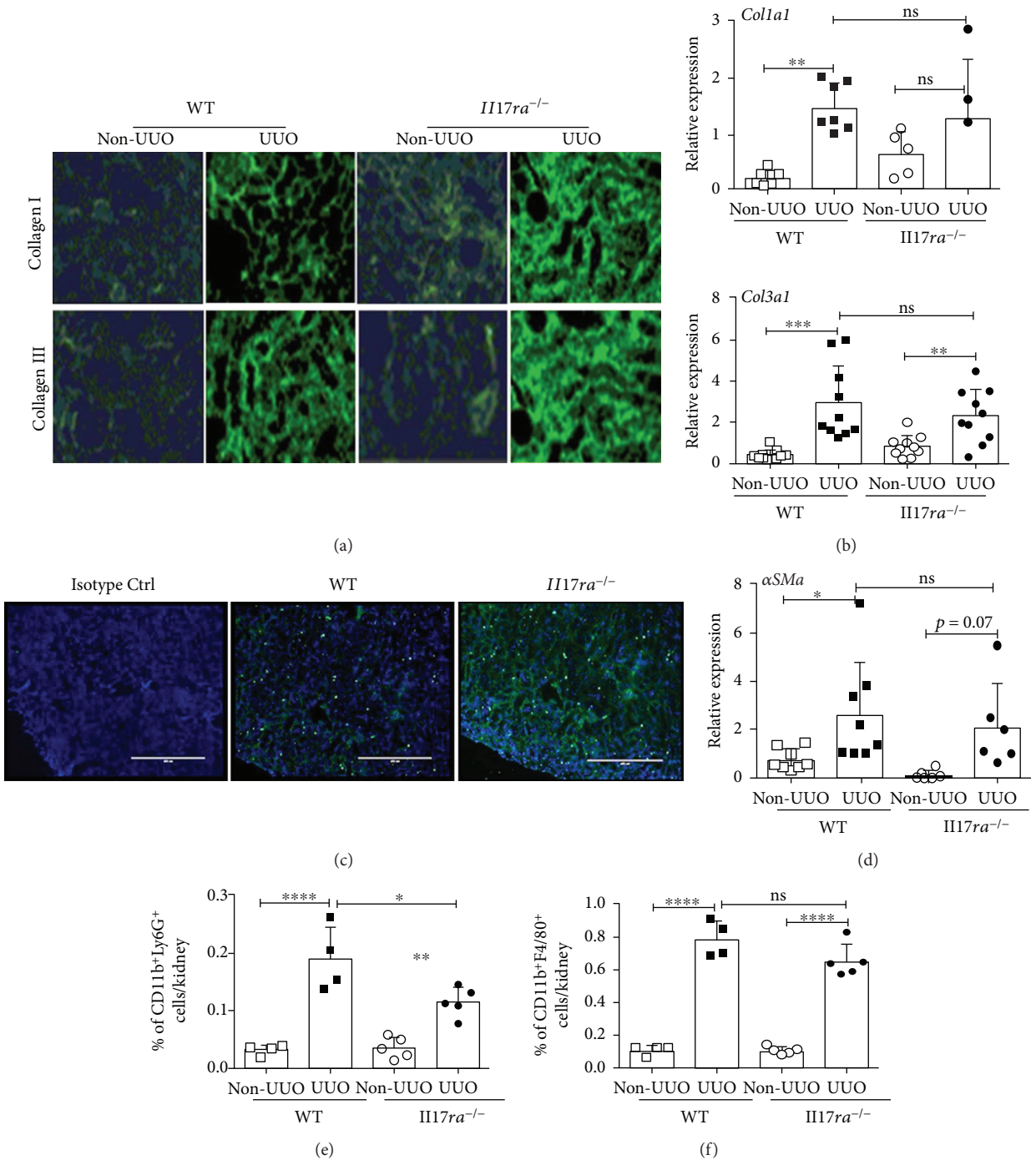


Figure 4: Diminished ECM protein degradation and not synthesis in the kidney of mice deficient in IL-17RA signaling. UUO was performed in WT and Il17ra$^{-/-}$ mice ($n = 7$–12). At day 7 p.i., (a) the frozen kidney sections were assessed for collagen I and collagen III deposition by immunofluorescence staining. (b) UUO and non-UUO kidneys were evaluated for mRNA expression of Col1a1 and Col3a1 by qPCR. (c) Frequency of αSMA$^+$ cells was evaluated by immunofluorescence staining. Photomicrographs are representative of 2 independent experiments for (a) and (c). Original magnification: 10x for (a) and (c). (d) Kidneys were evaluated for αSMA mRNA expression by qPCR. Single-cell suspension from the perfused kidneys was subjected to flow cytometry analysis to determine the percentages of kidney infiltrating (e) neutrophils and (f) macrophages (gated on CD45$^+$ cells). In the scattered plots, each dot represents individual mouse and error bars indicate mean ± SD. The data are pooled from three independent experiments for (a–d) and (g) and two independent experiments for (e) and (f). p value ≤0.05 (*), ≤0.01 (**), ≤0.001 (***), and ≤0.0001 (****). ns: not significant.

IL-17RA signaling on infiltrating macrophages, infiltration of inflammatory cells was evaluated in the obstructed kidneys of WT and *Il17ra*$^{-/-}$ mice. Seven days after surgery, significantly increased percentages of neutrophils (CD45^{+} CD11b^{+}Ly6G^{+}) and macrophages (CD45^{+}CD11b^{+}F4/80^{+}) were seen in the WT and *Il17ra*$^{-/-}$ obstructed kidneys

(Figures 4(e) and 4(f)). While mice deficient in IL-17RA signaling showed diminished neutrophil infiltration, there was no difference in the frequency of macrophages (out of total kidney-infiltrating $CD45^+$ cells) between the groups following UUO.

When evaluated for IL-17-responsive inflammatory cytokines and chemokine gene expression, $Il17ra^{-/-}$ kidney exhibited a significant reduction in *Tnfα* mRNA expression than control animals following UUO (Figure 5(a)). However, *Il6*, *Cxcl1*, *Cxcl2*, and *Cxcl5* transcript levels were comparable between the groups. Additionally, renal transcript expression of *Tgfβ* were comparable between the $Il17ra^{-/-}$ and WT UUO kidneys (Figure 5(b)). Collectively, these results suggest that exaggerated ECM protein deposition cannot be attributed to increased gene expression of ECM proteins, number of myofibroblasts, macrophage infiltration, and *Tgfβ* expression in the $Il17ra^{-/-}$ mice following UUO.

3.4. Renal Gene Expression of Mmp2 and tPA Are Diminished in the Absence of IL-17R Signaling. Multiple studies have emphasized the essential role of ECM protein-degrading enzymes in the removal of fibrous tissue in various organs [32]. Thus, we sought to determine whether increased TF in $Il17ra^{-/-}$ mice is due to diminished degradation of ECM proteins in the kidney. We measured the expression of *Mmp2*, *Mmp9*, and *tPA* genes in the obstructed kidney of WT and $Il17ra^{-/-}$ mice. These genes are selected based on their known role in the development of organ fibrosis [32]. While *Mmp9* transcript expression was similar between the groups, renal mRNA levels of *Mmp2* and *tPA* were significantly reduced in the UUO kidneys of $Il17ra^{-/-}$ mice than WT animals (Figures 5(c)–5(e)). Accordingly, gelatin zymography of total kidney extracts revealed significantly reduced MMP2 but not MMP9 activity in the UUO kidney of $Il17ra^{-/-}$ mice than WT at day 7 p.i. (Figure 5(f)). Overall, this data suggests that diminished expression of MMP2 and tPA may result in the reduced degradation of ECM proteins and drive aberrant TF in $Il17ra^{-/-}$ mice.

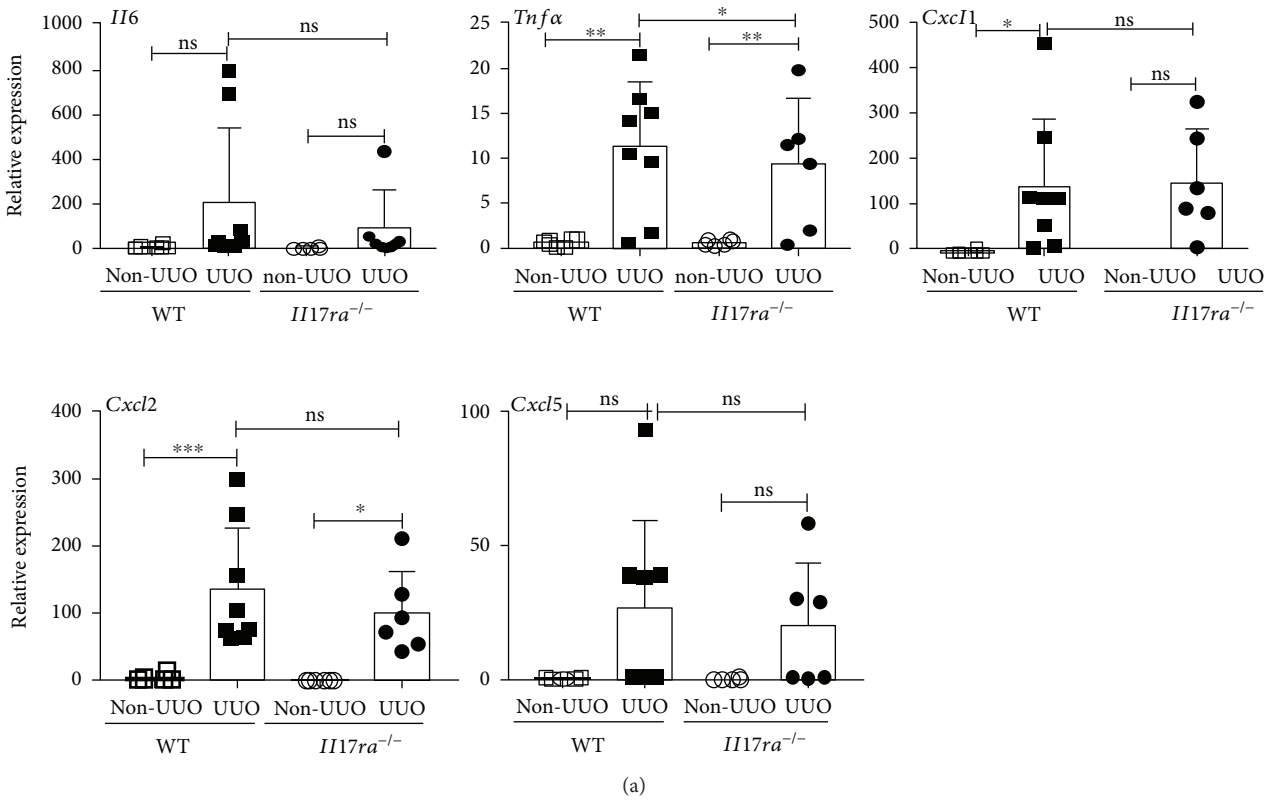
3.5. Activation of IL-17-KKS-Axis Protects against TF by Inducing the Expression of Matrix-Degrading Enzymes. Based on the known antifibrotic functions of KKS activation and its implication in IL-17-mediated renal immunity, we next interrogated the role of Klk1 in IL-17-driven protection against TF [11]. At day 7 post-surgery, immunoblot analysis of kidney whole-cell extract revealed a strong suppression of Klk1 protein expression in the $Il17ra^{-/-}$ mice compared to WT animals (Figure 6(a)). Collectively, this result confirms our prior findings and indicates that KKS is activated in the obstructed kidney in an IL-17-dependent manner.

Kallikreins cleave "kininogens" to generate kinins, namely, bradykinin and kallidin [18]. Numerous studies have identified an essential role of bradykinin in protection against TF following UUO [25, 26]. In this setting, bradykinin induced the renal expression of matrix-degrading enzymes such as Mmp2 and tPA, with minimal impact on ECM synthesis and macrophage influx in the UUO kidney [25, 26]. To define the contribution of IL-17-KKS-axis driven renal protection against TF, $Il17ra^{-/-}$ mice were either treated with bradykinin or left untreated starting 3 days prior to UUO and then daily for the next 7 days. $Il17ra^{-/-}$ mice treated with bradykinin showed reduced TF as evidenced by diminished fibrotic changes and total kidney collagen content compared to untreated $Il17ra^{-/-}$ mice (Figures 6(b) and 6(c)). The level of total collagen content in the bradykinin treated kidney was comparable to untreated WT mice. Interestingly, the protective phenotype observed in $Il17ra^{-/-}$ mice receiving bradykinin correlated with increased renal expression of *Mmp2* and *tPA* genes following UUO (Figures 6(d) and 6(e)).

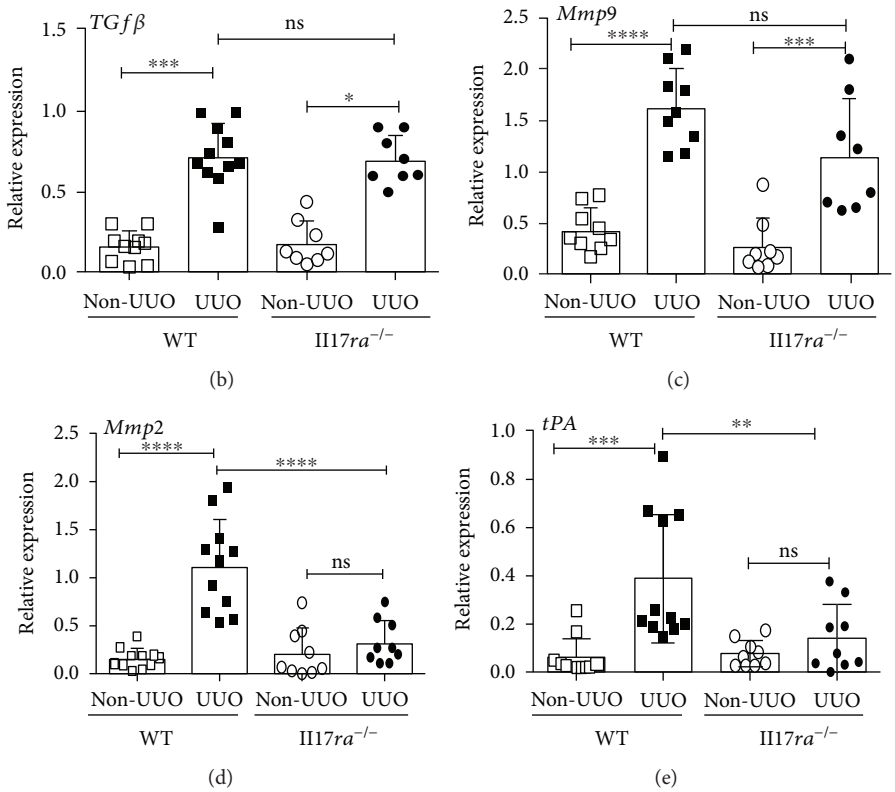
Bradykinin and its metabolite des-Ard-bradykinin activate Bdkrb2 and Bdkrb1, respectively. Based on our finding that the IL-17/KKS axis confers protection against TF, we next wanted to test the preclinical efficacy of Bdkrb agonists in treating mice with renal fibrosis. Accordingly, UUO was induced in WT mice and they were either treated with Bdkrb1 agonist or Bdkrb2 agonist or left untreated. At day 7 post-surgery, mice were evaluated for TF development by Masson's trichome staining of kidney sections and measuring the total collagen content in the obstructed kidney. As shown in Figures 7(a) and 7(b), we observed that treatment with either Bdkrb1 or Bdkrb2 agonists confers similar protection against TF. These data indicate that the IL-17-KKS axis facilitates the degradation of ECM protein in a Mmp2- and tPA-dependent manner.

4. Discussion

IL-17 promotes tissue inflammation and autoimmunity but also plays an important role in host defense against pathogens [33]. Consistent with its proinflammatory function, several studies have implicated IL-17 as a profibrotic cytokine. For example, IL-17 drives skin and pulmonary fibrosis, dilated cardiomyopathy, atherosclerosis, and hepatic fibrosis in experimental models. However, nagging discrepancies argue against these interpretations and experimental evidence also suggests an antifibrotic role of IL-17 in lung and skin fibrosis [34–38]. Compared to lung, skin, liver, and heart, studies investigating the role of IL-17 in renal fibrosis are surprisingly scarce. The few studies that have been performed have yielded contradictory results [14–16, 31]. Despite obvious parallels between fibrosis in the kidney and other organs, there are also a number of important differences in kidney and kidney-specific consequences. Renal specific factors, including poor regenerative capacity, toxins (uraemia), hypoxia, and arterial blood pressure significantly contribute to kidney disease outcomes [4]. Thus, lessons from skin, lung, or liver fibrosis cannot necessarily be applied to kidney. Understanding organ-specific differences has obvious therapeutic implications, since targeting kidney-specific factors would likely spare other organs from unwanted side effects. Here, we show that IL-17 is rapidly upregulated and plays an unappreciated antifibrotic role in the kidney following UUO. We have shown that IL-17R activation *in vivo* reduced UUO-induced TF and overexpression of IL-17 is beneficial in preventing renal fibrosis. IL-17 activates KKS, which in turn induces the expression of matrix-degrading enzymes in the obstructed kidney. These



(a)



(b) (c) (d) (e)

Figure 5: Continued.

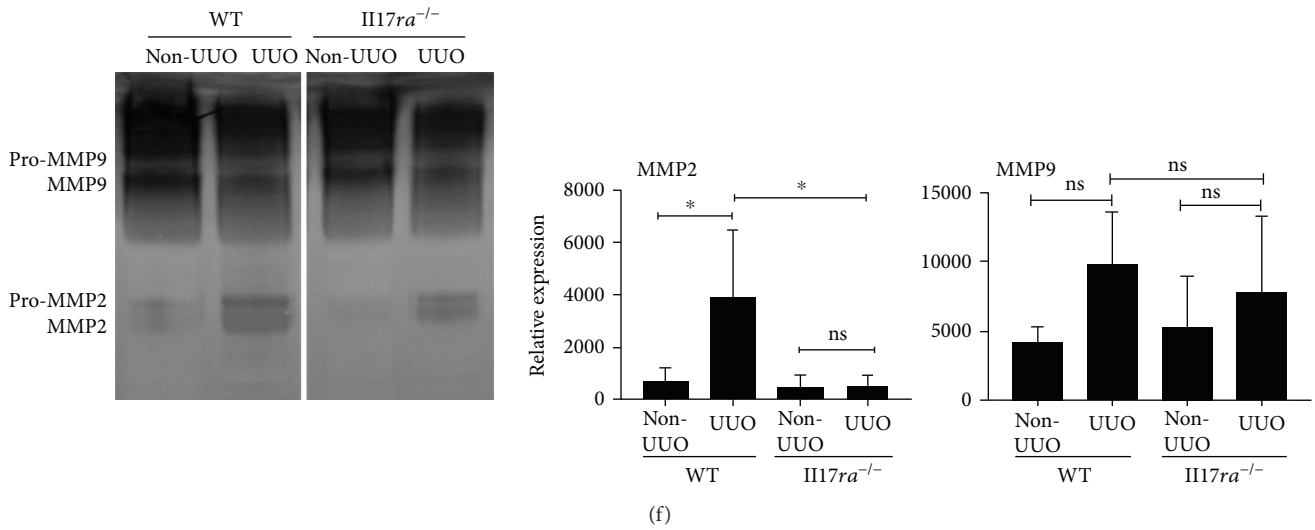


Figure 5: MMP2 and tPA expressions are compromised in the obstructed kidney of Il17ra$^{-/-}$ mice. WT and Il17ra$^{-/-}$ mice ($n = 6$–11) were subjected to UUO. At day 7 p.i., obstructed and non-UUO kidneys were evaluated for mRNA expression of (a) Il6, Tnfα, Cxcl1, Cxcl2, and Cxcl5, (b) Tgfβ, (c) Mmp9, (d) Mmp2, and (e) tPA by qPCR. (f) Total kidney extracts were subjected to gelatin zymography to assess for MMP9 and MMP2 activities at day 7 post-UUO. The lanes were run on the same gel but were noncontiguous (white line). In the scattered plots, each dot represents individual mouse and error bars indicate mean ± SD. The data are pooled from three independent experiments. p value ≤0.01 (**), ≤0.001 (***), and ≤0.0001 (****). ns: not significant.

observations are consistent with the antifibrotic effect of KKS and particularly bradykinin in TF development.

In the past decade, major emphasis in the field of IL-17 has been placed on understanding how IL-17-producing cells are generated. Fewer resources have been dedicated on defining downstream signaling via IL-17R on target cells [33, 39]. The specific IL-17R signaling pathways and downstream cell targets involved in mediating protection against TF are unknown. Although IL-17R is ubiquitously expressed, most studies to date indicate that the essential IL-17 target cells are nonhematopoietic [40, 41]. A recent study reported that IL-17 may affect renal fibrosis by directly impacting macrophage development [31]. Here, we show that there is no contribution for direct IL-17R signaling in the recruitment of macrophages in the obstructed kidney. Rather, we show that IL-17 is produced locally in the obstructed kidney and renal cells can respond to IL-17 directly [11]. Future studies should take advantage of *Il17ra* conditional knockout mice to dissect downstream signaling events in kidney-resident cells in the pathogenesis of TF.

A published study suggests that IL-17 is important for driving TF in the mouse model of UUO. In this paper, the authors showed that IL-17 acts on T cells to produce RANTES, a chemokine required for inflammatory cell infiltration in the obstructed kidney [14]. Since RANTES can be induced by numerous cytokines, failure to show that *in vitro* stimulation of T cells with IL-17 drives RANTES production weakens the overall interpretation of this report. Additionally, T cells use preexisting mRNA to produce and secrete RANTES rapidly following TCR stimulation [42]. TF occurs in rapid response to a nonimmune stimulus. Thus, it is unlikely that in this short time frame kidney-migrating CD3$^+$ T cells would encounter self-antigens to produce RANTES and drive disease pathogenesis in a UUO model of TF. In contrast, data from Sun et al. and our lab show that IL-17 plays an unappreciated kidney protective role in TF [15]. The apparent discordance between Peng et al. and our findings is currently unclear. However, we show that the seemingly opposite phenotype observed between these studies cannot be attributed to a difference in IL-17-driving gut microbiota between mice, as antibiotic treatment has minimal impact on TF development following UUO.

The accumulation of ECM proteins in pathological states results from an imbalance between both synthesis and degradation. We show here for the first time how the IL-17-KKS axis reduced ECM protein deposition by studying both ECM protein synthesis and degradation. In line with prior observations in bradykinin receptor-deficient mice, we observed no difference in collagen I and III protein synthesis between the obstructed kidneys of *Il17ra*$^{-/-}$ and WT mice [25]. Rather, the alteration was noted in the degradation of ECM components due to diminished expression of matrix-degrading enzymes including Mmp2 and tPA. It has been reported that bradykinin is a potent stimulus for tPA and Mmp2 production in the kidney [25]. In addition, plasmin transforms metalloproteinases from their latent to active forms [43]. The paralleled decrease in tPA and Mmp2 production related to decreased ECM protein degradation, thus suggesting a role for the IL-17-Bradykinin/tPA/Mmp2 activity in protection against TF.

An increase in the aging population and incidence of diabetes and hypertension has contributed to an alarming rise in the prevalence of ESRD [4]. Antibodies against IL-17 and its receptor have been approved for the treatment of autoimmune diseases, but the potential side effects of blocking IL-17 are not well defined, particularly in the context of

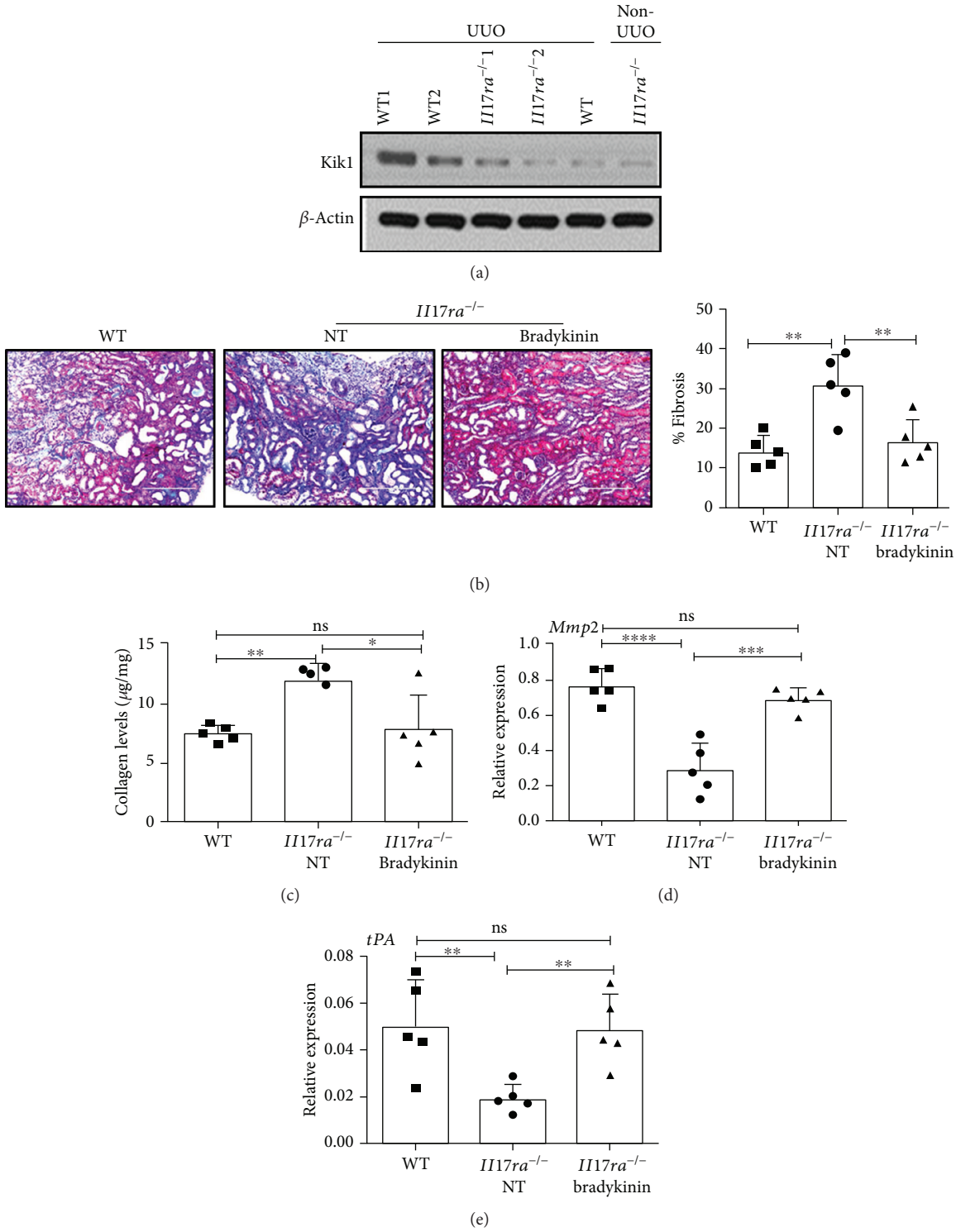


FIGURE 6: Diminished activation of KKS in the obstructed kidney of Il17ra$^{-/-}$ mice. Surgically unilateral ureteral obstruction was performed in WT and Il17ra$^{-/-}$ mice ($n = 5$). At day 7 post-surgery, (a) total kidney homogenate was assessed for Klk1 expression by immunoblot analyses. β-Actin is used as loading control. Representative immunoblot image from two independent experiments. (b) Il17ra$^{-/-}$ mice ($n = 5$) were either treated with bradykinin (300 nmol/kg/day) or left untreated (PBS injected) starting on day −1 (relative to surgery) and then daily for the next 8 days. On day 0, mice were subjected to UUO and evaluated for TF at day 7 post-surgery. WT mice ($n = 5$) were subject to UUO and left untreated. The TF development was evaluated by Masson's trichome staining of UUO kidney sections. Photomicrographs are representative of 2 independent experiments. Original magnification: 20x. (c) The total collagen content in the kidney was measured by hydroxyproline assay. The renal transcript expression of (d) Mmp2 and (e) tPA was assessed by qPCR. In the scattered plots, each dot represents individual mouse and error bars indicate mean ± SD. The data are pooled from two independent experiments for (a–e). p value ≤0.05 (*), ≤0.01 (**), ≤0.001 (***), and ≤0.0001 (****). ns: not significant.

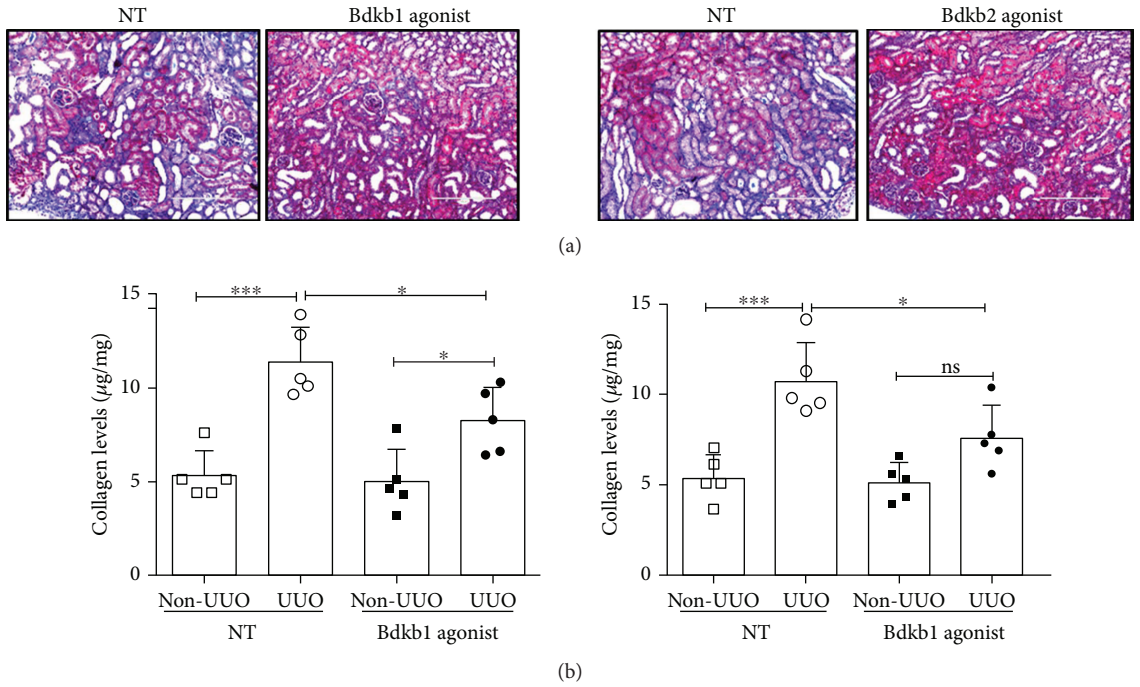


Figure 7: Activation of Bdkrb2 and Bdkrb1 which are key downstream mediators of IL-17-KKS axis-driven renal protection against TF. Groups of WT mice ($n = 5$) were either treated with selective agonist for Bdkrb1 or Bdkrb2 or left untreated (NT). Mice were evaluated for fibrosis development over a period of 7 days post-UUO surgery by (a) Masson's trichome staining and (b) measuring total collagen content in the UUO and non-UUO kidneys. Photomicrographs are representative of 2 independent experiments. Original magnification: 20x. In the scattered plots, each dot represents individual mouse and error bars indicate mean ± SD. The data are pooled from two independent experiments. p value ≤0.05 (*) and ≤0.001 (***). ns: not significant.

chronic kidney diseases [44]. While our data provide good evidence that IL-17 is needed for resistance against TF, the mechanisms that mediate this protection are poorly understood. Our new data implicate Klk1 providing an intriguing link between IL-17-mediated renal protection and activation of KKS. Treatment of TF patients with a combination of ACE inhibitor and angiotensin II receptor blockers shows limited efficacy and can be associated with persistent cough, angioedema, stenosis, birth defects, and renal failure [45]. Moreover, parenteral IL-17 as a possible therapy is likely to be associated with undesirable systemic inflammatory responses. Therefore, a comprehensive understanding of the inflammatory events in the kidney, and particularly the details of IL-17 signaling *in vivo*, is likely to be beneficial in designing new therapeutic or preventive approaches to treat TF.

Our discovery of an unrecognized connection between IL-17 and Klk1 suggests a previously unanticipated avenue for treatment of TF and is a major advance in our understanding of the function of IL-17 in the kidney. ACE inhibitors serve to increase levels of bradykinin and are routinely used to treat chronic kidney diseases [46]. Bdkrb2 agonist (lobradimil) has either been used or currently in clinical trials for treating brain tumors and HIV-infected individuals with cryptococcal meningitis (ClinicalTrials.gov: NCT00005602, NCT00019422, NCT00001502, and NCT00002316). Our data show a preclinical efficacy of treating TF with selective Bdkrb1 or Bdkrb2 agonists. Thus, exploiting IL-17-Klk1 pathways in preclinical immunotherapeutic modalities may dictate the development of new, safe, inexpensive, and rapidly implementable treatment options for TF with already available drugs.

5. Conclusions

(i) IL-17 plays an antifibrotic role in tubulointerstitial fibrosis following ureteral obstruction.

(ii) IL-17 activates the kallikrein-kinin system and facilitates the degradation of ECM proteins via upregulation of matrix-degrading enzymes such as matrix-degrading enzyme-2 and tissue plasminogen activator.

(iii) IL-17-kallikrein-kinin system axis-driven renal protection against TF is mediated by both bradykinin receptors 1 and 2.

Data Availability

All the data used to support the findings of this study are included within the article. The raw data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest exists.

Acknowledgments

This work is supported by the American Association of Immunologists Trainee/Mentor Award to KR and PSB and NIH grant (# DK104680) to PSB. RJT was supported by an American Heart Association Award 13FTF160990086 and NIH P30 DK079307. The authors thank the Unified Flow Core, Department of Immunology, University of Pittsburgh, for flow cytometry. We thank Drs. Sarah Gaffen and Mandy McGeachy for helpful suggestions and discussions.

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 3952526, 9 pages
https://doi.org/10.1155/2018/3952526

Research Article

sST2 as a New Biomarker of Chronic Kidney Disease-Induced Cardiac Remodeling: Impact on Risk Prediction

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Received 18 May 2018; Accepted 5 September 2018; Published 8 October 2018

Guest Editor: Marta Ruiz-Ortega

Heart failure is the most frequent cardiac complication of chronic kidney disease (CKD). Biomarkers help identify high-risk patients. Natriuretic peptides (BNP and NT-proBNP) are largely used for monitoring patients with cardiac failure but are highly dependent on glomerular filtration rate (GFR). Soluble suppression of tumorigenicity 2 (sST2) biomarker is well identified in risk stratification of cardiovascular (CV) events in heart failure. Furthermore, sST2 is included in a bioclinical score to stratify mortality risk. The aims of this study were to evaluate (i) the interest of circulating sST2 level in heart dysfunction and (ii) the bioclinical score (Barcelona Bio-Heart Failure risk calculator) to predict the risk of composite outcome (major adverse coronary events) and mortality in the CKD population. A retrospective study was carried out on 218 CKD patients enrolled from 2004 to 2015 at Montpellier University Hospital. sST2 was measured by ELISA (Presage ST2® kit). GFR was estimated by the CKD-EPI equation (eGFR). Indices of cardiac parameters were performed by cardiac echography. No patient had reduced ejection fraction. 112 patients had left ventricular hypertrophy, and 184 presented cardiac dysfunction, with structural, functional abnormalities or both. sST2 was independent of age and eGFR ($\rho = 0.05$, $p = 0.44$, and $\rho = -0.07$, $p = 0.3$, respectively). Regarding echocardiogram data, sST2 was correlated with left ventricular mass index ($\rho = 0.16$, $p = 0.02$), left atrial diameter ($\rho = 0.14$, $p = 0.04$), and volume index ($\rho = 0.13$, $p = 0.05$). sST2 alone did not change risk prediction of death and/or CV events compared to natriuretic peptides. Included in the Barcelona Bio-Heart Failure (BCN Bio-HF) score, sST2 added value and better stratified the risk of CV events and/or death in CKD patients ($p < 0.0001$). To conclude, sST2 was associated with cardiac remodeling independently of eGFR, unlike other cardiac biomarkers. Added to the BCN Bio-HF score, the risk stratification of death and/or CV events in nondialyzed CKD patients was highly improved.

1. Introduction

CV events and death are associated with reduced eGFR [1]. The prevalence of most comorbid conditions, including heart failure (HF), increases with decreasing eGFR [2]. Heart failure with preserved ejection fraction (HFpEF) constitutes the main feature of uremic cardiopathy and is often referred to as type 4 cardiorenal syndrome [3, 4]. In May 2016, the European Society of Cardiology developed guidelines to help diagnosis of chronic HFpEF, including cardiac structural or functional alterations underlying HF [5]. Left ventricular hypertrophy (LVH) represents the major event in type 4 cardiorenal syndrome (chronic renocardiac damage). The prevalence of LVH is estimated between 16% and 31% in CKD patients with eGFR > 30 mL/min to reach 60 to 75% before dialysis and 90% after dialysis [6]. Diastolic dysfunction, defined by pseudonormal or restrictive pattern through tissue Doppler imaging (E/e' ≥ 10) which appears in the early

stages of CKD, now emerges as an independent predictor of mortality and development of HF in a CKD patient [7].

Recent studies have identified new biomarkers involved in the pathogenesis of remodeling and cardiac fibrosis. Among them is sST2, an emerging biomarker predictive of fibrosis and cardiac remodeling in HF patients without CKD. This is a marker of interest in the stratification of patients at risk as well as in the therapeutic response of HF patients [8–11]. ST2 belongs to the family of interleukin receptors of type-1 (IL-1) and exists as membrane-bound (ST2L) and soluble (sST2) isoforms. By binding interleukin-33 (IL-33), ST2L is responsible for antihypertrophic, antifibrotic, and antiapoptotic effects [12]. sST2 is the soluble circulating form which acts as a decoy receptor, sequesters IL-33, and prevents its binding to ST2L, thereby neutralizing the beneficial effects of the ST2L/IL-33 signaling pathway [13]. sST2 is mainly secreted by cardiomyocytes when the cells are subjected to biomechanical overload. Nevertheless, the main source of sST2 secretion is still controversial, and in human cardiac disease, the vascular endothelial cells were shown to be the predominant source of sST2, rather than the human myocardium [14].

In patients with chronic HF episodes, sST2 acts as a predictor of both all-cause and cardiovascular death [15]. sST2 was included in a novel bioclinical algorithm (Barcelona Bio-Heart Failure (BCN Bio-HF) risk calculator) in association with NT-proBNP and high-sensitivity cardiac troponin T (hs-cTnT), which allowed accurate prediction of death at 1, 2, and 3 years in HF patients [16]. High levels of sST2 associated with NT-proBNP and identified risk factors improve prognosis performance independently of left ventricular ejection fraction and renal function in HF [17]. In this context, sST2 measurement can identify patients with left ventricular remodeling and decompensated hemodynamic profile [18].

To our knowledge, only few data are available regarding the prognosis value of fibrosis and myocardial remodeling biomarker in CKD patients for which the risk of a CV event constitutes the main cause of mortality. Therefore, objectives of this study were to evaluate sST2 in cardiac remodeling and to assess its role alone or in combination with other common biological parameters of HF for risk stratification of CV events or/and mortality in a nondialyzed CKD population (BCN Bio-HF score).

2. Population and Methods

2.1. Patients and Study Design. 218 patients were enrolled in the Montpellier University Hospital between 2004 and 2015. Main inclusion criteria were the ability to give informed consent, age > 18 years, cardiac echography at inclusion, and a confirmed diagnosis of CKD according to the National Kidney Foundation and KDIGO Guidelines [19, 20]. Stages of CKD were determined using eGFR calculated using the CKD-EPI equation [21]. None of the patients in stage 5 were on hemodialysis or on peritoneal dialysis. Regarding antihypertensive treatment, 16 patients had no treatment, 39 were on mono-, 71 on bi-, 70 on tri-, and 22 on quadritherapy.

At the time of enrollment, all patients had an echocardiogram performed by a trained physician. Dry and heparinized blood samples were drawn, and serum/plasma stored at −80°C for further analyses.

The follow-up of all included patients was approximatively 3 years with time-to-event analysis until the occurrence of fatal or nonfatal CV events, defined as major adverse coronary events (MACE).

Written informed consent was obtained for all patients. The protocol was approved by local authorities (Ethics Committee of Montpellier) according to standards currently applied in France (Commission Nationale de l'Informatique et des Libertés, CNIL, N°MR001). A biological collection was also registered by the French government (research ministry, # DC 2008-417 and # DC 2013-2027). The study was done in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

2.2. Cardiac Echography. Cardiac echocardiography was performed by a trained physician at inclusion. No patients presented signs of heart failure at inclusion. Subclinical cardiac dysfunction was defined as left ventricular ejection fraction (LVEF) > 40% with structural abnormality (left ventricular mass index (LVMI) ≥ 115 g/m^2 for men and ≥95 g/m^2 for women or left atrial volume index (LAVI) > 34 mL/m^2) or functional abnormality with impaired relaxation (E/A < 1) [5].

2.3. Laboratory Analyses. Biochemical parameters, including classical cardiac variables (NT-proBNP and high-sensitivity troponin T (hs-cTnT)), were performed on a Cobas 8000/e602 immunochemistry system (Roche Diagnostics, Meylan, France). C-reactive protein (CRP), urea, and IDMS traceable enzymatic creatinine were determined on a Cobas 8000/c701 (Roche Diagnostics, Meylan, France). Intact aminoterminal propeptide of type I procollagen (PINP) as a biomarker of collagen synthesis was determined by chemiluminescence technology using the IDS-iSYS Multi-Discipline automated analyser (IDS, Boldon, England).

2.4. sST2 Measurement. sST2 was measured using a sandwich ELISA kit (Presage© ST2 assay, Critical Diagnostics, San Diego, California, distributed in France by Eurobio Laboratories). In chronic HF patients, the upper reference limit for sST2 was 35 ng/mL [22]. A recombinant human sST2 standard calibrator was provided for this assay. sST2 concentrations were measured according to sST2 assay procedures and adapted on Evolis (France). Briefly, 100 μl of standard, diluted samples (1 : 20 in sample diluent) was added to the well of a ready-to-use microtiter plate coated with mouse monoclonal anti-human sST2 antibody. The standard curve was in the concentration range 2.8–100 ng/mL. Then, the plate was incubated for 60 min at room temperature. After washing, 100 μl of biotinylated antibody reagent was added into each well and incubated for 60 min at room temperature. After washing, 100 μl of streptavidin-HRP conjugated was added into each well and incubated for 30 min at room temperature. After washing, the TMB substrate was added to each well and incubated for 20 min at room temperature in the dark. Then, stop solution was added and absorbance was read at 450 nm.

2.5. Barcelona Bio-Heart Failure Score. The Barcelona Bio-Heart Failure risk calculator (BCN Bio-HF calculator) estimates the risk of death in patients with HF described by Lupón et al. [16]. The BCN Bio-HF calculator is an algorithm based on eight independent models, depending on available data. It is derived from a real-life cohort and includes, in addition to classical prediction factors, serum NT-proBNP, hs-cTnT, and sST2 reflecting different pathophysiological pathways. The models account for clinical and biological characteristics and treatments to predict the risk of mortality at 1, 2, and 3 years. Pharmacological treatments include beta-blockers, ARBs/ACEI, statins, and furosemide. In our study, all clinical and biomarker variable models were taken into account (hs-cTnT, NT-proBNP, and sST2). Using this model, prognostic indices were computed for each patient in our population.

2.6. Statistical Analysis. Descriptive statistics are presented as numbers (percentages) for categorical data and as medians (interquartile range (IQR)) for continuous variables. χ^2 test was performed to investigate the presence of differences between proportions. The Mann-Whitney U test and Kruskal-Wallis test were used to compare groups, as appropriate. Since their distributions were skewed, logarithmic transformations of sST2, troponin, NT-proBNP, and PINP biomarkers were used. For correlation analyses, Spearman's rank correlation coefficients were computed. Composite outcome was defined as any of the following events (MACE) during follow-up: death, myocardial infarction, ischemic cardiomyopathy, angioplasty, valvular cardiomyopathy, stroke, vascular angioplasty, or cardiac arrhythmias. The Kaplan-Meier estimator of event-free survival was used to assess the ability of biomarkers to predict adverse outcome in the population. Potential predictors of composite outcome were further evaluated using the Cox proportional hazard regression. The net reclassification improvement (NRI) was used to assess the incremental value by adding a biomarker over the BCN Bio-HF score. No treatment adjustment in relation to cardiac biomarkers and kidney dysfunction was assessed in the BCN Bio-HF score analysis since they were already included in the initial score calculation.

3. Results

3.1. Population Characteristics. This study included a total of 218 patients at different stages of CKD (i.e., 36 patients at stages 1–2, 42 patients at stage 3A, 57 patients at stage 3B, 62 patients at stage 4, and 21 patients at stage 5). Median eGFR level was 37 mL/min/1.73 m^2 (IQR 23–52). During the follow-up period, 85 out of 218 patients presented composite outcome. Demographic data, laboratory findings, and echocardiogram parameters in the global population presenting or not presenting composite outcome are shown in Table 1. Among the population, the median LVEF was 62%, and none of the patients had reduced LVEF. 112 (51%) patients presented LVH and 184 (84%) a cardiac dysfunction. Cardiac dysfunction was as follows: 45 patients with structure abnormality only, 72 with function abnormality only, and 67 with both. Median follow-up was 3.0 years (IQR 1.3–6.4) after initial evaluation. Compared to patients free of major adverse coronary events (MACE), eGFR and LVEF were lower in patients with MACE, whereas age, LAVI, left atrial diameter (LAD), LVMI, levels of CRP, NT-proBNP, and hs-cTnT were higher. No significant difference in PINP levels was observed between groups. Median sST2 was 29.5 ng/mL (IQR 22.6–35.1), and 55 out of 218 patients (25%) had an elevated level of sST2 (upper reference limit is 35 ng/mL in chronic HF).

3.2. sST2 Is Associated with Cardiac Remodeling Feature. Correlation between sST2 and inflammatory and cardiac biomarkers (CRP, NT-proBNP, and hs-cTnT) was significant ($\rho = 0.17$, $p = 0.01$; $\rho = 0.14$, $p = 0.03$; and $\rho = 0.15$, $p = 0.03$, respectively).

Regarding echocardiogram data, sST2 was correlated with LVEF ($\rho = -0.14$, $p = 0.04$) and cardiac remodeling (i.e., LAD ($\rho = 0.14$, $p = 0.04$), LAVI ($\rho = 0.13$, $p = 0.05$), and LVMI ($\rho = 0.16$, $p = 0.02$)). No association with functional abnormality was observed (E/A ($\rho = 0.08$, $p = 0.26$)). PINP was not correlated with any echocardiographic data (Figure 1). Other cardiac biomarkers (hs-cTnt and NT-proBNP) were correlated with structural abnormality parameters. Concerning patient treatments, no correlation was observed between sST2 and beta-blockers, ARBs/ACEI, statins, or furosemide (data not shown).

3.3. sST2 Is Independent of GFR and Age. No correlation between sST2 and both age ($\rho = 0.05$, $p = 0.44$) or eGFR ($\rho = -0.07$, $p = 0.3$) was observed, in contrast to classical cardiac biomarkers such as hs-cTnT ($\rho = 0.55$, $p < 0.001$, and $\rho = -0.59$, $p < 0.001$, respectively) and NT-proBNP ($\rho = 0.51$, $p < 0.001$, and $\rho = -0.56$, $p < 0.001$, respectively). Moreover, no relationship was observed between sST2 and CKD stages ($p = 0.9$) whereas NT-proBNP and hs-cTnT values increased from stages 1 to 5 ($p < 0.001$ for both parameters) (Figure 2).

3.4. Predictors of Outcome. During the 3 years of median follow-up (IQR 1.3–6.4), 85 (39%) patients experienced the composite outcome of death and/or CV events.

In univariate Cox analysis, older age (HR 1.052 (1.031–1.072)), male gender (female HR 0.420 (0.251–0.704)), increased LAVI (HR 1.050 (1.015–1.086)) and LAD (HR 1.074 (1.030–1.119)), elevated hs-cTnT (HR 5.152 (2.659–9.983)), NT-proBNP (HR = 1.650 (1.116–2.439)), and CRP (HR 2.155 (1.388–3.346)) were related to composite outcome (Table 2).

3.5. Multimarker Strategy Based on Barcelona Bio-HF Score. The Barcelona Bio-HF score was applied to assess the predictive composite outcome in our cohort. Taken together, NT-proBNP, hs-cTnT, and sST2 were highly predictive of the composite outcome of cardiovascular events and/or death ($p < 0.0001$) (Figure 3). A combination of CRP with the Barcelona Bio-HF score was performed in order to identify a high-risk subgroup and to improve the risk of CV events or death composite outcome. The risk classification analysis including the CRP level did not allow

Table 1: Baseline characteristics of all patients, with and without MACE.

Variable	Study population ($n = 218$)	No MACE ($n = 133$)	MACE ($n = 85$)	p
Age (years)	68.31 [57.62–75.47]	63.35 [49.18–71.69]	71.75 [67.44–79.37]	<0.001
Gender				0.005
Male	139 (64%)	75 (56%)	64 (75%)	
Female	79 (36%)	58 (44%)	21 (25%)	
Follow-up (years)	3.0 [1.3–6.4]	3.0 [1.3–6.5]	2.8 [1.4-5.8]	0.869
eGFR (mL/min/1.73 m^2)	37 [23–52]	40 [26–57]	35 [22–44]	0.014
SBP (mmHg)	134 [120–146]	135 [122–146]	130 [120–146]	0.214
DBP (mmHg)	73 [69–80]	75 [70–80]	70 [65–80]	0.051
Echocardiography				
LVEF (%)	62 [58–65]	65 [60–67]	60 [54–64]	<0.001
E/A	0.85 [0.70–1.11]	0.87 [0.73–1.12]	0.8 [0.69–1.11]	0.204
LAVI (mL/m^2)	11.4 [7.6–14.9]	10.4 [7.0–14.1]	13.3 [9.1–16.3]	0.001
LAD (mm)	34 [30–37]	33 [29–36]	36 [32–39]	<0.001
LVMI (g/m^2)	109.9 [85.3–130.5]	106.8 [79.0–125.0]	118.0 [97.2–139.3]	0.004
Biomarkers				
NT-proBNP (ng/L)	182.5 [75.0–445.3]	129.0 [61.0–379.0]	287.0 [121.2–623.5]	<0.001
sST2 (ng/mL)	29.5 [22.6–35.1]	28.2 [21.7–34.3]	30.5 [24.3–36.7]	0.100
PINP (ng/mL)	52.2 [38.2–77.5]	51.95 [38.2–77.2]	55.3 [38.4–77.5]	0.522
hs-cTnT (ng/L)	14.3 [7.7–24.9]	11.7 [6.2–19.5]	19.1 [12.4–34.3]	<0.001
CRP (mg/L)	2.2 [1.1–4.7]	1.8 [0.8–3.6]	3.2 [1.6–6.9]	<0.001
Na (mmol/L)	141 [139–142]	141 [139–142]	140 [139–142]	0.829
Hb (g/dL)	13.2 [12.3–14.2]	13.4 [12.2–14.4]	13.0 [12.4–14.0]	0.702
Treatments				
Beta-blockers				0.054
No	118 (54.1%)	79 (59.4%)	39 (45.9%)	
Yes	100 (45.9%)	54 (40.6%)	46 (54.1%)	
ARBs/ACEI				0.274
No	55 (25.2%)	30 (22.6%)	25 (29.4%)	
Yes	163 (74.8%)	103 (77.4%)	60 (70.6%)	
Statins				0.061
No	120 (55.0%)	80 (60.2%)	40 (47.1%)	
Yes	98 (45.0%)	53 (39.8%)	45 (52.9%)	
Furosemide				0.001
No	100 (45.9%)	73 (54.9%)	27 (31.8%)	
Yes	118 (54.1%)	60 (45.1%)	58 (68.2%)	

Data presented as median [1st quartile–3rd quartile] for quantitative variables and proportions for categorical variables. ACEI: angiotensin converting enzyme inhibitors, ARBs: angiotensin receptor blockers, DBP: diastolic blood pressure, eGFR: estimated glomerular filtration rate, LAD: left atrial diameter, LAVI: left atrial volume index, LVEF: left ventricular ejection fraction, LVMI: left ventricular mass index, MACE: major adverse coronary events, SBP: systolic blood pressure. p value was determined by χ^2 and Mann-Whitney U tests.

better patient classification (continuous NRI = 16% (−11.7–32.8%), $p = 0.27$).

4. Discussion

This study shows that the sST2 level is associated with cardiac remodeling features, and unlike common cardiac biomarkers, this biomarker is independent of eGFR and age. A multimarker approach including sST2 is thus reported as an appealing tool in CV risk stratification of nondialyzed CKD patients.

4.1. sST2 and Heart Dysfunction in CKD Patients. HFpEF is associated with increased cardiac remodeling, abnormal cardiac mechanics, and poor outcomes in CKD patients [1, 23]. Brain natriuretic peptides and hs-cTnT can facilitate the diagnosis of HF among patients with CKD. Elevation of these biomarkers is related to cardiac modifications contributing to HF [24]. Vickery et al. reported that eGFR and cardiac dysfunction have independent effects on brain natriuretic peptide concentrations in CKD patients [25]. A reduced renal excretion provokes elevated levels of cardiac troponins, NT-proBNP, and in a lesser extent BNP limiting the utility

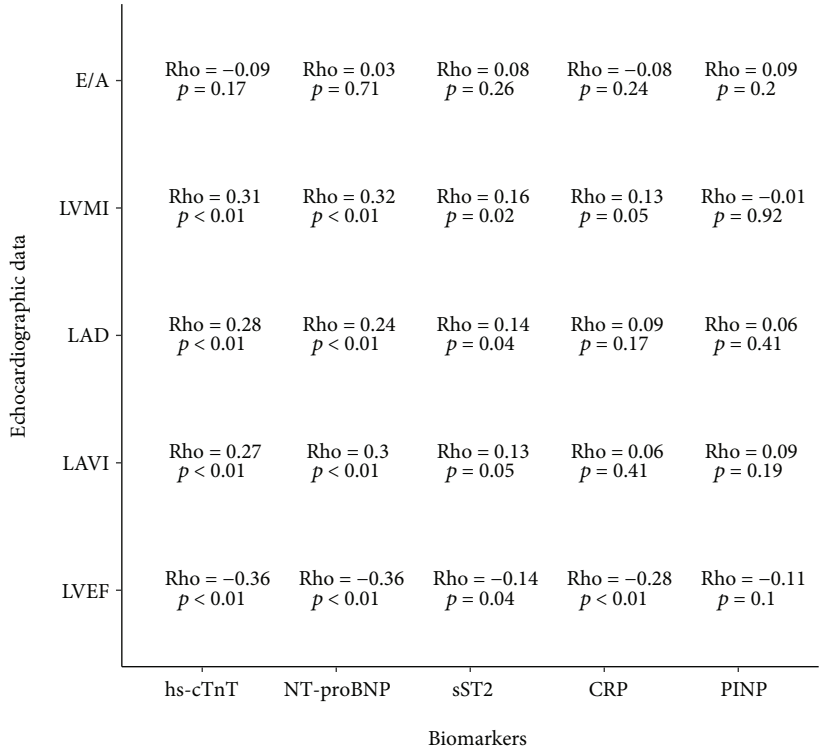

Figure 1: Correlation analysis between echocardiogram data, variables, and biomarkers.

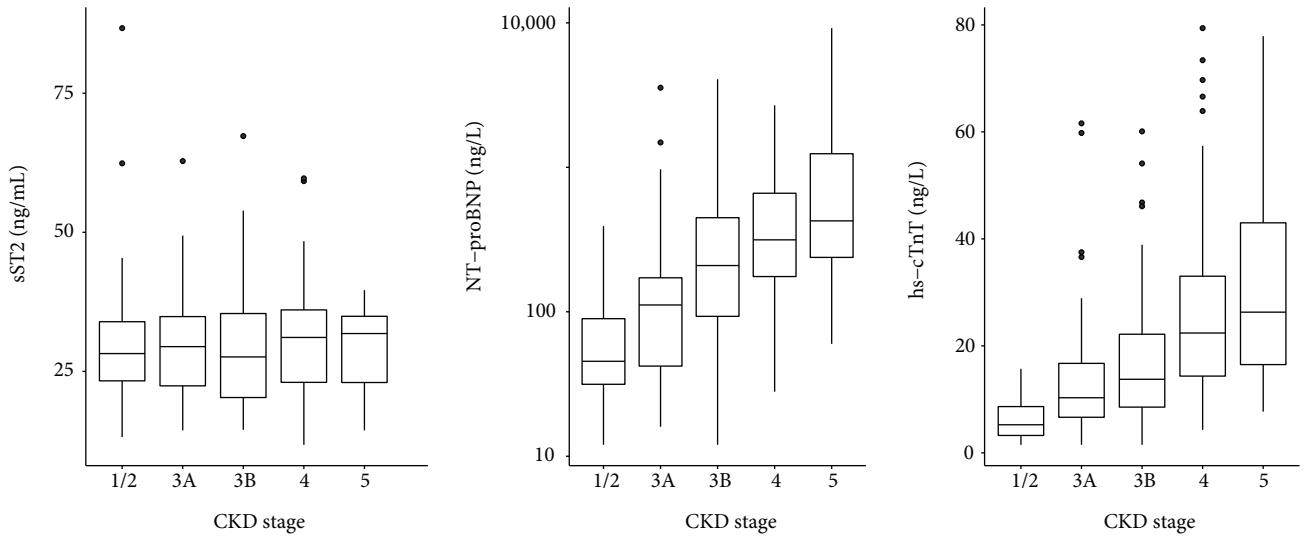

Figure 2: sST2, NT-proBNP, and hs-cTnT levels according to CKD stages (Kruskal-Wallis test: $p = 0.9$, $p < 0.001$, and $p < 0.001$, respectively).

of these markers although higher brain natriuretic peptide levels remain predictive of increased mortality in CKD [26–28]. sST2 is an interesting biomarker, and most studies have described sST2 as an independent marker of renal function and hemodialysis [29–31]. We confirmed here that sST2 is not correlated with eGFR, unlike NT-proBNP, and no difference was observed among CKD stages. Therefore, the weak correlation observed between NT-proBNP and sST2 ($\rho = 0.14$, $p = 0.03$) can be explained by renal dysfunction as a confusing factor (Figure 2). In a study conducted by Bao et al., sST2 levels were higher in CKD patients compared to healthy controls and were correlated with disease severity [32]. More recently, Gungor et al. observed that sST2 levels increased with CKD stages [33]. Indeed, these investigations did not evaluate cardiac function, and sST2 elevation was reported to be involved in the inflammatory state. Moreover, sST2 measurements are not directly comparable because ELISA kits with different standards or antibodies were used.

Table 2: Univariate Cox analysis predictive of cardiovascular events and/or death composite outcome.

Variable	HR [95% CI]	p
Age (years)	1.052 [1.031–1.072]	<0.001
Female	0.420 [0.251–0.704]	0.001
SBP (mmHg)	0.992 [0.981–1.004]	0.209
DBP (mmHg)	0.986 [0.966–1.006]	0.167
LAVI (mL/m^2)	1.050 [1.015–1.086]	0.005
LAD (mm)	1.074 [1.030–1.119]	0.001
LVMI (g/m^2)	1.004 [0.998–1.009]	0.183
E/A	1.010 [0.607–1.681]	0.969
Log hs-cTnT (ng/L)	5.152 [2.659–9.983]	<0.001
Log NT-proBNP (ng/L)	1.650 [1.116–2.439]	0.012
Log CRP (mg/L)	2.155 [1.388–3.346]	0.001
Log sST2 (ng/mL)	2.836 [0.532–15.134]	0.222
Log PINP (ng/mL)	0.836 [0.340–2.057]	0.696
eGFR (mL/min/1.73 m^2)	0.992 [0.981–1.003]	0.172
Na (mmol/L)	0.985 [0.908–1.069]	0.720
Hb (g/dL)	0.991 [0.852–1.153]	0.909

DBP: diastolic blood pressure, eGFR: estimated glomerular filtration rate, Hb: hemoglobin, LAD: left atrial diameter, LAVI: left atrial volume index, LVMI: left ventricular mass index, SBP: systolic blood pressure.

Lastly, in both studies, GFR was estimated through the Modification of Diet in Renal Disease Equation, and this could lead to different GFR values depending on the estimation method used.

sST2 is involved in pathophysiology of cardiac fibrosis, and its increase is considered as an indirect circulating marker of cumulative fibrotic processes [34]. Regarding echocardiogram results, sST2 correlates significantly with variables that describe structural alterations, and no correlation with functional abnormalities was observed. We can thus speculate that in CKD patients, sST2 by decreasing the availability of IL-33 may be involved in cardiac remodeling features typically observed in HFpEF. Although other cardiac biomarkers showed better correlation with cardiac dysfunction, sST2 is quite relevant because it does not depend on age and renal function. These results represent an important step in early detection of cardiac performance alteration in CKD. Finally, no correlation between PINP and echocardiographic data was observed. This could be explained by the lack of specificity of this marker in cardiac fibrosis [35].

4.2. sST2 and Prognosis Value. To our knowledge, this is the first time we observe that a multimarker strategy including combined sST2, NT-proBNP, and hs-cTnT biomarkers is highly associated with cardiovascular events and/or mortality and suitable in nondialyzed CKD patient risk stratification.

sST2 represents a promising biomarker in prognosis mortality and CV events in chronic HF [36, 37]. Recently, an update of ACC/AHA guidelines stated that the use of myocardial fibrosis biomarkers such as sST2 might be considered for predicting risk of hospitalization and death in patients with chronic HF and potentially added to natriuretic peptide biomarker levels in their prognostic value [34]. Our study confirms that hs-cTnT, CRP, and to a lesser extent NT-proBNP alone are predictive of poor outcome, as described in a hemodialysis population [38]. Yet, sST2 alone does not allow CV events or death composite outcome prognosis in nondialyzed CKD patients. Our findings are in line with Keddis et al., who found that sST2 level did not change CV risk prediction compared to cardiac troponin T in patients considered for kidney transplant [39].

A multimarker strategy approach was developed and proven to be more informative than a single biomarker in HF prognosis. To date, only few clinical scores evaluating risk stratification of HF have been developed. The BCN Bio-HF score is a unique tool combining a panel of biomarkers and clinical variables [40]. Taken together, biomarkers provide information about myocyte necrosis (hs-cTnT), fibrosis and inflammation (sST2), and chamber strain (NT-proBNP). In our study, NT-proBNP, hs-cTnT, and sST2 in combination with clinical variables and treatments were highly predictive of composite outcome. We showed that the BCN Bio-HF score can also be applied to the CKD population and is highly predictive of CV events and mortality. Beside, adding CRP to the BCN Bio-HF score did not provide better reclassification of CKD patients, as observed for HF [41]. Although CRP is considered as a marker of early inflammation and a low level of hs-CRP may be associated with a more favorable prognosis in patients with coronary heart disease [42], its level remains stable in CKD before dialysis and unlikely plays a major role in subacute inflammation [43]. Our results suggest that CRP does not improve the prognosis of patients at nondialyzed CKD stages, presenting mainly a subclinical cardiac dysfunction. In this population, a multimarker strategy such as the BCN Bio-HF score is definitely helpful to better stratify risk of death and CV events. Further longitudinal studies in CKD patients are needed to better characterize the interest of these combined markers involved in HF pathophysiology.

5. Limitations

This study presents several limitations. First of all, tissue Doppler imaging was not taken into account at the time of inclusion; consequently, the e' wave was not available. Then, a relatively small cohort of patients from a single medical center was enrolled. We only measured biomarkers at time of recruitment and did not evaluate the long-term trends of biomarkers, which could be useful for patient follow-up. Moreover, sST2 assay is not available in all laboratories, which reduces the use of the multimarker strategy.

6. Conclusion

To our knowledge, this is the first study showing that sST2 is a good biomarker to evaluate the cardiac remodeling feature in nondialyzed CKD patients. Although sST2 alone is not predictive of CV events and death in this population, it plays an important role to stratify risk of

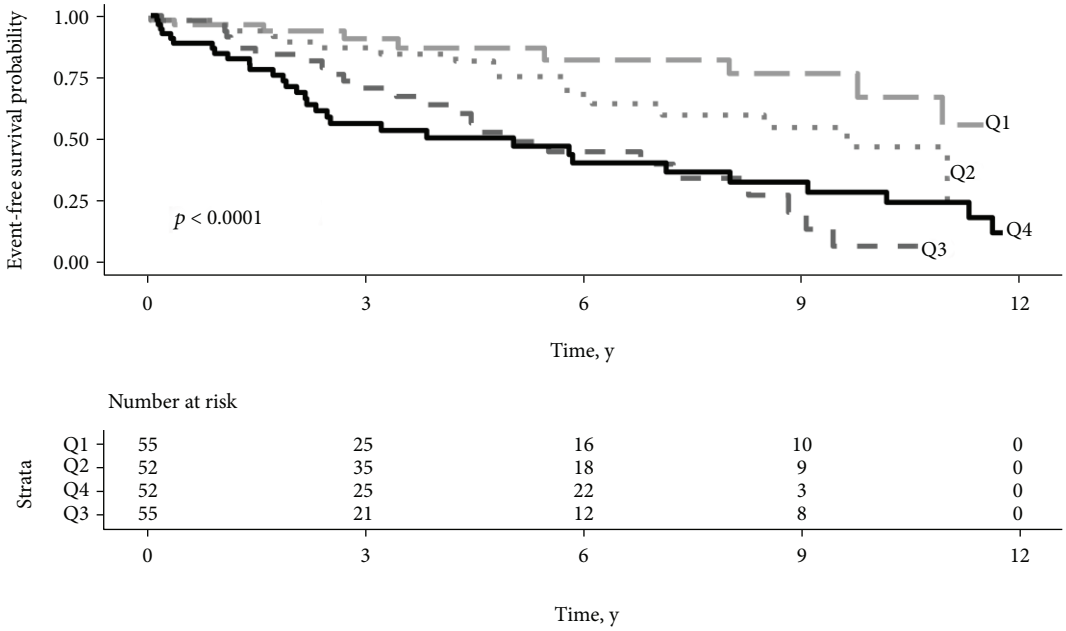

Figure 3: Barcelona Bio-HF score and composite outcome prediction. Event-free survival probability according to mortality risk predicted by the Barcelona Bio-Heart Failure score. Study population was stratified by quartiles of 1-year mortality risk (Q1: risk < 1.56%, Q2: 1.56 ≤ risk < 3.45%, Q3: 3.45 ≤ risk < 6.68%, and Q4: risk > 6.68%). p value refers to the log-rank test.

all-cause mortality and CV events in a multimarker strategy combined with clinical variables.

Data Availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Disclosure

Part of the study was presented at the meeting "Printemps de la Cardiologie 2018" and published as an abstract in the Archives of Cardiovascular Diseases Supplements 2018, 10-2:202.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This work was supported by a grant from the Ministère de la Santé (PHRC-UF 9763).

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 7659243, 12 pages
https://doi.org/10.1155/2018/7659243

Research Article

Markers of Glomerular and Tubular Damage in the Early Stage of Kidney Disease in Type 2 Diabetic Patients

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Received 13 February 2018; Revised 25 June 2018; Accepted 10 July 2018; Published 9 August 2018

Academic Editor: Sandra Rayego-Mateos

Diabetic kidney disease develops in half of genetically predisposed patients with type 2 diabetes (T2DM). Early diagnosis of kidney damage and nephroprotective treatment are the ways of preventing the disease progression. Our aim was to evaluate selected laboratory markers of glomerular and tubular damage in T2DM patients with early stages of chronic kidney disease (G1/G2, A1/A2) for their associations with A2 albuminuria and early decline in the estimated glomerular filtration rate (eGFR). Among 80 T2DM patients with median eGFR of 92.4 ml/min/1.73 m^2 and median urinary albumin to creatinine ratio (uACR) of 4.69 mg/g, 19 had uACR > 30 mg/g (A2). Higher serum cystatin C, serum and urine neutrophil gelatinase associated lipocalin (NGAL), urine kidney injury molecule 1 (KIM-1), detectable urine transferrin and IgG, and lower serum uromodulin significantly predicted A2 albuminuria, urine KIM-1/creatinine ratio, and IgG being the best predictors. Albuminuria, urine NGAL/creatinine, and IgG correlated with diabetes duration. Albuminuria, urine NGAL, transferrin, IgG, and uromodulin correlated with diabetes control. In a subgroup of 29 patients, retrospective data were available on changes in eGFR and uACR over one year. Decline in eGFR was observed in 17 patients and increase in uACR in 10 patients. Serum and urine NGAL correlated with eGFR changes. Higher urine NGAL, KIM-1/creatinine ratio, and detectable IgG were significantly associated with the increase in uACR. Widely available markers, serum cystatin C, urine IgG, transferrin, and NGAL, may help in early assessment of kidney disease in T2DM patients; however, large prospective studies are needed to confirm the conclusion.

1. Introduction

Diabetes is the most prevalent metabolic disease worldwide, and its complications are among the most important public health issues [1]. About 30–40% of diabetic patients, especially the genetically predisposed ones, develop diabetic kidney disease (DKD), which makes it the most frequent cause of end-stage kidney disease and renal replacement therapy [2, 3].

Early diagnosis and early initiation of nephroprotective therapy have the potential to prevent the progression of DKD toward end-stage renal disease and to improve patients' prognosis. Based on the guidelines issued by Kidney Disease Outcomes Quality Initiative (KDOQI) in 2007 [4], the

repeated assessment of urine albumin/creatinine ratio (uACR) in two to three samples of morning urine together with the estimation of glomerular filtration rate (eGFR) has been recognized as the best standard screening for DKD. However, currently, it is well known that kidney damage in course of type 2 diabetes mellitus (T2DM) may occur without increased albuminuria [5]. Moreover, decrease in eGFR is not an early indicator of diabetic renal damage. Therefore, in some patients, albuminuria and eGFR are not sensitive markers of early DKD. Diagnostic imaging produces nonspecific results in DKD patients, and the thick needle biopsy is very rarely used due to invasiveness and the lack of strict clinical indications in the early stage of DKD, usually characterized by very few symptoms. Thus, there is a need to seek new biomarkers of early renal damage in patients with T2DM.

Morphological changes observed in kidneys in the course of DKD affect almost all nephron structures: glycocalyx and glomerular endothelial cells, glomerular basement membrane, podocytes and slit membranes, mesangial matrix, renal interstitium, and renal tubules [6]. To properly qualify the potential markers of renal damage in T2DM, it is useful to classify them according to the renal structure affected by the pathological process [7]. On this basis, we distinguish markers of glomerular damage including transferrin, immunoglobulin G (IgG), ceruloplasmin, type IV collagen, laminin, glycosaminoglycans, lipocalin-type prostaglandin D synthase, fibronectin, podocalyxin, vascular endothelial growth factor, cystatin C (CysC), or nephrin and markers of tubular damage such as neutrophil gelatinase-associated lipocalin (NGAL), alpha-1-microglobulin, kidney injury molecule-1 (KIM-1), N-acetyl-beta-D-glucosaminidase, angiotensinogen, uromodulin, liver-type fatty acid-binding protein (L-FABP), heart-type fatty acid-binding protein (H-FABP), the products of advanced glycation, or inflammatory markers [8]. Some biomarkers, including albumin, are filtered in the glomeruli and then reabsorbed in the proximal tubule of the nephron; increased urine excretion of such markers may indicate damage of both glomerular and tubular structures [7].

The aim of the study was the assessment of selected laboratory markers of glomerular and tubular damage in serum and urine of T2DM patients without significant decrease in the glomerular filtration rate and without significantly increased albuminuria. We evaluated the studied biomarkers as the predictors of moderately increased albuminuria and evaluated their associations with early decline of kidney function in T2DM.

2. Materials and Methods

2.1. Study Group. The cross-sectional study recruited consecutive patients with T2DM consulted at the ambulatory clinic of Nephrology Department of Clinical District Hospital No. 2 in Rzeszów, Poland, between October 2014 and November 2015. The study visit was a part of a standard care and was either arranged to assess kidney function in newly diagnosed T2DM patients or was a control visit of patients with single benign renal cysts or following treatment of urinary tract infection. A subgroup of patients attended a control visit as a part of longitudinal observation of changes in eGFR and urine albumin/creatinine ratio (uACR) [9]. Inclusion criteria were T2DM diagnosis, eGFR > 60 ml/min/1.73 m^2, and uACR < 300 mg/g. We excluded patients diagnosed with anemia, neoplasm, connective tissue disease, infection, allergy, treated with potentially nephrotoxic drugs, and with known renal disease other than DKD. Moreover, patients with poorly controlled hypertension, decompensated heart failure, urinary tract infection, after increased physical activity, and women during menstruation as well as pregnant women were excluded to avoid nonspecific albuminuria.

Patients were subjected to detailed medical examination. Patient's history was collected focusing on the presence of comorbidities, their duration, and treatment. Weight, height, and blood pressure were measured; body mass index (BMI) was calculated, and laboratory tests were ordered. According to 2012 KDOQI guidelines [10], kidney impairment was assessed based on uACR and eGFR estimated using 2009 Chronic Kidney Disease Epidemiology Collaboration formula based on serum creatinine (CKD-EPI_{Cr}). Moderately increased albuminuria (uACR between 30 and 300 mg/g or A2 albuminuria category) was considered the objective evidence of kidney disease, according to the definition of chronic kidney disease issued by Kidney Disease: Improving Global Outcomes (KDIGO) [11].

In a subgroup of patients, retrospective data were available on eGFR values and uACR changes over one-year observation. These patients were seen a year and 6 months before the study visit. During the previous visits, patients were instructed to maintain physical activity and diabetic diet. The doses of insulin or oral hypoglycemic drugs were adjusted to maintain good glycemic control. Renin-angiotensin-aldosterone system inhibitors were used as antihypertensive drugs, and statins were used in treatment of dyslipidemia if not contraindicated [12]. We assumed the decrease in eGFR based on serum creatinine and the increase in urine albumin/creatinine ratio after one year of observation as the indicators of kidney function decline [13, 14].

All patients provided informed consent for the study. The study protocol was approved by the Bioethics Committee of the Regional Medical Chamber in Rzeszów, Poland (approval number 70/2017/B issued on 19th September 2014).

2.2. Laboratory Tests. First morning urine and fasting blood sample were collected for laboratory measurements at the day of the study visit. The laboratory tests included routine tests used for the assessment of the patients' health status, and a set of additional tests was performed for the purpose of this study.

The routine tests included fasting serum glucose, glycated hemoglobin A_{1c} (HbA_{1c}), complete blood count, lipid profile, serum C-reactive protein (CRP), and creatinine. GFR was estimated based on serum creatinine according to CKD-EPI_{Cr} formula [8]. Urinalysis with urine sediment analysis was performed using the first morning urine sample to exclude urinary tract infection. First morning urine samples

were also tested for the concentration of NGAL, albumin, and creatinine, and then, uACR and urine NGAL/creatinine ratios (uNGAL/Cr) were calculated. The measurements of uNGAL were conducted using chemiluminescent microparticle immunoassay and Architect analyzer (Abbott Diagnostics, Lake Forest, IL, USA). Urine albumin concentrations were measured using an immunoturbidimetric method, and creatinine concentration was assessed using an enzymatic method on Olympus/Beckman Coulter Chemistry Analyzer AU680 (Beckman Coulter, Brea, USA). Peripheral blood counts were performed using a hematology analyzer ADVIA 2120i (Siemens Healthcare, Erlangen, Germany). Urinalysis was performed using LabUMat-UriSed 2 analyzer. The routine tests and uNGAL measurements were performed on the day of samples' collection in the Department of Diagnostics of St. Queen Jadwiga Clinical District Hospital, Rzeszów, Poland.

The remaining serum and urine were aliquoted and frozen in −80°C until the complete set of samples was collected. The tests were performed in series to avoid the cycles of repeated freezing/defreezing. The preserved material was used to determine the concentrations of transferrin, IgG, KIM-1, and uromodulin (uUMOD) in urine and NGAL, CysC, and uromodulin (sUMOD) in serum. Transferrin, IgG, and CysC were measured using an immunonephelometric method on a Nephelometer II analyzer (Siemens Healthcare, Erlangen, Germany) at the Diagnostics Department, University Hospital, Kraków, Poland. The upper reference limits were, respectively, 2.17 mg/l for urine transferrin and 3.36 mg/l for urine IgG. The reference range for serum cystatin C was 0.59–1.04 mg/l. The concentrations of sUMOD, uUMOD, serum NGAL, and urine KIM-1 were determined using commercially available enzyme-linked immunosorbent assays with a Human Uromodulin ELISA kit (BioVendor, Brno, Czech Republic), a Human Lipocalin-2/NGAL ELISA kit (BioVendor, Brno, Czech Republic), and Quantikine ELISA Human TIM-1/KIM-1/HAVCR Immunoassay (R&D Systems, McKinley Place, MN, USA), respectively. The readings were done with an automatic microplate reader Automatic Micro ELISA Reader ELX 808 (BioTek Instruments Inc., Winooski, VT, USA). The reference range for sUMOD determined by the manufacturer of the kit was 37.0–501.0 ng/ml (mean 241 ng/ml); the limit of detection was 0.12 ng/ml. The reference range for urine KIM-1 values determined by the manufacturer of the kit was 0.156–5.33 ng/ml, and mean detectable concentration was 0.009 ng/ml. The limit of detection of NGAL in serum was 0.02 ng/ml, and the reference values were 63.5 ± 33.4 for men and 64.9 ± 46.5 ng/ml for women. Uromodulin, serum NGAL, and KIM-1 measurements were performed in the Department of Diagnostics, Chair of Clinical Biochemistry, Jagiellonian University Medical College, Kraków, Poland.

2.3. Statistical Analysis. Data were presented as number of patients (percentage of the group) for categories, mean ± standard deviation or median (lower-upper quartile) for quantitative variables with normal and nonnormal distributions (as checked using Shapiro-Wilk's test). Spearman's rank correlation coefficient was used to study correlations. Differences between groups were tested with t-test and Mann–Whitney's test, according to distributions. Simple and multiple logistic regressions were used to assess predictors of G1/G2 A2 CKD. Odds ratios (OR) with 95% confidence intervals (95% CI) were reported as results of logistic regression. The variables that proved significant predictors in simple logistic regression were studied further using receiver operating characteristic (ROC) curves' analysis to assess the diagnostic accuracy. Cut-off values were chosen by maximizing the Youden index. The statistical tests were two-tailed, and the results were considered significant at $p < 0.05$. Statistica 12.0 (StatSoft, Tulsa, USA) was used for computations.

3. Results

3.1. Characteristics of Patients. The study recruited 80 patients with T2DM with median eGFR (CKD-EPI$_{Cr}$) of 92.4 ml/min/1.73 m^2 (79.7–101.0 ml/min/1.73 m^2) and median uACR of 4.69 mg/g (2.86–19.17 mg/g). The patients presented with typical comorbidities, including hypertension in (67) 84%, ischemic heart disease in 10 (12%), and heart failure in 6 (7%). In most patients, diabetes was well controlled: median HbA$_{1c}$ concentrations equaled 6.4% (5.9–7.9%). The treatment of diabetes included metformin in 74 patients (93%). Insulin therapy was necessary in 25 patients (31%).

Most patients ($N = 61$; 77%) had normal to mildly increased albuminuria (uACR < 30 mg/g). Moderately increased albuminuria (uACR between 30 and 300 mg/g) was observed in 19 patients (23%) (Table 1). On average, these patients were older and had longer known duration of diabetes, as well as higher HbA$_{1c}$ (Table 1). Ischemic heart disease was more commonly associated with moderately increased albuminuria; however, no significant differences regarding other comorbidities nor treatment modalities were observed between patients with normal and moderately increased albuminuria (Table 1). Moreover, white blood cell counts and C-reactive protein concentrations were higher among patients with uACR between 30 and 300 mg/g (Table 1).

3.2. The Associations of Studied Markers with Moderately Increased Albuminuria. Serum cystatin C, serum NGAL, urine NGAL and NGAL/creatinine ratios, urine KIM-1 and KIM-1/creatinine ratio, urine transferrin, and urine IgG were significantly higher among patients with moderately increased albuminuria while serum uromodulin was significantly lower in this group of patients (Table 1). High serum cystatin C, serum NGAL, urine NGAL and NGAL/creatinine ratio, urine KIM-1 and KIM-1/creatinine ratio, detectable urine transferrin, and urine IgG as well as low serum uromodulin were also significant predictors of moderately increased albuminuria in simple logistic regression (Table 2). Most of these predictors (except for serum concentrations of cystatin C, NGAL, and uromodulin) were independent of age, diabetes duration, the presence of hypertension, heart failure, and the treatment with

Table 1: Clinical characteristics and the results of laboratory tests of T2DM patients with eGFR > 60 ml/min/1.73 m^2 according to albuminuria categories.

Characteristic	Normal to mildly increased albuminuria: uACR < 30 mg/g ($N = 61$)	Moderately increased albuminuria: uACR 30–300 mg/g ($N = 19$)	p value
Age, years	59 ± 11	67 ± 12	0.007#
Male sex, N (%)	32 (52)	6 (32)	0.1
Known diabetes duration, years	5 (2–10)	10 (6–15)	0.009#
Hypertension, N (%)	50 (82)	17 (89)	0.4
Ischemic heart disease, N (%)	5 (8)	5 (26)	0.037#
Heart failure, N (%)	3 (5)	3 (16)	0.1
BMI, kg/m^2	31.5 ± 5.0	31.9 ± 7.5	0.8
Insulin treatment, N (%)	17 (28)	8 (42)	0.2
Statin use, N (%)	27 (44)	13 (68)	0.07
ACEI/ARB use, N (%)	40 (66)	13 (68)	0.8
Hemoglobin, g/dl	14.0 ± 1.4	13.2 ± 1.7	0.045#
White blood cell count, $\times 10^3/\mu$l	7.20 (6.20–8.33)	8.51 (6.65–10.30)	0.039#
Fasting glucose, mmol/l	6.95 ± 0.84	7.25 ± 0.70	0.2
HbA_{1c}, %	6.30 (5.90–7.80)	7.35 (6.30–8.40)	0.049#
Total cholesterol, mmol/l	4.68 (3.85–5.74)	4.84 (3.98–5.72)	0.8
HDL-cholesterol, mmol/l	1.19 (1.06–1.42)	1.22 (1.01–1.40)	0.7
LDL-cholesterol, mmol/l	2.66 (1.86–3.49)	2.49 (1.94–3.54)	0.8
Triglycerides, mmol/l	1.53 (1.15–2.03)	1.83 (1.40–2.80)	0.07
C-reactive protein, mg/l	2.80 (1.30–5.80)	6.90 (3.40–14.60)	0.008#
Serum creatinine, μmol/l	68.1 (59.2–78.7)	64.5 (53.0–79.6)	0.6
eGFR (CKD-EPI_{Cr}), ml/min/1.73 m^2	94 (81–101)	86 (72–97)	0.2
Serum cystatin C, mg/l	0.86 (0.78–1.01)	1.15 (0.93–1.37)	<0.001#
eGFR (CKD-EPI_{CysC}), ml/min/1.73 m^2	93 (78–103)	71 (62–95)	0.002#
Serum NGAL, μg/l	53.8 (43.3–70.4)	67.2 (61.0–103.1)	0.013#
Urine NGAL, μg/l	10.3 (3.1–21.8)	24.7 (14.3–43.9)	0.008#
Urine NGAL/creatinine, μg/g	9.02 (2.97–18.84)	35.1 (8.89–74.92)	0.012#
Urine KIM-1, μg/l	0.73 (0.32–1.54)	1.26 (0.73–2.92)	0.022#
Urine KIM-1/creatinine, μg/g	0.98 (0.30–1.31)	1.91 (1.20–3.29)	<0.001#
Urine transferrin, mg/l	<2.17	<2.17 (<2.17–6.43)	<0.001#
Detectable urine transferrin (≥2.17 mg/l), N (%)	1 (2)	9 (47)	<0.001#
Urine IgG, mg/l	<3.36	5.88 (<3.36–12.60)	<0.001#
Detectable urine IgG (≥3.36 mg/l), N (%)	4 (7)	14 (74)	<0.001#
Serum uromodulin, μg/l	127 (95–173)	100 (58–138)	0.031#
Urine uromodulin, mg/l	6.56 (2.19–14.38)	5.60 (2.09–13.41)	0.5
Urine uromodulin/creatinine, mg/g	7.62 (2.18–15.95)	9.19 (3.31–12.79)	0.8

#Statistically significant difference between the groups. Abbreviations: T2DM: type 2 diabetes mellitus; uACR: urine albumin to creatinine ratio; BMI: body mass index; ACEI: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker; HbA_{1c}: glycated hemoglobin A1c; HDL: high-density lipoprotein; LDL: low-density lipoprotein; eGFR: estimated glomerular filtration rate; CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; Cr: creatinine; CysC: cystatin C; NGAL: neutrophil gelatinase-associated lipocalin; KIM-1: kidney injury molecule-1; IgG: immunoglobulin G.

renin-angiotensin-aldosterone system inhibitors (Table 2). In ROC curves' analysis, urine KIM-1/creatinine and urine IgG showed highest values of the area under the ROC curve, being the best markers to discriminate between patients with and without moderately increased albuminuria (Figure 1, Table 3). In case of most studied markers, the cut-off values selected to best discriminate between patients with normal to mildly increased and moderately increased albuminuria fell within the values observed in healthy individuals. However, this was not the case for serum cystatin C, urine transferrin, and urine IgG, where the selected cut-off values were slightly higher than the upper reference limit established by our laboratory. Among 61 patients with uACR < 30 mg/g, 11 patients (18%) had serum cystatin C above the upper reference limit of 1.04 mg/l, 4 (7%) had urine IgG above the upper reference limit of 3.36 mg/l, and 1 (2%) had urine transferrin above the upper reference limit of 2.17 mg/l.

Table 2: Odds ratios for moderately increased albuminuria (uACR between 30 and 300 mg/g) among T2DM patients with eGFR > 60 ml/min/1.73 m^2 in simple and multiple logistic regressions adjusted for age, diabetes duration, the presence of hypertension, heart failure, and the treatment with renin-angiotensin-aldosterone system inhibitors.

Predictor variable	Simple analysis		Multiple analysis	
	Odds ratio (95% confidence interval)	p value	Odds ratio (95% confidence interval)	p value
Serum creatinine, per 1 μmol/l	0.99 (0.96–1.02)	0.6	0.98 (0.94–1.01)	0.2
Serum cystatin C, per 1 mg/l	33.09 (2.82–387.83)	0.005#	14.98 (0.64–353.13)	0.09
Serum NGAL, per 1 μg/l	1.02 (1.00-1.04)	0.018#	1.02 (1.00-1.04)	0.059
Urine NGAL, per 1 μg/l	1.04 (1.01–1.07)	0.016#	1.04 (1.00-1.07)	0.035#
Urine NGAL/creatinine, per 1 μg/g	1.02 (1.00-1.035)	0.018#	1.02 (1.00-1.04)	0.035#
Urine KIM-1, per 1 μg/l	1.64 (1.09–2.45)	0.020#	1.87 (1.11–3.15)	0.016#
Urine KIM-1/creatinine, per 1 μg/g	5.63 (2.16–14.68)	<0.001#	7.12 (2.22–22.87)	<0.001#
Detectable urine transferrin	54.00 (5.95–490.37)	<0.001#	54.90 (4.70–640.90)	0.001#
Detectable urine IgG	39.90 (9.25–172.10)	<0.001#	59.37 (8.54–412.79)	<0.001#
Serum uromodulin, per 1 μg/l	0.99 (0.98–1.00)	0.049#	0.99 (0.98–1.00)	0.09
Urine uromodulin, per 1 mg/l	0.96 (0.89–1.02)	0.2	0.95 (0.88–1.03)	0.2
Urine uromodulin/creatinine, per 1 mg/g	1.00 (0.95–1.06)	0.9	1.01 (0.95–1.07)	0.8

#Statistically significant result. Abbreviations: see Table 1.

3.3. Correlations of Studied Markers with eGFR, Albuminuria, Diabetes Duration, and Diabetes Control. Except for serum cystatin C, the studied serum and urine markers of renal function did not significantly correlate with eGFR based on serum creatinine (Table 4). Patients with detectable IgG and those with detectable transferrin had on average lower eGFR values (median 86 and 83 ml/min/1.73 m^2, resp.) than those with undetectable concentrations (median 93 ml/min/1.73 m^2 for both patients with undetectable IgG and those with undetectable transferrin); however, the differences were not statistically significant ($p = 0.2$ in case of transferrin, $p = 0.3$ in case of IgG).

Urine albumin, albumin/creatinine ratio, NGAL/creatinine ratio, and IgG significantly correlated with known duration of diabetes (Table 4). However, only serum cystatin C ($R = 0.61$; $p < 0{,}001$) and serum uromodulin ($R = -0.26$; $p = 0.018$) were significantly correlated with age. Moreover, urine albumin, uACR, urine NGAL and NGAL/creatinine ratio, urine transferrin, urine IgG, urine uromodulin, and uromodulin/creatinine ratio significantly correlated with diabetes control as reflected by HbA_{1c} concentrations. Urine markers NGAL, KIM-1, IgG, and transferrin were correlated with albuminuria (Table 4). Except for serum NGAL ($R = 0.45$; $p < 0.001$), the studied markers were not correlated with C-reactive protein. Also, none correlated with BMI. None of the studied markers differed significantly between men and women, between patients with and without hypertension, or between those treated and not treated with renin-angiotensin-aldosterone inhibitors.

3.4. Associations between Concentrations of Studied Markers and Follow-Up Data. Among 29 patients (36% of the study group), the retrospective data were available that had been recorded one year before the study visit. The retrospective data included eGFR and uACR values. During the year, GFR decreased in 17 patients (59%) (Table 5). Median decrease equaled 5.2 ml/min/1.73 m^2, that is, 7.6% of the initial eGFR value. Among studied markers, only higher NGAL concentrations both in serum and in urine were significantly associated with decrease in eGFR (Table 5). Moreover, the concentrations of serum NGAL, urine NGAL, and urine NGAL/creatinine ratios correlated negatively with the percentage changes in eGFR over the year (Figure 2).

During the one-year observation, uACR increased in 10 patients (34%), with median increase of 12.2 mg/g. We observed no relationship between the decrease in eGFR and the increase in albuminuria ($p = 0.9$). However, higher urine NGAL, urine NGAL/creatinine ratio, urine KIM-1/creatinine ratio, and detectable urine IgG were significantly associated with the increase in uACR (Figure 3).

We compared the longitudinal data (i.e., the incidence of decrease in eGFR and increase in uACR over one-year observation) between patients with studied markers below and above the cut-off values selected in a cross-sectional study (as presented in Table 3). Serum NGAL above 61 μg/l was the only marker significantly associated with more prevalent decrease in eGFR (it occurred in 53% of patients with serum NGAL above the cut-off versus 17% of those with serum NGAL below the cut-off; $p = 0.047$). However, several markers were associated with more prevalent increase in uACR, that is, urine NGAL above 14.3 μg/l (70% versus 32%; $p = 0.048$), urine NGAL/creatinine above 28.3 μg/g (50% versus 11%; $p = 0.023$), urine KIM-1/creatinine above 1.81 μg/g (50% versus 11%; $p = 0.018$), and urine IgG above 3.49 mg/l (50% versus 5%; $p = 0.005$).

4. Discussion

Based on KDOQI definitions [4, 10], DKD diagnosis is based on increased albuminuria and decreased eGFR. However, there are several weaknesses of albuminuria as a marker of (early) DKD. Most importantly, among T2DM who develop kidney disease associated with decrease in glomerular filtration, there are about 30–45%

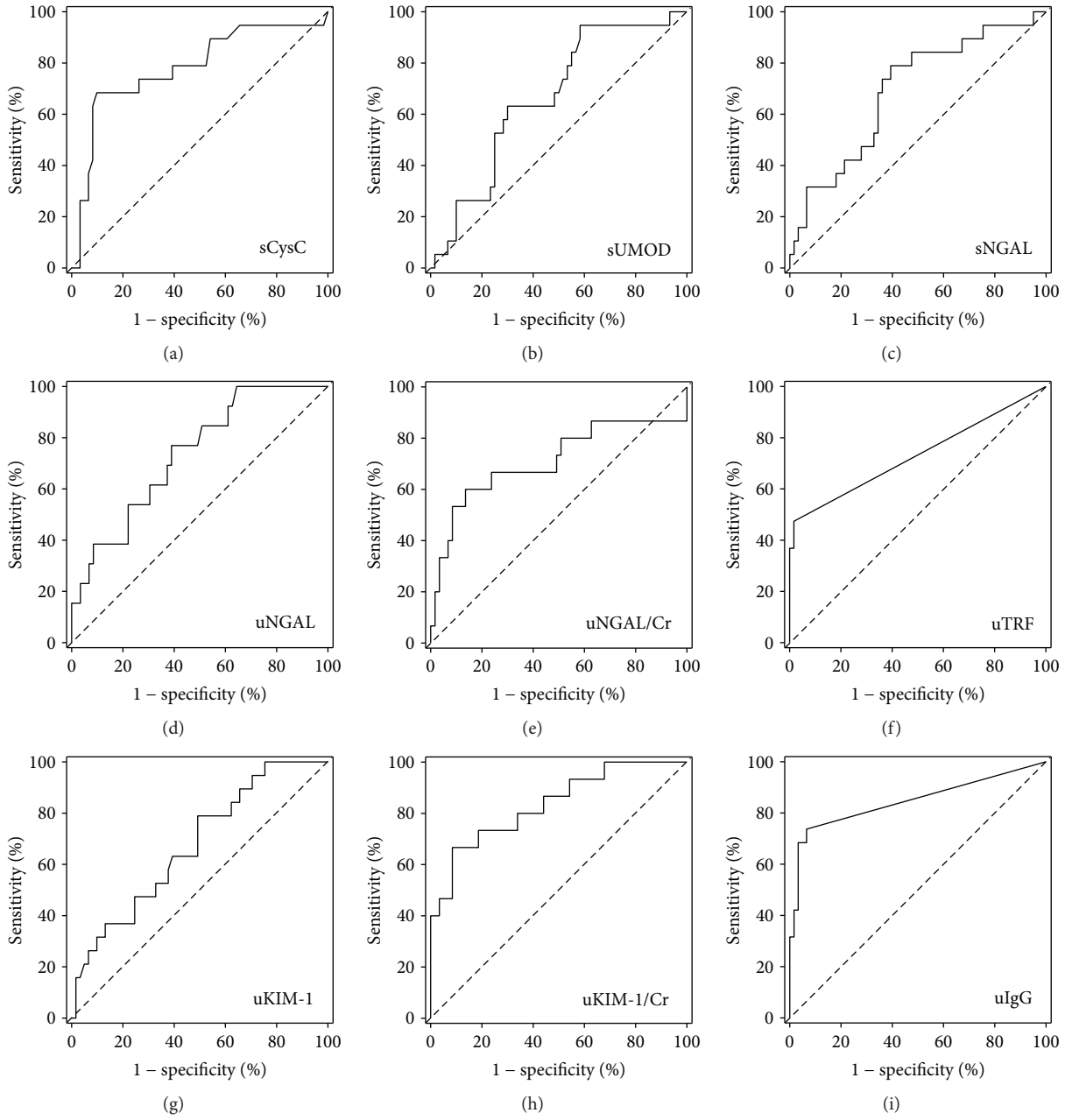

Figure 1: Receiver operating characteristic curves for selected serum and urine markers used to diagnose moderately increased albuminuria (uACR between 30 and 300 mg/g) among T2DM patients with eGFR > 60 ml/min/1.73 m^2: (a) serum cystatin C (sCysC); (b) serum uromodulin (sUMOD); (c) serum NGAL (sNGAL); (d) urine NGAL (uNGAL); (e) urine NGAL/creatinine (uNGAL/Cr); (f) urine transferrin (uTRF); (g) urine KIM-1 (uKIM-1); (h) urine KIM-1/creatinine (uKIM-1/Cr); (i) urine IgG (uIgG).

in whom no increased albuminuria (of above 30 mg/g creatinine) is observed [15, 16]. Further, albuminuria is not specific for DKD, and the comorbidities often observed in T2DM (such as hypertension or obesity) may also affect the filtration barrier of the glomeruli leading to increased albuminuria [17]. Moreover, treatment of hypertension often includes renin-angiotensin-aldosterone inhibitors (angiotensin-converting enzyme inhibitors and angiotensin receptor blockers) that lower hydrostatic pressure in glomeruli and thus normalize albuminuria. Although beneficial, this effect influences the diagnosis of DKD based on current guidelines [10]. There is an ongoing debate on lowering the cut-off value for albuminuria; evidence exists that the urine albumin/creatinine ratio exceeding 15 mg/g should be used to define chronic kidney disease as it better predicts kidney disease-related cardiovascular complications [18]. On the other hand, decreased eGFR occurs rather late following changes in the kidney in DKD, the early damage being often accompanied by hyperfiltration [19]. Both routine markers of DKD (albuminuria and eGFR) are altered in consequence of the damage to renal glomeruli. However, in about 30% of T2DM patients with kidney impairment,

Table 3: Diagnostic accuracy data for selected serum and urine markers used to diagnose moderately increased albuminuria (uACR between 30 and 300 mg/g) among T2DM patients with eGFR > 60 ml/min/1.73 m^2. Values observed in healthy individuals are shown to enable comparison with selected cut-off values.

Marker	Reference values previously associated with healthy individuals	Detection of moderately increased albuminuria in T2DM			
		AUC (95% CI)	Selected cut-off value	Sensitivity, %	Specificity, %
Serum cystatin C, mg/l	0.59–1.04[a]	0.78 (0.65–0.91)	1.09	68	90
Serum NGAL, μg/l	Men 63.5 ± 33.4 Women 64.9 ± 46.5[b]	0.69 (0.56–0.83)	61.0	79	61
Urine NGAL, μg/l	10.9 (6.0–38.2)[c]	0.74 (0.60–0.87)	14.3	80	61
Urine NGAL/creatinine, μg/g	12.2 (5.9–27.9)[c]	0.71 (0.53–0.89)	28.3	60	87
Urine KIM-1, μg/l	0.156–5.33[b]	0.68 (0.54–0.81)	0.73	79	51
Urine KIM-1/creatinine, μg/g	0.225–3.20[b]	0.84 (0.72–0.95)	1.81	67	92
Urine transferrin, mg/l	<2.17[a]	0.73 (0.58–0.88)	2.41	47	98
Urine IgG, mg/l	<3.36[a]	0.85 (0.72–0.97)	3.49	74	93
Serum uromodulin, μg/l*	191.2 (89.1–299.1)[c]	0.66 (0.53–0.80)	144	95	43

[a]Reference interval used in the laboratory that performed the measurement for the present study. [b]Reference values reported by the manufacturer of the test used in the present study [43–45]. [c]Previously reported values measured in the same laboratory and with the same tests as in the present study: urine NGAL, urine NGAL/creatinine [46], and serum uromodulin [38]. *Low concentrations are associated with renal impairment. Abbreviations: see Table 1.

Table 4: Correlations between studied serum and urine markers and eGFR, diabetes duration, and HbA_{1c} concentrations.

Marker	eGFR (CKD-EPI_{Cr})		Diabetes duration		HbA_{1c}		uACR	
	R	p value	R	p value	R	p value	R	p value
Serum cystatin C	−0.72	<0.001#	0.17	0.1	−0.05	0.7	0.21	0.08
Urine albumin	0.02	0.9	0.21	0.071	0.28	0.020#		NA
Urine albumin/creatinine	−0.01	0.9	0.25	0.037#	0.29	0.015#		NA
Serum NGAL	−0.15	0.2	0.09	0.4	0.10	0.4	0.13	0.3
Urine NGAL	−0.06	0.6	0.19	0.1	0.24	0.048#	0.39	<0.001#
Urine NGAL/creatinine	−0.05	0.7	0.24	0.046#	0.25	0.035#	0.36	0.002#
Urine KIM-1	0.11	0.4	−0.03	0.8	0.08	0.5	0.32	0.005#
Urine KIM-1/creatinine	0.05	0.7	0.05	0.7	0.16	0.2	0.45	<0.001#
Urine transferrin	−0.17	0.1	0.19	0.1	0.32	0.006#	0.48	<0.001#
Urine IgG	−0.14	0.2	0.27	0.016#	0.33	0.005#	0.61	<0.001#
Serum uromodulin	0.16	0.2	0.06	0.6	0.03	0.8	−0.09	0.4
Urine uromodulin	0.08	0.5	−0.14	0.2	−0.35	0.002#	−0.03	0.8
Urine uromodulin/creatinine	0.04	0.7	−0.12	0.3	−0.32	0.009#	0.01	0.9

#Statistically significant correlation. Abbreviations: see Table 1; NA: not applicable.

changes in renal interstitium and tubules precede damage of glomeruli [20]. Nevertheless, markers of tubular damage are currently not a part of routine assessment of T2DM patients. Our study is a part of extensive efforts aiming at the evaluation of other urinary and serum markers for their possible usefulness in early diagnosis of DKD. However, we used eGFR and albuminuria as the standard indicators of renal function at baseline and after one year of follow-up.

Our study recruited patients without significant decrease in eGFR and without severely increased albuminuria. According to currently accepted definitions [11], in individuals with eGFR > 60 ml/min/1.73 m^2, moderately increased albuminuria serves as the evidence of stage A2 chronic kidney disease. In our study, patients with A2 albuminuria presented higher concentrations of most studied markers of both glomerular (serum cystatin C, urine IgG, and transferrin) and tubular damage (urine and serum NGAL, urine KIM-1), as well as lower serum concentrations of uromodulin. A2 patients were older, with longer history of diabetes, and there was a positive correlation between albuminuria and diabetes control, similar to previous reports [21]. Still, in case of urine IgG, transferrin, NGAL, and KIM-1, the difference between A1 and A2 subgroups remained significant after adjustment for age, diabetes duration, and comorbidities (hypertension and heart failure) as well as treatment with renin-angiotensin-aldosterone system inhibitors.

Using ROC curves analysis, we determined the cut-off values that best discriminate between A1 and A2 patients. The cut-off values for studied markers of glomerular damage, that is, serum cystatin C, urine IgG, and transferrin were higher as compared with upper reference limits

Table 5: Characteristics of 29 patients for whom retrospective longitudinal data were available. The group was divided according to changes in eGFR over one-year observation.

Characteristic	Patients with decrease in eGFR ($N = 17$)	Patients without decrease in eGFR ($N = 12$)	p value
Age, years	64 ± 15	63 ± 11	0.4
Male sex, N (%)	11 (65)	6 (50)	0.4
Known diabetes duration, years	7 (2–11)	10 (4–11)	0.4
HbA_{1c}, %	6.10 (5.70–6.45)	6.20 (5.70–7.00)	0.7
Serum creatinine, μmol/l	61.9 (59.2–72.5)	68.9 (61.4–77.8)	0.5
eGFR (CKD-EPI_{Cr}), ml/min/1.73 m^2	91 (81–98)	96 (80–99)	0.8
Serum cystatin C, mg/l	0.92 (0.82–1.14)	0.86 (0.76–1.10)	0.2
eGFR (CKD-EPI_{CysC}), ml/min/1.73 m^2	87 (67–96)	96 (70–105)	0.2
Urine albumin, mg/l	8.06 (6.76–13.53)	10.18 (6.21–14.65)	0.9
Urine albumin/creatinine, mg/g	7.93 (3.38–13.38)	8.07 (5.50–16.4)	0.6
Serum NGAL, μg/l	66.7 (49.7–72.3)	44.4 (39.7–55.0)	0.028[#]
Urine NGAL, μg/l	15.3 (4.0–31.5)	2.9 (1.9–18.5)	0.048[#]
Urine NGAL/creatinine, μg/g	6.47 (3.36–45.78)	8.82 (2.42–20.3)	0.3
Urine KIM-1, μg/l	1.05 (0.42–1.64)	1.12 (0.35–1.69)	0.9
Urine KIM-1/creatinine, μg/g	1.01 (0.55–1.58)	1.34 (0.59–2.23)	0.5
Urine transferrin, mg/l	<2.17	<2.17	0.1
Detectable urine transferrin (≥2.17 mg/l), N (%)	0	2 (17)	0.08
Urine IgG, mg/l	<3.36	<3.36 (<3.36–4.00)	0.2
Detectable urine IgG (≥3.36 mg/l), N (%)	2 (12)	4 (33)	0.2
Serum uromodulin, μg/l	125 (97–142)	105 (61–144)	0.5
Urine uromodulin, mg/l	11.30 (6.38–15.50)	4.94 (2.99–14.41)	0.3
Urine uromodulin/creatinine, mg/g	11.20 (6.67–23.17)	10.07 (4.51–15.34)	0.5

[#]Statistically significant difference between the groups. Abbreviations: see Table 1.

established in healthy individuals. A substantial proportion (18%) of G1-G2/A1 patients presented with serum cystatin C above the upper reference limit, in line with previous reports [22, 23]. In our study, neither of the tubular markers was significantly correlated with eGFR based on serum creatinine. Moreover, serum creatinine and eGFR based on creatinine did not differ between our A1 and A2 patients, while serum cystatin C and eGFR based on cystatin C differed significantly. The KDIGO guidelines [11] recommend the estimation of GFR based on serum cystatin C in adult patients with suspected stage G3a of chronic kidney disease (GFR between 45 and 60 ml/min/1.73 m^2). Our results suggest that measurements of serum cystatin C accompanied by calculation of eGFR with the use of CKD-EPI_{CycC} equation may be useful already at the earlier stages of diabetic kidney disease. The recently performed laboratory standardization of cystatin C measurements is also the advantage that must be remembered.

Urine IgG and transferrin excretion has been previously observed in normoalbuminuric patients with diabetes and has been associated with hypertension and hyperfiltration [24, 25]. In accordance with previous studies [26, 27], we observed increased urine IgG and transferrin in a subgroup with moderately increased albuminuria and both proteins were specific markers of kidney disease. These are notable observations, especially considering high molecular weight of IgG (150 kDa) that preserves the protein from being filtered by healthy glomeruli [28].

In our study, both urine NGAL and KIM-1 correlated positively with albuminuria, consistently with previous reports regarding patients with T2DM [29–31] and were significantly higher in patients with moderately increased albuminuria. Nauta et al. [32] observed higher concentrations of urine NGAL and KIM-1 in normoalbuminuric diabetic patients comparing to healthy subjects. Also, we have previously reported increased urine NGAL concentrations among patients with early stages or risk for DKD [33], similarly to other groups [34, 35].

Tamm-Horsfall's protein or uromodulin is the main protein present in normal urine. This glycoprotein is exclusively expressed in the thick ascending limb of the loop of Henle and proximal part of the distal renal tubule and is released to urine by partial proteolysis [36]. It was demonstrated that the damage of renal tubules in chronic kidney disease (including early stages: G1 and G2) was associated with reduced concentrations of uromodulin in both urine and serum [37–39], which reflects a pathophysiological difference of this glycoprotein compared to other renal tubule injury markers. In our study, neither serum nor urine concentrations of uromodulin were correlated with albuminuria. Moreover, we have not observed correlations between uromodulin and eGFR. However, an interesting observation

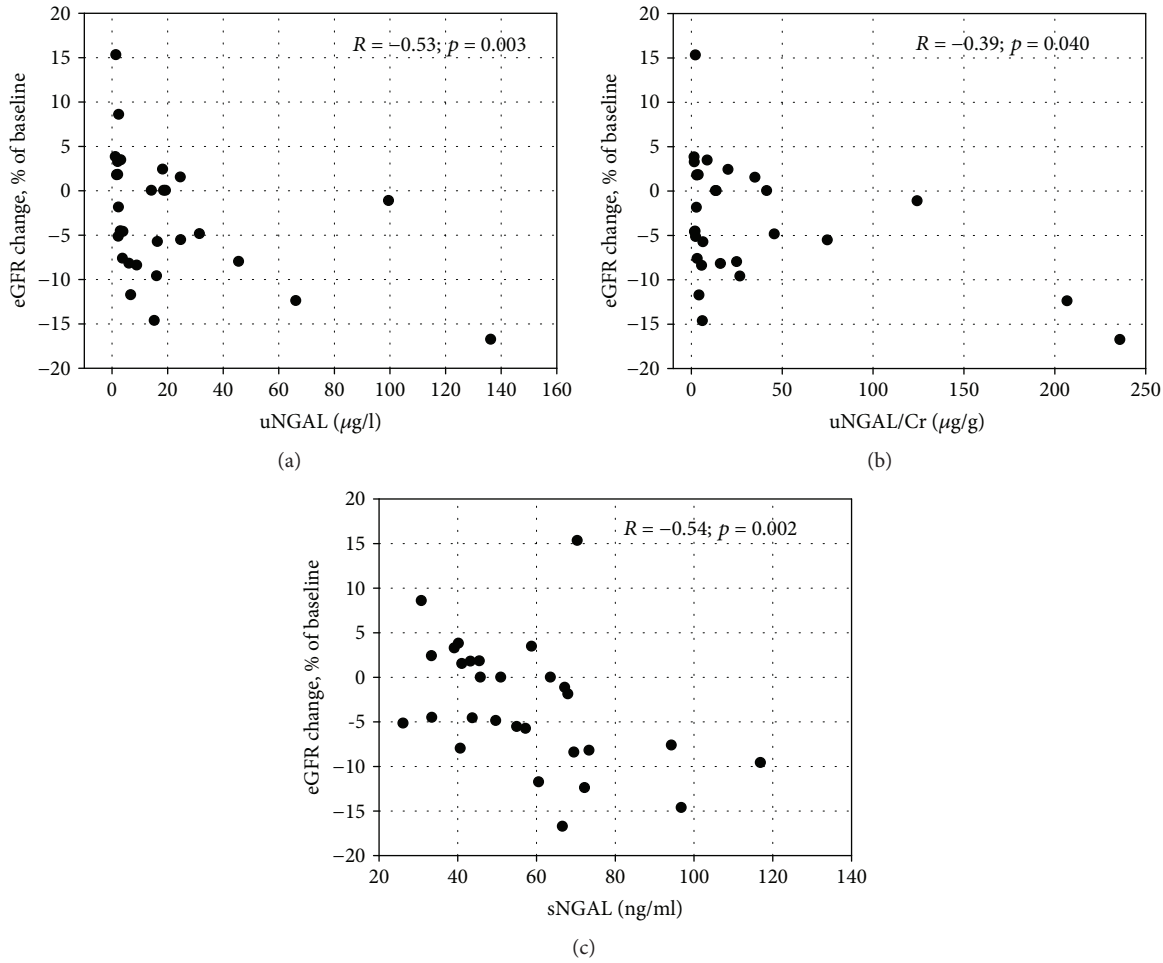

Figure 2: The correlations between percentage change in eGFR values over one-year observation and urine NGAL concentrations (a), urine NGAL/creatinine ratios (b), serum NGAL concentrations (c) among 29 patients for whom retrospective data were available. Change in eGFR was calculated as [(control eGFR − baseline eGFR)/baseline eGFR] ∗ 100%. Spearman R coefficients and p values are shown on the graphs.

is negative correlation between HbA_{1c} and urine uromodulin, suggesting tubular impairment among patients with worse glycemic control. Previously, Leiherer et al. [40] demonstrated decreased concentrations of uromodulin in urine of patients with impaired carbohydrate metabolism, including ones with T2DM.

From practical point of view, it is important for clinical practitioners that both glomerular (albumin, uACR) and tubular (urine NGAL, transferrin, IgG, and uromodulin) markers positively correlated with glycemic control, as assessed with HbA_{1c} measurements. Hence, good glycemic control is important both for the glomeruli and tubules in the early stages of diabetic nephropathy.

In a subgroup of patients, we were able to obtain longitudinal data on changes in standard measures of renal function (eGFR and uACR) over one-year observation. During the year, patients were treated according to current recommendations [12]. The concentrations of NGAL in both serum and urine as well as urine NGAL/creatinine ratios were correlated with decline in eGFR. We did not observe significant correlations between eGFR changes and other studied glomerular or tubular biomarkers. This may be caused by the small number of patients in the longitudinally observed subgroup, as well as by the relatively short period of observation [14]. However, we were able to show significant associations between the concentrations of KIM-1, NGAL, and IgG in urine and the increase in uACR in longitudinal observation. Urine KIM-1/creatinine ratio and NGAL/creatinine ratio were also examined by Nowak et al. [41] who studied the association of the markers with decline in kidney function in T2DM patients over a follow-up period of 5–12 years. Both markers were significantly associated with renal decline in simple analysis; however, in case of the NGAL/creatinine ratio, the association was weak and only KIM-1 remained a significant predictor in multiple analysis. To the contrary, we have not observed the correlation between urine KIM-1 or KIM-1/creatinine ratio and eGFR decline, although it correlated with the increase in albuminuria.

Our study, in accordance to previous ones, suggests that various pathways may be involved in early renal damage among T2DM patients [35, 41, 42]. Although albuminuria remains one of the most significant predictors of renal decline [42], other markers, such as serum and urine NGAL,

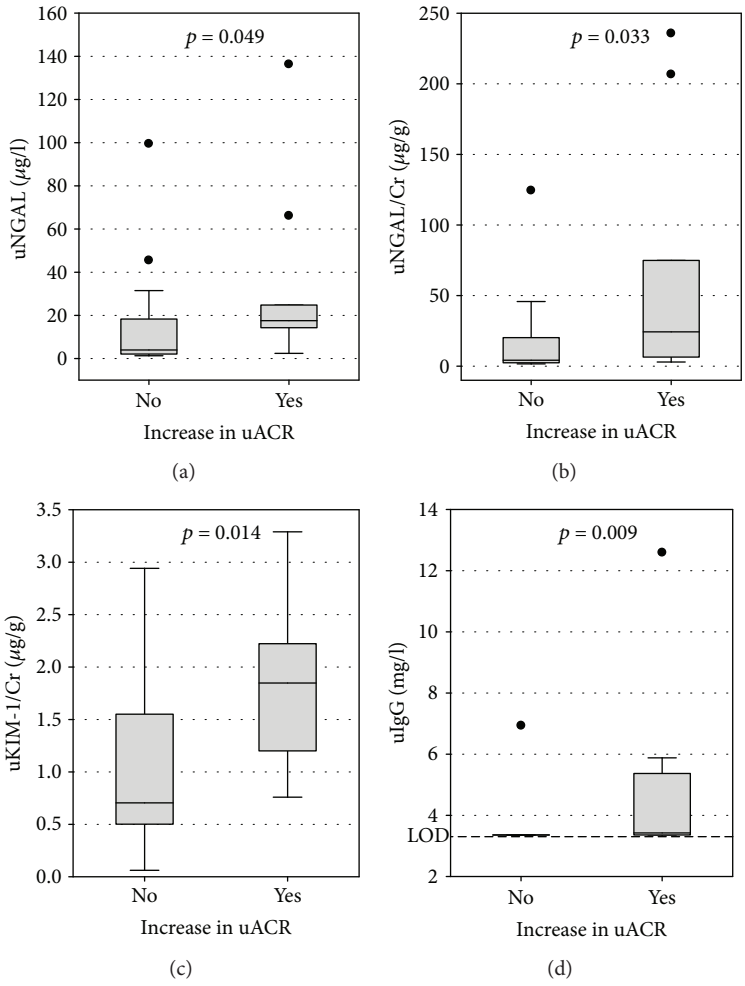

Figure 3: The associations between increase in urine albumin/creatinine ratio (uACR) over one-year observation and urine NGAL concentrations (a), urine NGAL/creatinine ratios (b), urine KIM-1/creatinine ratios (c), and urine IgG concentrations (d) among 29 patients for whom retrospective data were available. Data are shown as median, interquartile range (box), nonoutlier range (whiskers), and outliers (points). LOD indicates the limit of detection of urine IgG; p values in Mann–Whitney's test are shown on the graphs.

serum cystatin C, urine KIM-1, IgG, and transferrin, may add to the early diagnosis of diabetic kidney disease.

The main limitations of our study are the cross-sectional setting and relatively low number of patients. In a subgroup of patients, we were able to obtain the longitudinal data on changes in standard measures of renal function (eGFR, uACR); however, these are retrospective data, and the number of patients is small. In this subgroup of patients, the studied markers were measured at the end of the follow-up, so we were only able to evaluate the associations between the studied markers and the changes in renal function during the preceding year. The diagnosis of kidney disease was not confirmed by renal biopsy, as our patients did not have clinical indications for biopsy. Therefore, we adopted surrogate markers of kidney disease, that is, the presence of moderately increased albuminuria, decrease in eGFR, and increase in uACR. Also, lack of the control group studied simultaneously with the patients' group limits our findings; however, we relied on previous experience of our laboratory with most studied markers.

In summary, the recruitment of the uniform group of T2DM patients with eGFR > 60 ml/min/1.73 m^2 and uACR < 300 mg/g, with exclusion of important interfering conditions, enabled us to study the concentrations of selected glomerular and tubular markers in early stages of DKD. In most of our patients, the diabetes duration was below 10 years and the diabetes was well controlled. Both glomerular (serum cystatin C, urine IgG, and transferrin) and tubular (urine NGAL, KIM-1 and uromodulin) markers differed significantly between patients with moderately increased albuminuria comparing to those with normal to mildly increased albuminuria. Serum and urine NGAL were significantly associated with eGFR decline. Our findings indicate that the widely available markers such as serum cystatin C, urine IgG, transferrin, and NGAL may help in early assessment of kidney disease in T2DM patients, although large prospective studies are needed to confirm the conclusion.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

The work was partly financed from Jagiellonian University Medical College, Grant no. K/ZDS/006347.

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 6909035, 10 pages
https://doi.org/10.1155/2018/6909035

Research Article

Apoptosis-Associated Speck-Like Protein Containing a CARD Deletion Ameliorates Unilateral Ureteral Obstruction Induced Renal Fibrosis and Endoplasmic Reticulum Stress in Mice

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Received 5 February 2018; Revised 9 May 2018; Accepted 31 May 2018; Published 2 July 2018

Academic Editor: Sandra Rayego-Mateos

Inflammation might be one of the essential underlying mechanisms of renal fibrosis, which is considered a key pathological feature of end-stage renal disease and is closely associated with proteinuria and decreased renal function. Apoptosis-associated speck-like protein containing a CARD (ASC), identified as the central structure of inflammasome, is involved in the progression of interstitial fibrosis; however, its signal transduction pathways remain unclear. In the present study, we performed unilateral ureter obstruction (UUO) in both wild-type and ASC deletion mice to determine the contribution of ASC to renal fibrosis. Compared with control groups, UUO significantly induced renal fibrosis and collagen deposition, as evidenced by photomicrographs. ASC deletion attenuated renal injury, reduced cell infiltration and the release of inflammatory cytokines, protected against apoptosis, and downregulated the PRKR-like endoplasmic reticulum kinase (PERK) pathway of endoplasmic reticulum (ER) stress. Our data identify a novel role of ASC in the regulation of renal fibrosis and ER stress after UUO, strongly indicating that ASC could serve as an attractive target in the treatment of chronic kidney disease.

1. Introduction

Chronic kidney disease (CKD) is a common concern associated with high mortality and disability worldwide, which leads to much lower quality of life and a substantial economic burden [1, 2]. Renal fibrosis, closely associated with proteinuria and the decreased glomerular filtration rate (GFR) [3], is considered as a key pathological feature of end-stage renal dysfunction. Interstitial fibrosis contributes to adverse long-term renal prognosis, and inflammation might be one of the essential underlying mechanisms [4]. Inflammatory mediators, such as chemokines or cytokines, interact with myofibroblasts, recruiting more inflammatory cells that migrate into the renal interstitium, thereby causing progressive glomerulosclerosis, mesangial proliferation, and collagen deposition [5]. Despite recent rapid advances in research into CKD, the detailed pathways remain to be revealed. Such insights might lead to new therapeutic targets for CKD.

ASC (apoptosis-associated speck-like protein containing a CARD), characterized as the central structure of inflammasome, contains a pyrin domain (PYD) and a caspase recruitment domain to link pattern recognition receptors (PRRs) with procaspase 1. Consequently, prointerleukin (IL)-1β and pro-IL-18 are cleaved into their activated forms to trigger the downstream inflammation cascade [6]. Unsurprisingly, given its critical role under multiple pathophysiological conditions, ASC is broadly involved in the progression of CKD; however, its signal transduction pathways remain unknown [7].

The endoplasmic reticulum (ER) is responsible for modifying and folding proteins correctly, serving as a quality control system to maintain protein homeostasis [8]. Upon

various endogenous disturbances and external irritants, consequent accumulation of misfolded proteins in the lumen causes ER stress and induces downstream regulation, defined as the unfolded protein response (UPR). ER stress is related to kidney diseases; while, its interaction with ASC or the inflammasome requires further research [9–11].

In this study, we focused on the role of ASC in unilateral ureter obstruction- (UUO-) induced renal fibrosis and apoptosis, tried to detect its regulation potential on ER stress or UPR. Our findings expand the understanding of the pathogenesis of CKD.

2. Materials and Methods

2.1. Materials. Antibodies recognizing the following proteins were used in this study: caspase-3, α-SMA, and E-cadherin, Bax, Bcl-2, BIP, ATF-4, CHOP, P-eIF2a, and eIF2a (Cell Signaling Technology); IL-1β, IL-18, collagen I, fibronectin, CD11b, F4/80 (Abcam); Ly6G (Protein Specialists); and caspase 1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.2. Animal Studies. C57BL/6J (wild-type, WT) mice were purchased from Shanghai SLAC Laboratory Animals (Shanghai, China). ASC$^{-/-}$ mice (C57BL/6J genetic background) were generated as described previously [12]. Only age- and sex-matched mice were applied in this study. All animal experiments were performed under the approval of the Animal Care Committee at Jiao Tong University. Male WT and ASC$^{-/-}$ mice (aged 8–10 weeks) were divided equally into a control group (sham) and a model group (UUO); each group contained six mice. Complete ureteral obstruction was induced as previously described [13] on anesthetized animals. After isolation of the left kidney and ureter, the left ureter was ligated using 4–0 silk thread in the UUO group, while the ureter was simply isolated without ligation in the sham group. Mice were sacrificed at 14 days after surgery. Kidney tissues were collected and placed in 10% paraformaldehyde or immediately frozen in liquid nitrogen for storage.

2.3. Photomicrographs and Immunohistochemical Studies. Renal samples fixed with 10% formalin were dehydrated in alcohol and cut into 4 mm slices. Periodic acid-Schiff (PAS) and Masson trichrome staining were performed following the protocol. Specifically, the glomerular injury score was evaluated following PAS staining according to glomerulosclerosis and extracellular matrix expansion, which were scored from 0 to 4+ (0, no change; 1+, changes affecting 5–25% of the sample; 2+, changes affecting 25–50%; 3+, changes affecting 50–75%; and 4+, changes affecting 75–100%). At least 60 glomeruli were counted under microscope from 6 mice in each group, and the averaging scores were calculated as final glomerular injury score. As for tubulointerstitial fibrosis index, the areas of interstitial fibrosis were evaluated in 10 random ×400 magnification fields obtained from Masson trichrome staining. All of these observations were made by two independent researchers who were blinded to the experimental groups. The kidney samples were also stained with anticollagen I (1 : 1000) and antifibronectin antibodies (1 : 1000). The immunohistochemistry was quantified according to the method detailed in our previous study [13]. The areas of collagen I and fibronectin positivity were represented as a percentage of the total section, as analyzed using an image analyzer (Winroof; Mitani Corporation, Tokyo, Japan). The immunohistochemistry of CD11b, F4/80, and Ly6G was performed on paraffin-embedded renal tissue following protocols and imaged after incubated with secondary antibodies. Positive cells (brown pixels) were calculated from 10 high-power fields per group to indicate cell infiltration in kidney after UUO.

2.4. Western Blot Analysis. Protein lysates were separated by electrophoresis using 10% and 12% SDS gels. The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, which were blocked with 5% skim milk for 1 h and then incubated with primary antibodies against α-SMA, E-cadherin, caspase 1, IL-1β, IL-18, Bcl-2, Bax, caspase 3, p-eIF2α, ATF4, BIP, and CHOP overnight at 4°C. After washing with TBST (Tris-buffered saline Tween 20), the membranes were incubated with secondary antibodies for 1 h. Detection of the immunoreactive protein bands intensity was achieved using enhanced chemiluminescence and analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA).

2.5. Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from kidney tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed into cDNA following the instructions of PrimeScript RT reagent kit (Takara, Dalian, Liaoning, China). RT-PCR analysis was performed in the 7500 Fast Real-Time PCR System (Applied Biosystems, Rockford, IL, USA) as previously described, and the primer sequences were as follows: NLRP3-F, 5′ACATCTCCTTGGTCCTCAGC3′, NLRP3-R, 5′GCTTCAGTCCCACACACAGA 3′, IL-1β-F, 5′TTGTGGCTGTGGAGAAGCTG3′, IL-1β-R, 5′GCCGTC TTTCAT ACACAGG3′, IL-6-F, 5′GTGCCTCTTTGCTGC TTTCAC3′, IL-6-R, 5′GGTACATCCTCGACGGCATCT3′, IL-18-F, 5′GCTTGAATCTAAATTATCAGTC3′, IL-18-R, 5′GAAGATTCAAATTGCATCTTAT3′, TNF-α-F, 5′CCCT CACACTCAGATCATCTTCT3′, TNF-α-R, 5′GCTACGA CGTGGGCTACAG3′, 18S-F, 5′TTCGGAACTGAGGCCA TGATT3′, and 18S-R, 5′TTTCGCTCTGGTCCGTCTTG3′.

Relative mRNA expression was normalized to 18 S rRNA and presented as fold over control.

2.6. Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Assay. Apoptosis of renal tissue was detected using a TUNEL kit (Roche, Netley, NJ, USA) following the manufacturer's protocol [13]. After incubated with TUNEL reagent in dark for 60 min, 10 fields were selected randomly for each section and TUNEL-positive cells were counted and averaged.

2.7. Statistical Analyses. Data are expressed as the mean ± standard error of the mean (SEM). Comparisons between groups were performed using one-way analysis of variance

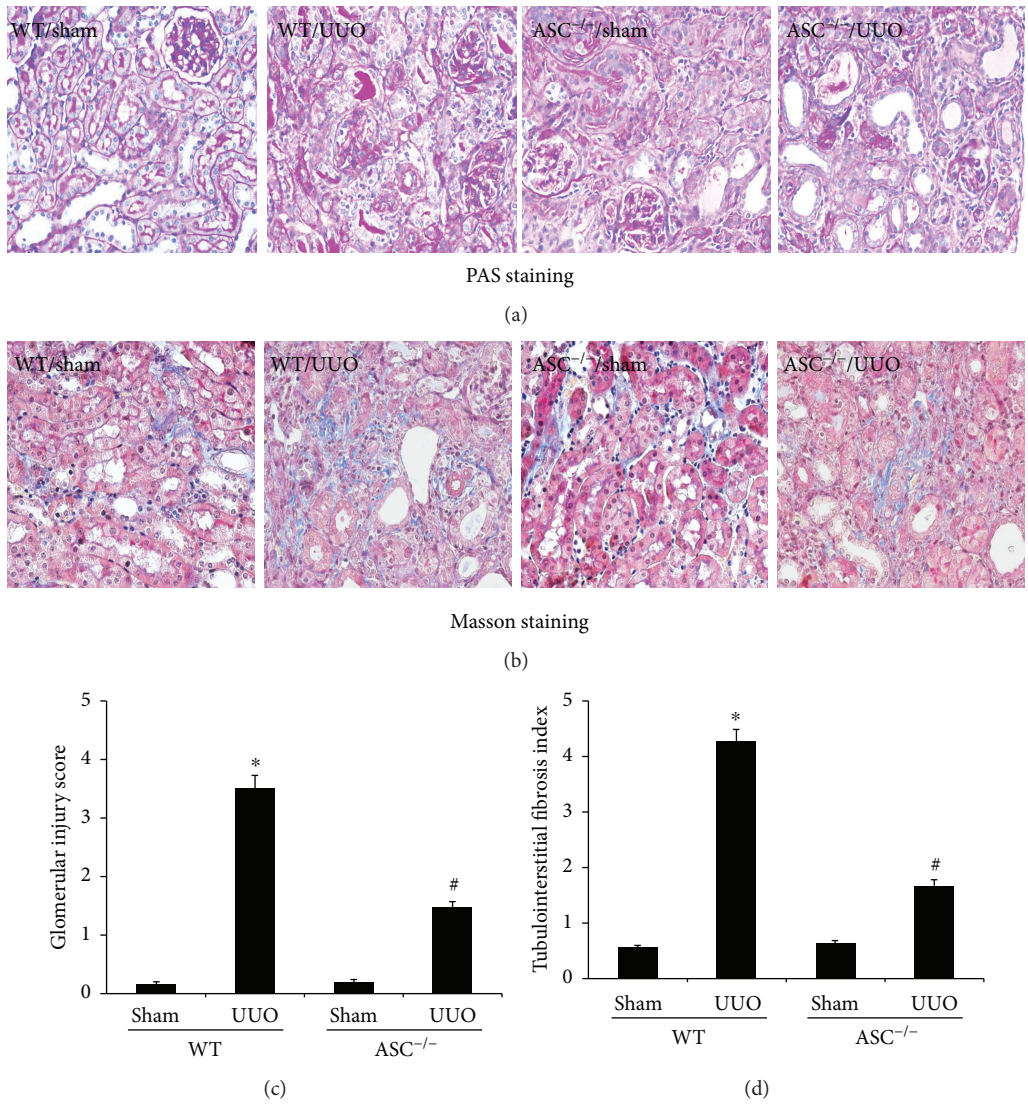

Figure 1: ASC deletion attenuates UUO-induced renal injury in mice. (a) Representative photomicrographs at 14 days after UUO of PAS-stained sections (magnification, ×400). (b) Masson trichrome-stained kidney sections (magnification, ×400). (c) Glomerular injury scores and (d) tubulointerstitial fibrosis indices evaluated based on PAS and Masson staining; over 20 sections for each group were counted. $^{*}P < 0.05$, WT/sham group versus WT/UUO group. $^{\#}P < 0.05$, ASC$^{-/-}$/UUO group versus WT/UUO group. Data represent the mean ± SEM.

(ANOVA) followed by Dunnett's multiple comparison tests or Student's t-test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 20.0 software (IBM, Armonk, NY, USA).

3. Results

3.1. ASC Deficiency Minimized Renal Injury after UUO. To determine whether ASC contributes to the progression of renal fibrosis, UUO was performed in both ASC$^{-/-}$ mice and WT mice. After 14 days, the model groups exhibited severe tubular necrosis, epithelial degeneration, and inflammatory infiltration compared with the sham groups, while ASC deletion markedly reduced the extent of injury, as observed through PAS and Masson trichrome staining of kidney sections (Figures 1(a) and 1(b)). Additionally, ASC$^{-/-}$ mice displayed a significantly lower glomerular injury score (1.46 ± 0.11 versus 3.51 ± 0.22) and interstitial fibrosis index (1.67 ± 0.195 versus 4.25 ± 0.518) compared with WT/UUO mice, which was in line with the pathological changes (Figures 1(c) and 1(d)). These findings indicated that UUO results in renal injury and ASC deficiency could minimize its effect *in vivo*.

3.2. ASC Deletion Alleviates Collagen Deposition and Renal Fibrosis. Previous studies showed that activation of profibrotic mediators, such as collagen (especially collagen I) and fibronectin, is the key feature of interstitial fibrosis in CKD [14]. Therefore, we detected collagen I and fibronectin deposition in renal tissue from the ligated site using immunohistochemical staining. A dramatic increase in collagen I (Figure 2(a)) and fibronectin-positive cells (Figure 2(b)) in

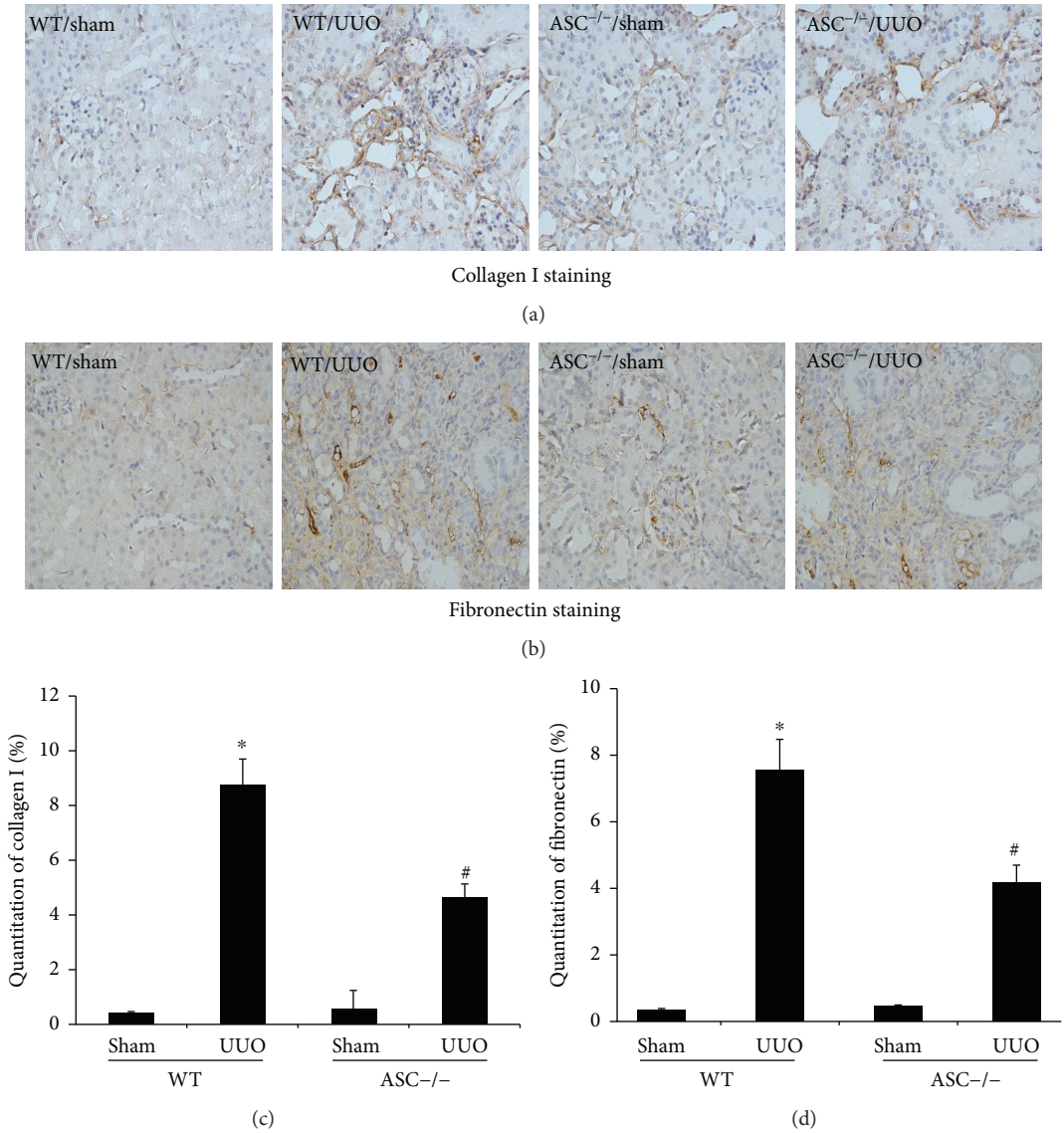

Figure 2: ASC deletion attenuates UUO-induced renal fibrotic cytokines in mice. (a) Representative photomicrographs at 14 days after UUO of collagen I staining (magnification, ×400). (b) Representative photomicrographs at 14 days after UUO of fibronectin staining (magnification, ×400). (c) Semiquantitative analysis of collagen I among the different groups. (d) Quantification of fibronectin-positive areas. $^{*}P < 0.05$, WT/sham group versus WT/UUO group. $^{\#}P < 0.05$, ASC$^{-/-}$/UUO group versus WT/UUO group. Data represent the mean ± SEM ($n = 6$).

WT mice was observed at 14 days after UUO. However, semiquantitative analysis demonstrated a marked reduction in collagen I (4.62 ± 0.51 versus 8.73 ± 0.96, $P < 0.05$) and fibronectin (4.17 ± 0.53 versus 7.55 ± 0.92) deposition in ASC deletion mice compared with WT/UUO group (Figures 2(c) and 2(d)).

3.3. ASC Regulates UUO-Induced Phenotypic Alterations and Apoptosis in Renal Fibrosis. During phenotypic alterations, tubular cells typically lose their epithelial characteristics and express protein characteristic of myofibroblasts. To further unravel the regulatory effect of ASC in this process, α-smooth muscle actin (α-SMA) and E-cadherin were chosen as the phenotypic change markers. Compared with the sham operation groups, UUO led to a remarkable increase in α-SMA (2.1-fold, WT/UUO) expression and a decrease in E-cadherin (0.268-fold, WT/UUO). Interestingly, ASC$^{-/-}$ mice exhibited a partial inhibition of tubular cell epithelial-mesenchymal transition (EMT), represented as relatively higher E-cadherin level and less α-SMA (Figure 3).

In addition, Western blotting analysis of Bcl-2 and Bax demonstrated that in UUO mice, the level of Bcl-2 was downregulated. By contrast, Bax, a common marker of apoptosis, was significantly upregulated and, in turn, caused a prominent increase in cleaved caspase 3 (Figures 4(c) and 4(d)). Importantly, ASC deletion consistently decreased the level of Bax and caspase 3. TUNEL staining also confirmed that the apoptosis induced by UUO was markedly rescued by ASC depletion, which indicated that ASC$^{-/-}$

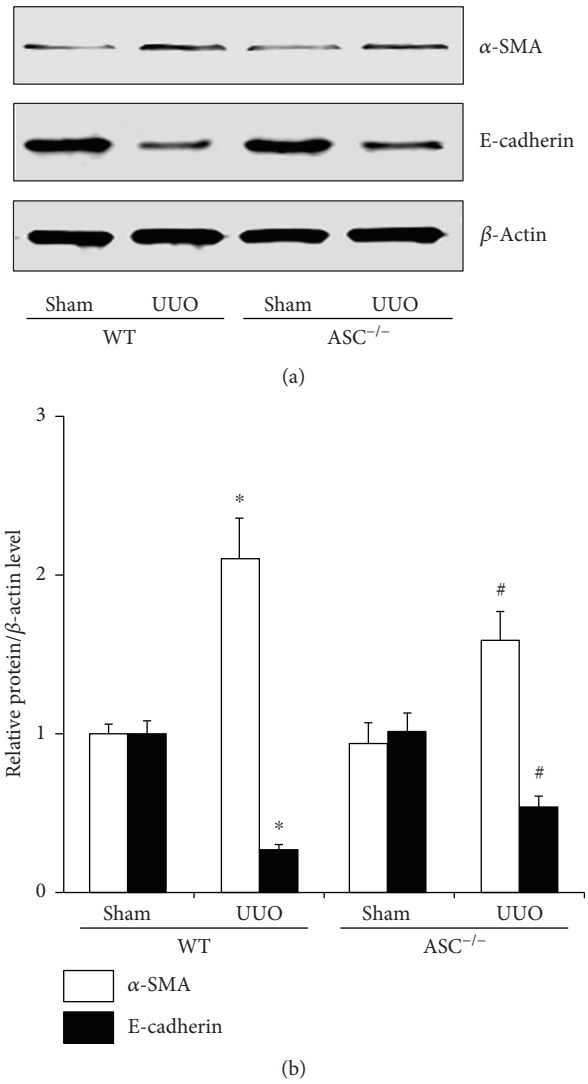

Figure 3: ASC regulates phenotypic alterations in UUO mice. (a) Immunoblotting for α-SMA and E-cadherin in WT and ASC$^{-/-}$ mice with or without UUO after 2 weeks. (b) Quantification of α-SMA and E-cadherin levels, normalized against those of β-actin. $^{*}P < 0.05$, WT/sham group versus WT/UUO group. $^{\#}P < 0.05$, ASC$^{-/-}$/UUO group versus WT/UUO group. Data represent the mean ± SEM ($n = 6$).

protected against cell apoptosis in the UUO model of CKD (Figures 4(a) and 4(b)).

3.4. ASC$^{-/-}$ Mice Display Reduced Inflammation after UUO. Inflammation is integrally associated with renal interstitial fibrosis; therefore, immunohistochemistry was performed to detect the influence of ASC deletion on inflammatory cell infiltration and whether it was connected with the restored renal function. Consistent with pathological changes, UUO mice appeared severely aggravated immune cell infiltration of CD11b-positive leukocytes, F4/80-positive macrophages, and Ly6G-positive granulocytes in renal interstitium at 14 days (Figure 5). Quantification analysis confirmed that absence of ASC significantly reduced the cellular infiltration mentioned above, compared with wild-type controls.

Since ASC serves as central functional component of inflammasome, cytokines such as IL-18 and IL-1β should be involved in the regulation process. Therefore, immunoblotting for the proinflammatory mediators was performed. In line with our hypothesis, the levels of caspase 1, cleaved IL-18, and IL-1β were markedly increased in the UUO groups compared with the sham controls. ASC deletion downregulated the level of caspase 1 and dramatically inhibited the expression and maturation of IL-18 and IL-1β, providing further evidence that ASC modulates inflammation during the disease process (Figures 6(a) and 6(b)). Similarly, gene expressions of NLRP3, IL-18, and IL-1β were upregulated after UUO. ASC$^{-/-}$ mice showed decreased mRNA levels of IL-18 and IL-1β but not NLRP3. However, the expression of other cytokines like IL-6 and TNF-α was not affected by ASC knockout as presented by RT-PCR (Figures 6(c) and 6(d)).

3.5. ASC Is a Regulator of the ER Stress Pathway. ASC and ER stress are both closely related to renal tubulointerstitial fibrosis, which prompted us to investigate the linkage between ASC and ER stress in UUO-induced CKD. To determine the possible underlying mechanism, the activation of CHOP, an ER stress marker, was evaluated. As shown by Western blotting analysis, CHOP and BIP were significantly increased at day 14 after UUO in the WT mice and the ASC$^{-/-}$/UUO mice showed significantly reduced CHOP and BIP levels. We then examined the PRKR-like endoplasmic reticulum kinase (PERK) pathway, one of the typical downstream pathways of ER stress and UPR. Similar to CHOP, the levels of both p-eIF2α/eIF2α and ATF4 increased markedly upon UUO stimulation, while the ASC$^{-/-}$ mice presented a much lower increase compared with that in the WT/UUO group (Figures 7(a) and 7(b)). Taken together, these data revealed that the prolonged activation of ER stress and/or UPR following UUO was inhibited by ASC deletion.

4. Discussion

In the current study, we identified ASC as a regulator that interacts with ER stress in the progression of renal fibrosis after UUO. ASC deficiency attenuated renal injury, decreased collagen deposition, reduced cellular infiltration, and downregulated inflammasome-activated cytokines associated with ER stress and UPR activation. Our study uncovered a new layer of the complex mechanisms underlying renal fibrosis and shed light on a potential therapeutic target for CKD.

During fibrinogenesis, upon the gradual nephron loss and suspended tissue remodeling, the injured tubular cells undergo apoptosis and the capillaries become pathologically permeable, thus triggering chronic interstitial inflammation. The activation of inflammasomes, especially the maturation and secretion of cytokines, are widely involved in renal fibrosis [15]. Vesey et al. revealed that IL-1β could stimulate human renal fibroblast proliferation and fibronectin production and induce proximal tubule cell injury [16]. Similarly, IL-18 neutralization ameliorated UUO-induced EMT and renal fibrosis [17]. In our study, we confirmed that the infiltration of leukocytes and F4/80+ macrophages was induced

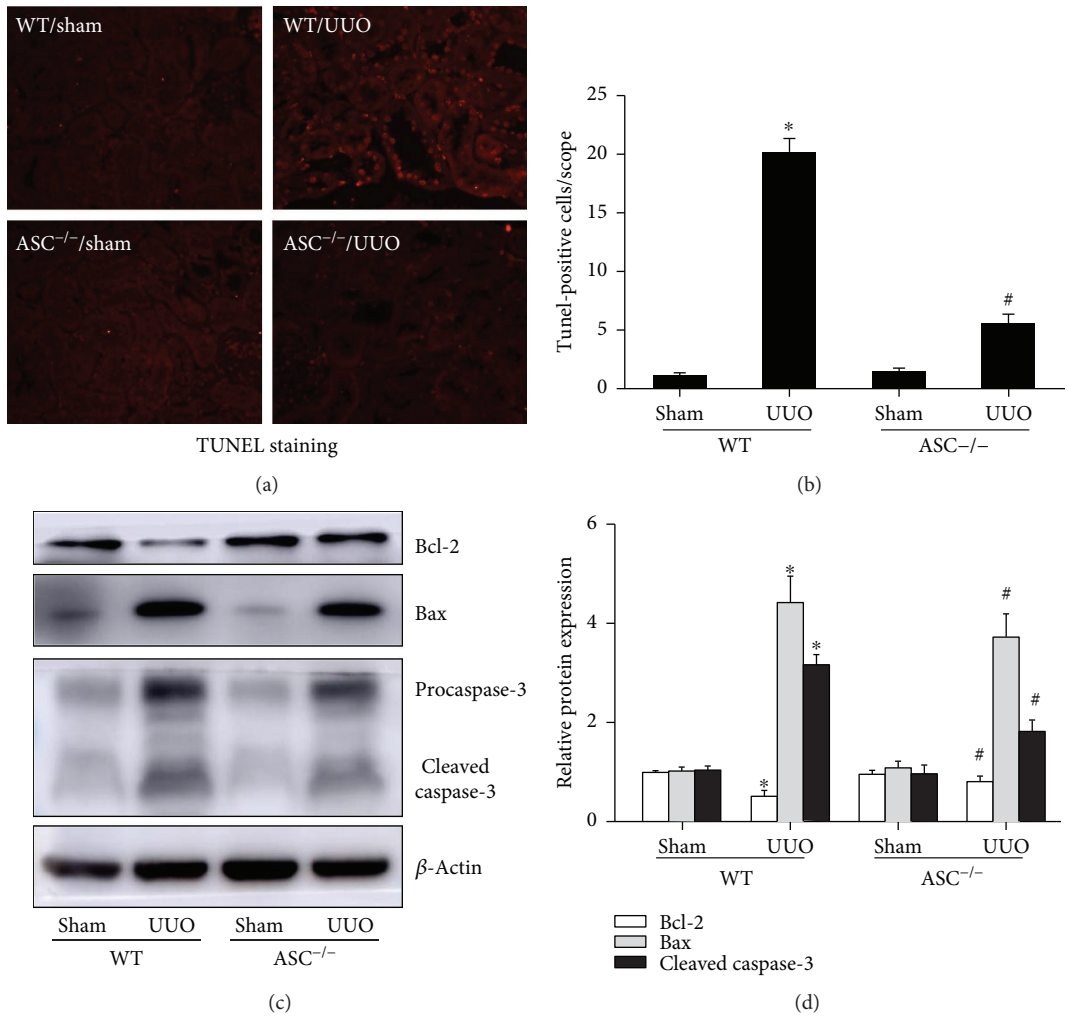

Figure 4: ASC$^{-/-}$ protects against apoptosis associated with UUO. (a) Images of TUNEL staining (magnification, ×200). (b) Average number of TUNEL-positive cells per scope, 10 fields were selected randomly over 6 mice for each group. (c) Immunoblotting for Bcl-2, Bax, and caspase 3 protein expression at day 14 after UUO. (d) Quantification of Bcl-2, Bax, and cleaved caspase 3 levels, normalized against those of β-actin. $^{*}P < 0.05$, WT/sham group versus WT/UUO group. $^{\#}P < 0.05$, ASC$^{-/-}$/UUO group versus WT/UUO group. Data represent the mean ± SEM ($n = 6$).

by UUO. And the expression and maturation of IL-1β and IL-18 were markedly increased in mice at day 14, which supported the results of previous studies. By contrast, ASC knockdown reversed this phenotype to some extent, prompting us to summarize that the protective effect of ASC deletion against renal injury and fibrosis functions partially through the reduction of inflammatory mediators. Notably, the translation of pro-IL-1β or IL-18 and the processing of cleaved cytokines via inflammasome are separated [18]. Our data showed the effects of ASC appeared on both transcripts and proforms evidenced by PCR and Western blot, which suggested that the regulation of ASC$^{-/-}$ on inflammation might be mostly through the inflammasome-independent pathway.

ASC is the major component of the inflammasome protein complex, which relocates into a cytosolic speck and collaborates with caspase 1 upon various PRRs stimuli. Among them, NLRP3 is the best characterized so far. NLRP3 deletion mice displayed less tubular injury, renal inflammation, and fibrosis 14 days after UUO [15]. In humans, NLRP3 expression was increased in renal biopsy samples from patients with CKD [19]. Our previous study also demonstrated that NLRP3$^{-/-}$ significantly decreased cytokine release and preserved kidney ultrastructure in glomerular injury and tubulointerstitial fibrosis [13]. Interestingly, other studies reported contradictory results. Pulskens et al. determined that Nlrp3 knockout mice developed improved early tubular damage and interstitial edema after UUO; however, no statistical difference was found for the level of renal fibrosis [20]. Furthermore, the protective effect of NLRP3 in the early stage in UUO mice was not reversed in the later phase. In the present study, marked glomerular injury and tubulointerstitial fibrosis were observed at day 14 after UUO according to PAS and Masson staining. ASC deletion preserved kidney function via decreasing collagen deposition and reducing epithelial EMT and apoptosis, as shown by immunohistochemistry, TUNEL staining, and Western blotting analyses. Meanwhile, the regulation of inflammatory cytokines was associated with the protective phenotype in ASC

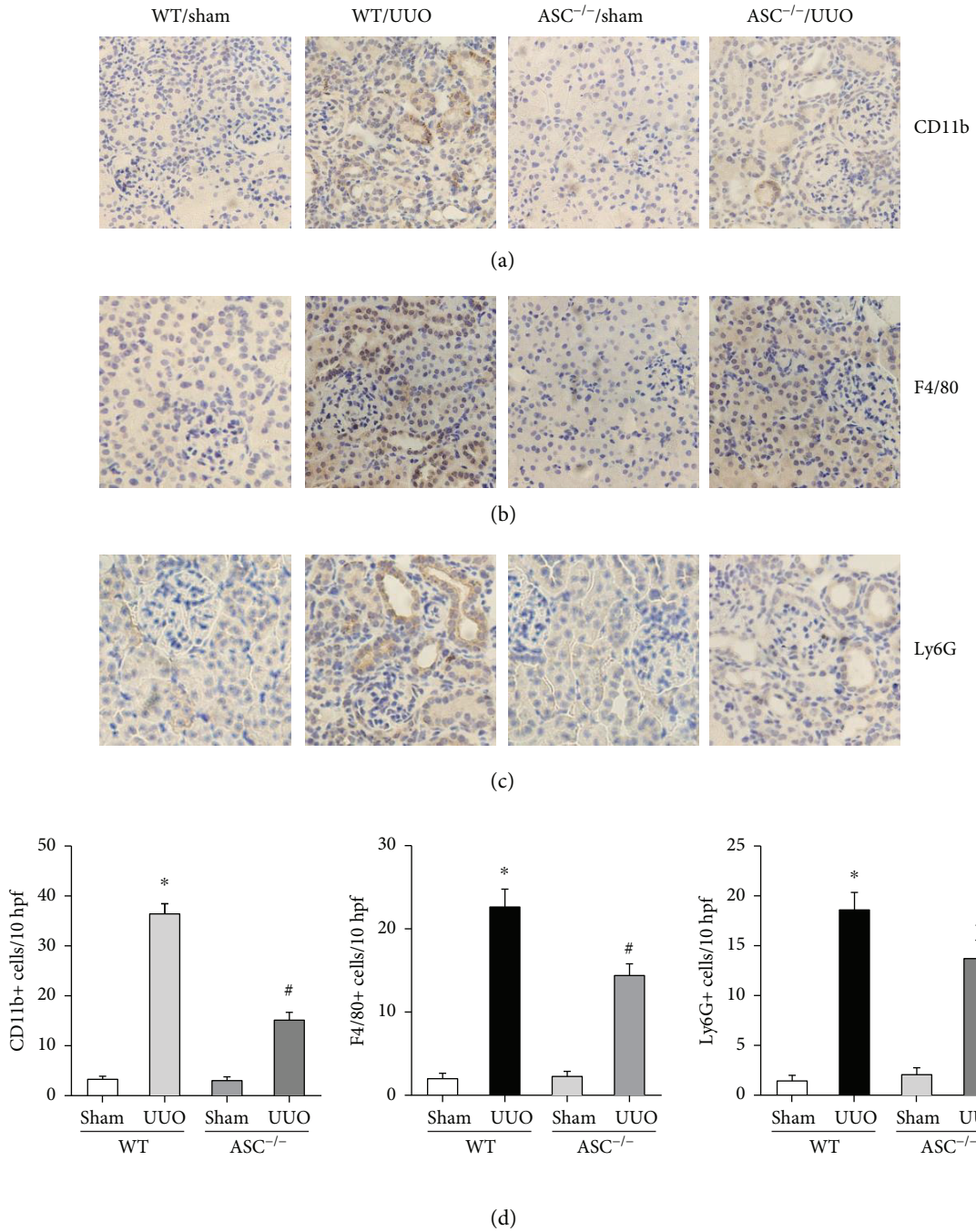

Figure 5: Renal inflammatory cell infiltration after UUO is reduced in ASC$^{-/-}$ mice. Immunohistochemistry (magnification, ×400) of (a) CD11b-positive leukocytes, (b) F4/80-positive macrophages, and (c) Ly6G-positive granulocytes. (d) Quantification of inflammatory cell infiltration (brown pixels) in renal interstitium. $^{*}P < 0.05$, WT/sham group versus WT/UUO group. $^{\#}P < 0.05$, ASC$^{-/-}$/UUO group versus WT/UUO group. Data represent the mean ± SEM ($n = 10$).

knockdown mice. Interestingly, the levels of IL-6 and TNF-α remained stable in ASC$^{-/-}$ mice, which suggested extensive pathways underlying inflammation after UUO independently of the assembly of NLRP3 inflammasome. Taken together, these data indicate that the role of ASC in renal fibrosis is specific for IL-1β and IL-18, which are known to be processed by the inflammasome, though our evidence does not fully support the conclusion that this effect is inflammasome-dependent.

Additionally, ASC could also act independent of NLRP3 or caspase 1. ASC affected MAPK phosphorylation by pathogens and Toll-like receptor agonists, distinct from inflammasome activation [21]. In multiple disease models, the epigenetic regulation of ASC/TMS1 expression was critical in the function of major host defense systems, cellular housekeeping, and carcinogenesis [22]. Ippagunta et al. also determined an independent role of ASC in T cell priming for collagen-induced arthritis [23]. Similarly, Wang et al. showed

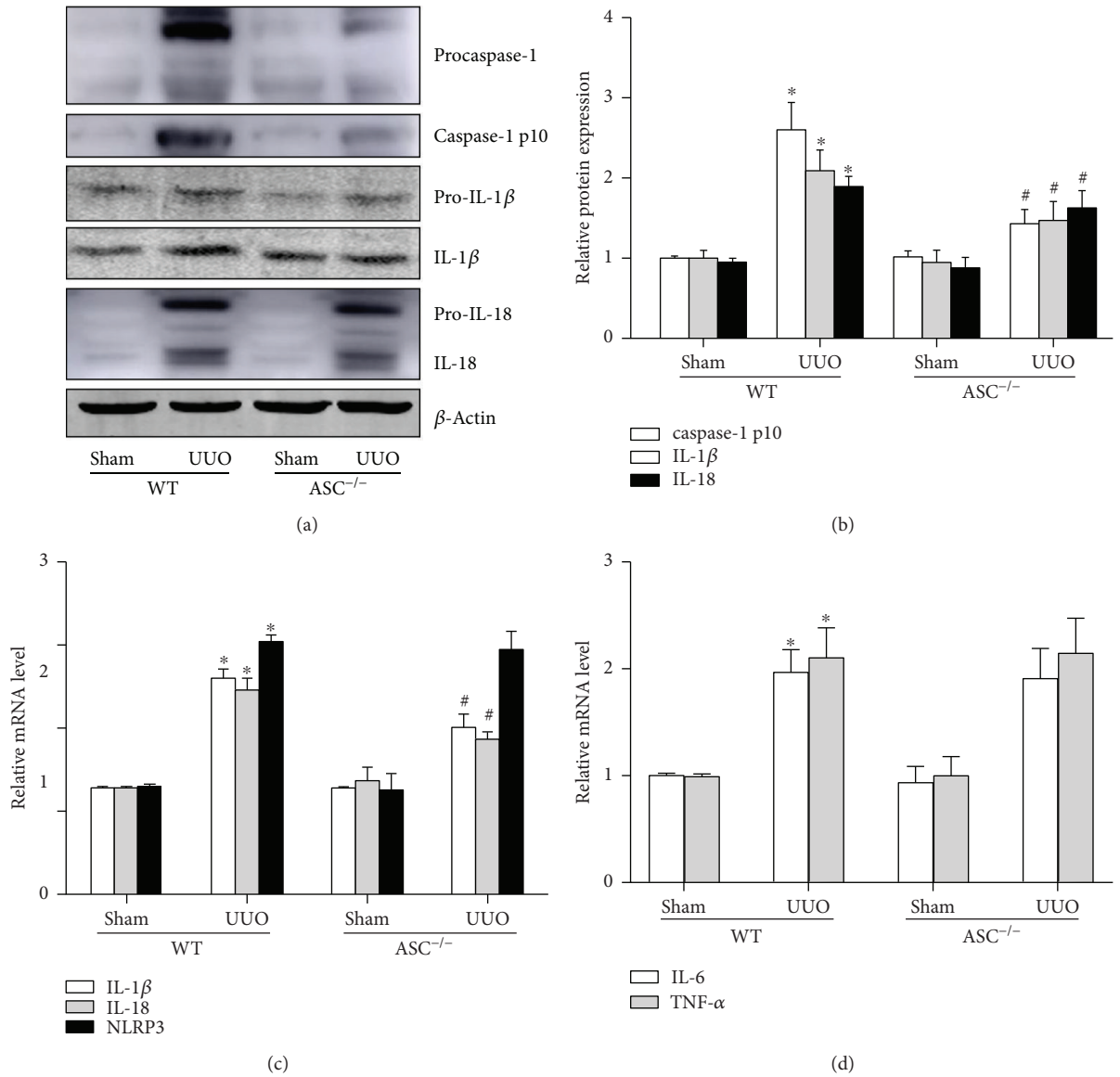

Figure 6: ASC deletion reduces the expression of cytokines that are upregulated by inflammasomes in the UUO model of CKD. (a) Immunoblotting for the level of caspase 1, IL-18, and IL-1β. (b) Quantification analysis of caspase 1, IL-18, and IL-1β levels, normalized against those of β-actin. Semiquantitative analysis of NLRP3, IL-18, and IL-1β (c) and IL-6 and TNF-α gene expression (d) normalized against 18S performed by real-time PCR. $^{*}P < 0.05$, WT/sham group versus WT/UUO group. $^{\#}P < 0.05$, ASC$^{-/-}$/UUO group versus WT/UUO group. Data represent the mean ± SEM ($n = 6$).

that NLRP3 promoted TGF-β signaling, R-Smad activation, and EMT in the kidney renal tubular epithelium. Both NLRP3$^{-/-}$ and ASC$^{-/-}$ would impair the pathway and reduce the expression of related genes in tubular cells, which occurred independently of caspase 1 or inflammasome-regulated cytokines [24]. Overall, the regulation of ASC in renal fibrosis must be tightly controlled and complex. More comprehensive investigations on the underlying mechanisms are required.

ER stress serves as a protective process that enhances the degradation of unfolded proteins and promotes the efficiency of correct folding to maintain protein homeostasis. However, under pathological insults, such as ischemia, infection, hypoxia, and reactive oxygen species (ROS) overload, persistent ER stress leads to ER dysfunction and the activation of the UPR. Numerous data have already identified ER stress as a key factor in tubular epithelial cell atrophy and interstitial fibrosis [25]. ER stress markers, including chaperones and CHOP, were increased in kidney biopsy samples of patients suffering from various kidney diseases [26]. CHOP deletion inhibited the UPR and attenuated aldosterone-induced interstitial fibrosis and apoptosis [27]. Our study also provided evidence of the regulation of ER stress in renal injury through ASC. CHOP was significantly increased in UUO mice, associated with elevated EMT and matrix expansion, while this effect was markedly ameliorated by ASC knockdown. Furthermore, we explored the expression of related pathways to detect the underlying mechanisms.

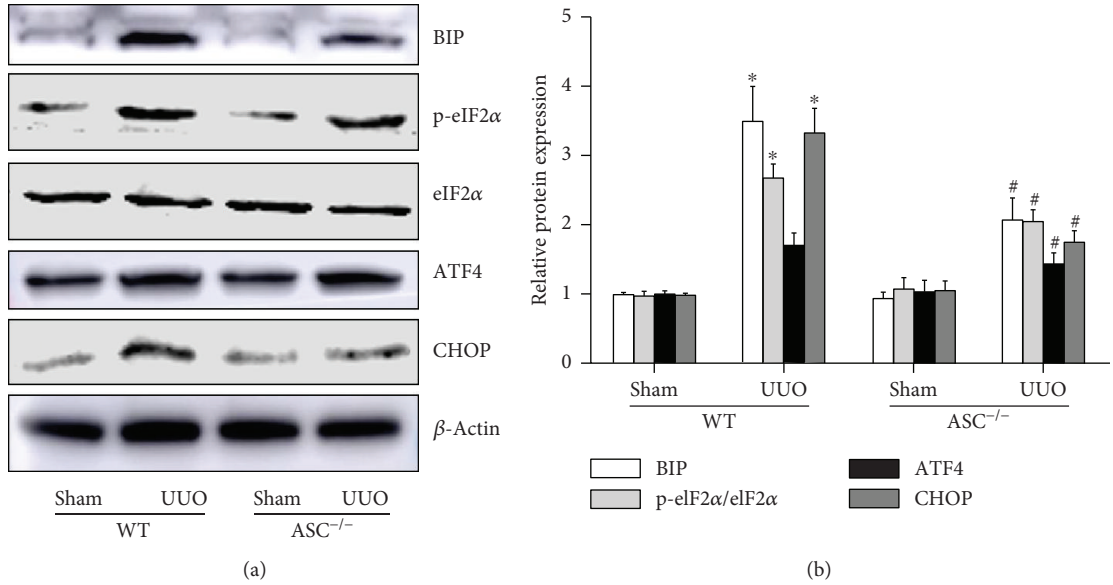

Figure 7: Activation of ER stress induced by UUO is inhibited by ASC deficiency. (a) Immunoblotting for the levels of BIP, p-eIF2α/eIF2α, ATF4, and CHOP. (b) Quantification analysis of BIP, p-eIF2α/eIF2α, ATF4, and CHOP levels, normalized against those of β-actin. $^{*}P < 0.05$, WT/sham group versus WT/UUO group. $^{\#}P < 0.05$, ASC$^{-/-}$/UUO group versus WT/UUO group. Data represent the mean ± SEM ($n = 6$).

There are three main protein sensors that prime ER stress, ATF6, IRE1α, and PERK. These sensors bind to BiP in physiological conditions and dissociate after stimulation, thus activating downstream signaling, including inhibition of translation, induction of apoptosis, and ER-associated degradation [28]. In our UUO mouse model, we evaluated the levels of the PERK pathway involving eukaryotic translation initiation factor 2α (eIF2α), ATF4, and CHOP. Compared with the WT, ASC$^{-/-}$ mice exhibited dramatically lower levels of p-eIF2α and ATF4, which were upregulated by UUO. ER stress-related apoptosis was also reduced. Interestingly, we also detected changes in Bcl-2, Bax, and caspase 3 levels induced by ASC deletion. The death receptor pathway, ER pathway, and mitochondrial pathway are known as the three major pathways of cell apoptosis; therefore, the role of mitochondria cannot be excluded. Consistent with our previous study, mitochondrial dysfunction contributed to renal fibrosis and chronic interstitial inflammation [19]. Similarly, recent reports elaborated an NLRP3-caspase-1-dependent mechanism that relayed ER stress to the mitochondria to promote inflammation [29]. However, the crosstalk between ER and mitochondria remains elusive and requires a more detailed investigation.

In conclusion, our study established an axis of ASC inflammation-ER stress in renal fibrosis and apoptosis. ASC deletion attenuates renal injury, extracellular matrix deposition, and tubular EMT and reduces cellular infiltration and the release of inflammatory cytokines, which are closely related to the regulation of ER stress. Our data strongly support the hypothesis that ASC could serve as an attractive target in the treatment of renal fibrosis in CKD.

Data Availability

No additional data are available.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was sponsored by the Shanghai Pujiang Program (17PJD021), the Interdisciplinary Program of Shanghai Jiao Tong University (YG2017MS07), and the Distinguished Young Scholar of Ninth People's Hospital (jyyq09201701).

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 5270657, 10 pages
https://doi.org/10.1155/2018/5270657

Research Article

Circulating $CD14^+CD163^+CD206^+$ M2 Monocytes Are Increased in Patients with Early Stage of Idiopathic Membranous Nephropathy

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Received 17 January 2018; Revised 2 April 2018; Accepted 16 April 2018; Published 21 June 2018

Academic Editor: Sandra Rayego-Mateos

Aim. To analyze changes in peripheral blood monocytes and their clinical significance in patients with early stage of idiopathic membranous nephropathy (IMN). *Methods.* A total of 27 patients with early stage of IMN and 16 age- and sex-matched healthy controls (HCs) were recruited for the study. The monocyte subset counts in circulation were measured by flow cytometry, and serum interleukin- (IL-) 10 and IL-12 concentrations were tested by enzyme-linked immunosorbent assay. The potential association between clinical signs and monocyte subset counts was analyzed statistically. *Results*. Compared with the HCs, the patients with early stage of IMN had higher counts of $CD14^+CD163^+$, $CD14^+CD163^+CD206^+$, and $CD14^+CD163^+CD206^+CD115^+$ M2-like monocytes. The $CD14^+CD163^+CD206^+$ M2-like cell counts and intracellular IL-10 concentrations in the monocytes were positively correlated with progression in proteinuria. The levels of serum IL-10 were significantly higher in early IMN patients than in the HCs. Furthermore, $CD14^+CD163^+CD206^+$ M2-like cell counts in the patients with incipient IMN were also positively related with 24 h urinary albumin levels and the values of serum M-type phospholipase A2 receptor (PLA2R). *Conclusion*. $CD14^+CD163^+CD206^+$ M2-like monocytes may contribute to the pathologic process in early-stage IMN and could serve as potential markers for evaluating the disease severity.

1. Introduction

Membranous nephropathy (MN) is a common pathological type of nephrotic syndrome in adults. Approximately 30–40% of patients with MN experience gradual progression to chronic kidney disease [1]. MN can generally be divided into idiopathic MN (IMN) and secondary MN according to the different pathogenic factors. IMN accounts for about 75 percent of cases of MN in adults, caused by the deposition of immune complexes of autoantibodies and M-type phospholipase A2 receptor (PLA2R) on the glomerular basement membrane [2, 3].

Macrophage infiltration is a common feature in the inflammatory process initiated by autoantibodies and complement activation. Macrophages play an important role in renal injury [4–6]. Macrophages in the tissue are differentiated from monocytes in the serum. Human peripheral blood monocytes can be classically activated as M1 monocytes and alternatively activated as M2 monocytes [7–10], initially put forward for macrophages. Monocytes highly express CD14,

a kind of Toll-like receptor, which can identify monocyte populations. CD163 is a classical symbol of M2 cells. Whereas there is no specific surface marker to identify M1 cells, $CD14^+CD163^-$ cells are considered to be M1-like cells [11]. Another characteristic of activated M2 monocytes is increased expression of CD206 and CD115 [12, 13]. Upon activation, M1 monocytes release proinflammatory cytokines, inducing a Th1 immune response, while M2 monocytes secrete anti-inflammatory mediators, such as interleukin- (IL-) 10, to trigger a Th2 immune response. M1 cells are considered to be antimicrobial and cytotoxic, while M2 monocytes are responsible for tissue repair and have profibrotic action [13, 14].

Previous reports have found that M2 macrophages participated in the pathogenesis of several renal diseases, including anti-neutrophil cytoplasmic antibody- (ANCA-) associated glomerulonephritis, IgA nephropathy [15, 16], proliferative glomerulonephritis [17], and human acute tubulointerstitial nephritis [18], which are closely associated with disease activity in patients with systemic lupus erythematosus (SLE) [19, 20]. Macrophage infiltration is part of the pathological process in IMN. However, the differences in the various types of polarized monocytes/macrophages in IMN have not been clarified.

In this study, we analyzed the counts of different monocyte subpopulations in peripheral blood in patients with early IMN and in healthy controls (HCs). Furthermore, we investigated the potential relationship between different monocyte subsets and the overall laboratory data.

2. Materials and Methods

2.1. Patients and Controls. A total of 27 patients with newly diagnosed IMN and 16 HCs were recruited for the study at the inpatient service of the Department of Nephrology, the First Hospital of Jilin University (Changchun, China), from January 2017 to December 2017. Patients met the criteria for IMN with pathology-confirmed diagnosis established by the World Health Organization, all in I-II stage histologically. The patients with IMN were classified into three groups according to the 24 h urine protein levels: <4 g, group A ($n = 9$); 4–8 g, group B ($n = 8$); and ≥8 g, group C ($n = 10$). Patients with secondary MN, such as lupus nephritis and other forms of primary nephritis, were excluded from the study. None of the participants had taken immunosuppressive drugs in the previous 6 months, and there was no history of autoimmune and inflammatory diseases, malignant tumors, diabetes mellitus, and atherosclerosis. The 16 age-, sex-, and ethnicity-matched HCs were recruited simultaneously. Written informed consent was provided from all subjects. The Human Ethics Committee of Jilin University approved the study protocol. Table 1 showed the demographic and clinical characteristics of the participants.

2.2. Treatment and Follow-Up. Patients were treated orally with a combination of prednisolone (Tianyao Pharmaceuticals, Tianjin, China) and tacrolimus (Astellas Pharmaceuticals, Ireland). Prednisone was initially administered at 0.5 mg/kg daily for the first two months, and the dose was gradually reduced according to the response. Tacrolimus was taken initially at 0.05 mg/kg daily and adjusted to 5–10 ng/mL in blood. The patients were followed up for 12 weeks. Overall, there were complete records available for six patients, and the other 21 patients were lost during follow-up. Blood samples from the six patients were collected again for subsequent laboratory examinations.

2.3. Flow Cytometry Analysis. Venous blood (8 mL) was obtained from all participants. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation with the Ficoll-Paque PLUS system (Amersham Biosciences, Little Chalfont, UK). PBMCs at a density of 1×10^6/tube were stained in duplicate with the following antibodies: BV510-anti-CD14, PE-anti-CD115 (BD Biosciences, US), PE/Cy7-anti-CD163, and APC/Cy7-anti-206 (BioLegend, US) at 4°C for 30 min. Then, we fixed and permeabilized the cells with a fixation/permeabilization kit (BD Biosciences).

To clarify the function of monocyte subsets, PBMCs (1×10^6/tube) were stimulated in duplicate with lipopolysaccharide, phorbol myristate acetate (50 ng/mL), and ionomycin (1.0 μg/mL, Sigma-Aldrich, St. Louis, US) in RPMI 1640 medium mixed with 10% fetal bovine serum for 2 h at 37°C in 5% CO_2 and exposed to brefeldin A (GolgiPlug; BD Biosciences) for 4 h, as described in previous studies [9, 10, 21, 22]. Then, the cells were washed, followed by staining with BV510-anti-CD14 and PE/Cy7-anti-CD163. Subsequently, they were fixed and permeabilized and stained with PE-CF594-anti-IL-10 and BV421-anti-IL-12 (BD Biosciences).

Fluorescence minus one (FMO) was used for identifying the positive and negative populations with flow cytometry. The frequencies of different monocyte subsets were assessed on a FACS Calibur instrument (BD, Franklin Lakes, US), and the data were dealt with FlowJo software (v5.7.2; TreeStar; Ashland, US).

2.4. Enzyme-Linked Immunosorbent Assay (ELISA). The serum IL-10 and IL-12 concentrations were measured using ELISA performed with a human IL-10 ELISA kit and a human IL-12 ELISA kit, according to the instructions from the manufacturer (MultiSciences, Hangzhou, China). Briefly, individual serum sample was subjected to ELISA, and the serum IL-10 and IL-12 concentrations were analyzed according to the standard curve established.

2.5. Statistical Analysis. Variables were shown as median and range values. We employed the Mann–Whitney U nonparametric test to evaluate the differences among groups. The relationship between variables was analyzed by the Spearman rank correlation test. All of the data were carried out with the SPSS version 19.0 software. P value of <0.05 represented statistically significant.

3. Results

3.1. Patient Characteristics. The demographic characteristics did not significantly differ with respect to the distribution of age, sex, serum uric acid concentrations, triglyceride concentrations, total cholesterol concentrations, estimated

Table 1: The demographic and clinical characteristics of participants.

	IMN group A $n = 9$	IMN group B $n = 8$	IMN group C $n = 10$	HC $n = 16$
Age, years	38 (27–58)	45 (28–64)	49 (25–60)	42 (25–63)
Female/male	5/4	3/5	6/4	7/9
Monocytes (10^9/L)	0.3 (0.2–0.45)	0.32 (0.21–0.5)	0.33 (0.2–0.6)	0.35 (0.18–0.35)
Serum uric acid (μmol/L)	311 (163–450)	363 (300–424)	376 (235–499)	330 (230–410)
Triglycerides (mmol/L)	1.6 (0.6–3.77)	2.0 (0.87–5.34)	2.1 (1.66–5.26)	1.3 (0.42–1.78)
Total cholesterol (mmol/L)	6.8 (5.8–13)	6.9 (4.4–9)	7.4 (4.8–9.1)	3.9(2.8–5.6)
Serum albumin (g/L)	25 (19.4–39)*	22 (15–30)*	21 (17–27)*	42 (40–52)
Urinary proteins (g/24 h)	2 (2–5.3)*	5.65 (4.4–7) *	9.8 (8–11)*	0.01 (0–0.05)
eGFR (CKD-EPI) (mL/min/1.73 m^2)	119 (99–127)	108 (96–124)	99.5 (90–125)	118 (102–130)
Serum PLA2R (RU/mL)	17.8 (3–76)*	26 (1.6–77.7)*	72 (16.9–170)*	1.5 (0–2.7)

Data shown are median and range. Normal values: monocytes: 0.1–0.6 (10^9/L), serum uric acid: 155–357 (μmol/L), triglycerides: 0.28–1.8 (mmol/L), total cholesterol (mmol/L): 2.6–6.0 (mmol/L), serum albumin: 40.00–55.00 (g/L), urinary proteins: 0–0.2 (g/24 h), eGFR: 80–120 (mL/min/1.73 m^2), and serum PLA2R: 0–14 (RU/mL). IMN: idiopathic membranous nephropathy; HC: healthy control; eGFR: estimated glomerular filtration rate. *$P < 0.05$ versus the controls.

glomerular filtration rate (eGFR), and monocyte counts among the different groups (Table 1). The levels of 24 h urinary protein and serum PLA2R were significantly higher in early-stage IMN patients than in the HCs, but serum albumin concentrations were lower in the patients with early IMN than in the HCs ($P < 0.05$).

3.2. Increased $CD14^+CD163^+$ M2 Monocyte Counts in Patients with Early-Stage IMN. We analyzed the circulating $CD14^+CD163^-$ M1-like and $CD14^+CD163^+$ M2-like monocyte counts by flow cytometry. The $CD14^+CD163^+$ M2 monocyte counts were significantly higher in the patients with early-stage IMN than in the HCs ($P = 0.021$; Figure 1), but there were no statistically significant difference among the three IMN subgroups. In addition, the $CD14^+CD163^-$ M1-like monocyte counts did not significantly differ between the IMN patients and controls, or among the three IMN subgroups. The data indicated that patients with incipient IMN had increased $CD14^+CD163^+$ M2-like monocyte counts.

3.3. Increased $CD14^+CD163^+CD206^+$ M2 Monocyte Counts in Patients with Early-Stage IMN. Subpopulations of monocytes have distinct surface markers, such as CD206 and CD115, and functions. To figure out the importance of these subpopulations of M2 monocytes, we further analyzed the $CD206^+$ and $CD115^+$ M2 monocyte counts in patients with IMN and HCs by flow cytometry. The $CD14^+CD163^+$ $CD206^+$ and $CD14^+$ $CD163^+$ $CD115^+$ $CD206^+$ M2 monocyte counts were higher in the patients of early-stage IMN than those in the HCs (Figure 2). However, no significant differences were shown in the $CD14^+CD163^+CD115^+$ M2 monocyte counts between the patients and the HCs, or among the three subgroups (all $P > 0.05$, Figure 2(b)). Furthermore, the blood $CD14^+CD163^+CD206^+$ and $CD14^+CD163^+$ $CD115^+CD206^+$ M2 monocyte counts were significantly higher in group C patients with IMN than in group B individuals with IMN. Likewise, the counts of those subsets of M2 cells were significantly higher in group B individuals with IMN than in group A subjects with IMN (all $P < 0.05$, Figures 2(b)). Collectively, the increased counts of $CD206^+$ M2-like monocytes were positively correlated with the development of IMN.

3.4. Intracellular and Serum Cytokine Concentrations in Patients with Early-Stage IMN. To regulate the function of T cell, M1 monocytes mainly exert a proinflammatory effect by secreting IL-12, whereas M2 monocytes produce IL-10, thereby exerting an anti-inflammatory effect. Therefore, we analyzed the IL-12^+ M1 and IL-10^+ M2 monocyte counts, as well as the serum IL-10 and IL-12 concentrations by flow cytometry and ELISA, respectively. As shown in Figures 3(a) and 3(b), patients with IMN had higher IL-10^+ M2 monocyte counts than the HCs, and the counts were increased in parallel with the severity of IMN (group C > group B > group A; all $P < 0.05$, Figure 3(b)). In contrast, the IL-12^+ M1 monocyte counts did not significantly differ between patients with IMN and HCs ($P > 0.05$, data not shown). Moreover, the serum IL-10 concentrations were generally higher in patients with IMN than in HCs (all $P < 0.05$, Figure 3(c)), but with no significant differences among the three IMN subgroups. By contrast, the IL-12 concentration did not significantly differ between the patients with IMN and HCs ($P > 0.05$, data not shown).

3.5. Correlation between Clinical Parameters and Different Monocyte Subsets. To further understand the roles of monocyte subsets in incipient IMN, we analyzed the potential association between the counts of various monocyte subpopulations and the clinical parameters. $CD14^+$ $CD163^+CD206^+$ cell counts were positively related with the 24 h urine protein level, 24 h urine albumin concentrations, and serum PLA2R levels (all $P < 0.05$, Figures 4(a)–4(c)). These data revealed that the increased $CD14^+$ $CD163^+CD206^+$ M2-like monocyte counts were closely associated with the severity of IMN.

3.6. Clinical Parameters and Numbers of M2 Monocytes in IMN Patients following Treatment. Next, we evaluated

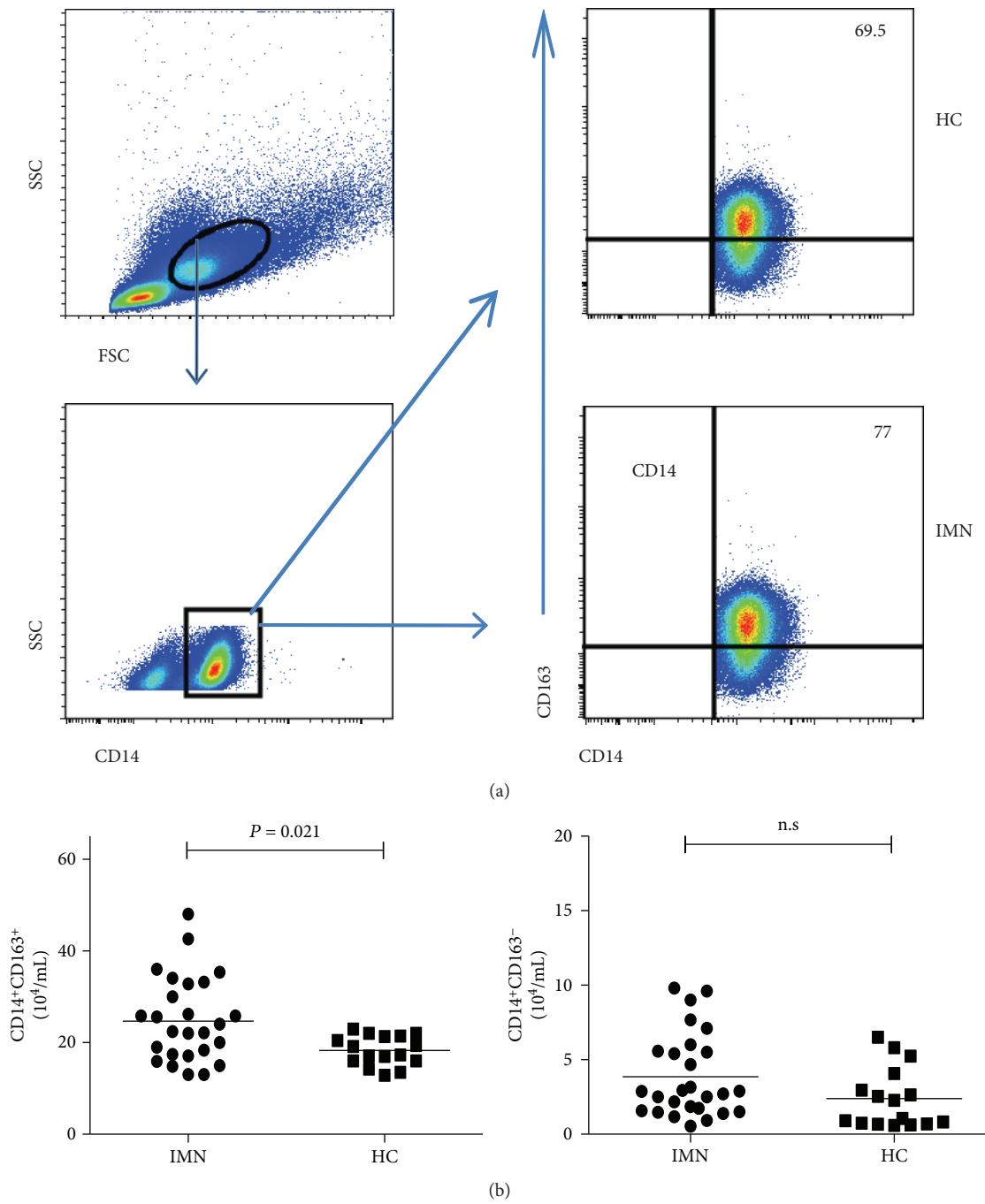

Figure 1: Flow cytometry analysis of the counts of different subpopulations of monocytes. PBMCs from IMN patients and HC were stained with anti-CD14 and anti-CD163. The cells were gated firstly on mononuclear cells and secondly on $CD14^+$ monocytes. Afterwards, the counts of $CD14^+CD163^-$ M1-like and $CD14^+CD163^+$ M2-like monocytes were determined by flow cytometry. (a) Representative frequency of monocyte subpopulations. (b) Quantitative analysis of monocyte subpopulations. Horizontal lines show the median values.

clinical responses and quantitative changes of M2 subsets in IMN patients following the prescribed therapy for 12 weeks. As shown in Table 2, the level of 24 h urine protein decreased, while the serum albumin levels increased significantly in these six patients. There was no significant change detected in the values of other clinical indexes. Unfortunately, there were no significant differences in the numbers of circulating $CD14^+CD163^+$, $CD14^+CD163^+CD206^+$, and $CD163^+CD206^+IL\text{-}10^+$ M2-like monocytes before and after treatment in patients with IMN. However, the counts of these M2 subsets significantly increased after treatment in the patients compared to those in the HCs (all $P < 0.05$, Figure 5).

4. Discussion

Monocytes, as progenitors of dendritic cells or macrophages in tissues, are important regulators of the immune response.

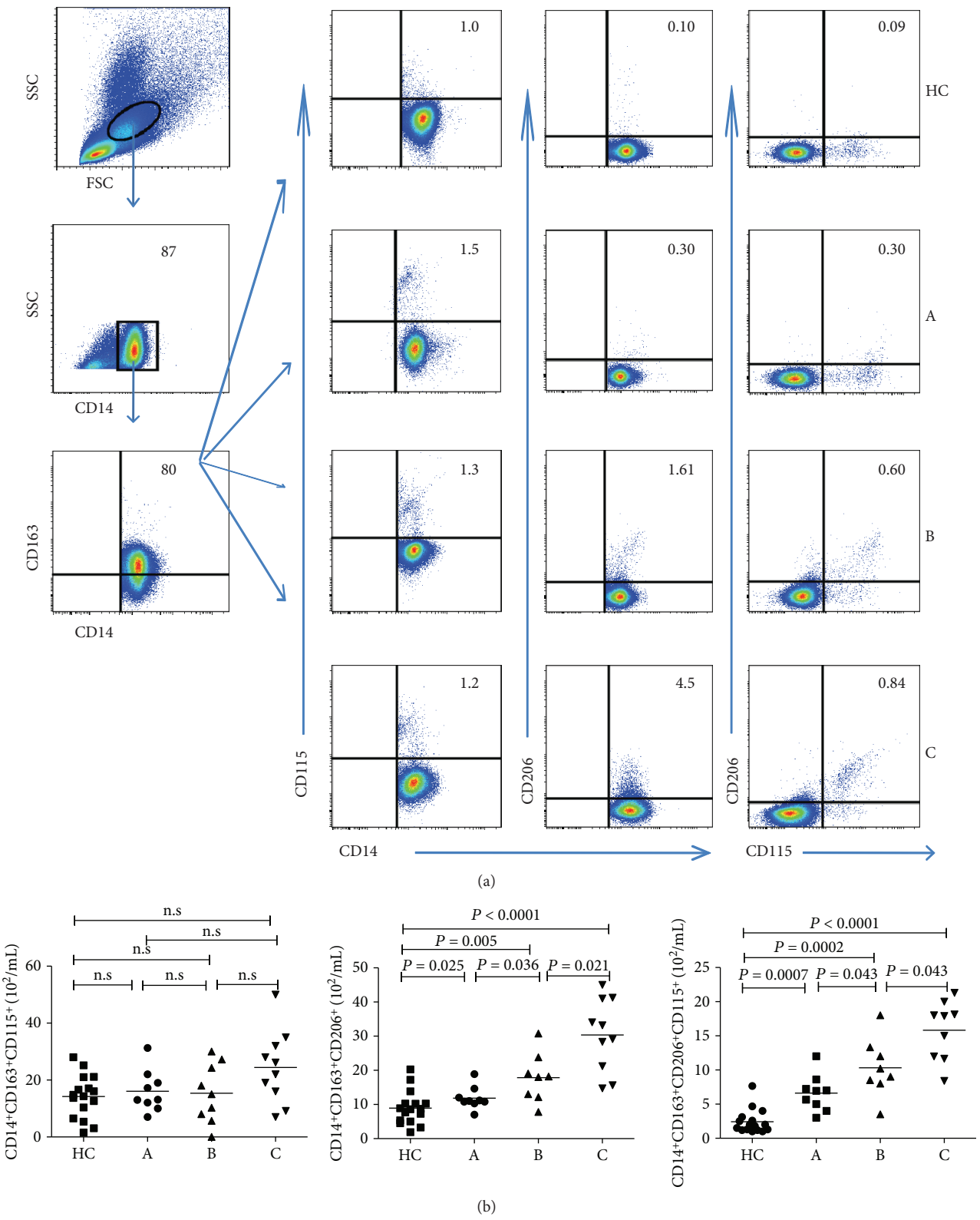

Figure 2: Flow cytometry analysis of CD206$^+$ and CD115$^+$ M2 monocytes. PBMCs from individual subjects were stained with anti-CD14, anti-CD163, anti-CD206, and anti-CD115. The numbers of CD14$^+$CD163$^+$CD206$^+$, CD14$^+$CD163$^+$CD115$^+$, and CD14$^+$CD163$^+$CD206$^+$CD115$^+$ M2-like monocytes were assessed by flow cytometry (a, b). Representative FACS charts and the median values for each group are shown.

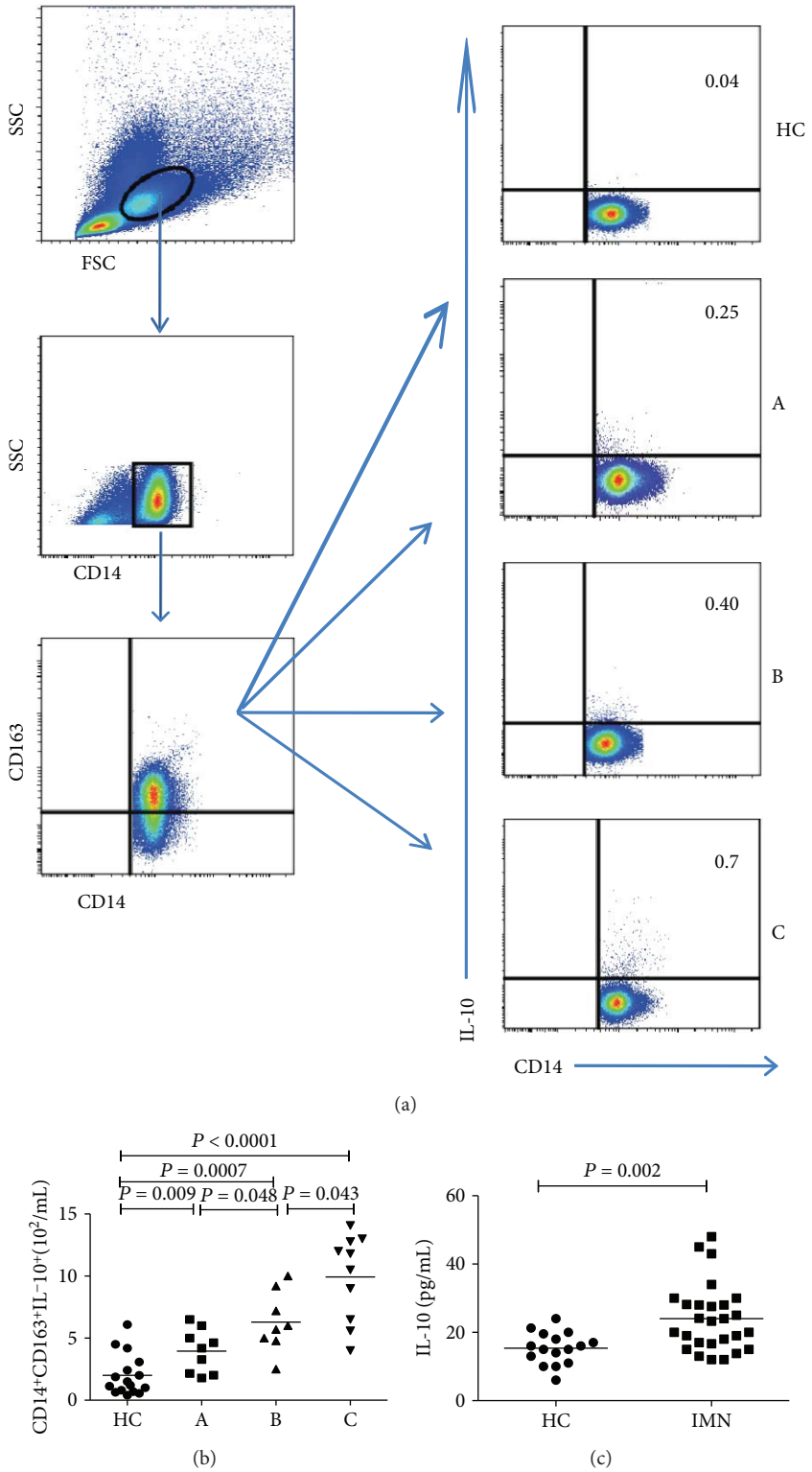

Figure 3: Analysis of IL-10$^+$ M2 monocytes and the concentrations of serum IL-10. PBMCs were obtained from all subjects and stimulated in vitro. The cells were stained in duplicate with anti-CD14 and anti-CD163, then fixed and permeabilized, following the staining with anti-IL-10. The numbers of CD14$^+$CD163$^+$IL-10$^+$ M2-like monocytes in all participants were analyzed by flow cytometry. The concentrations of serum IL-10 were determined by ELISA. Data are representative charts or the mean numbers of the monocyte subsets and the mean values of serum IL-10 in all groups. The horizontal lines indicate the median values.

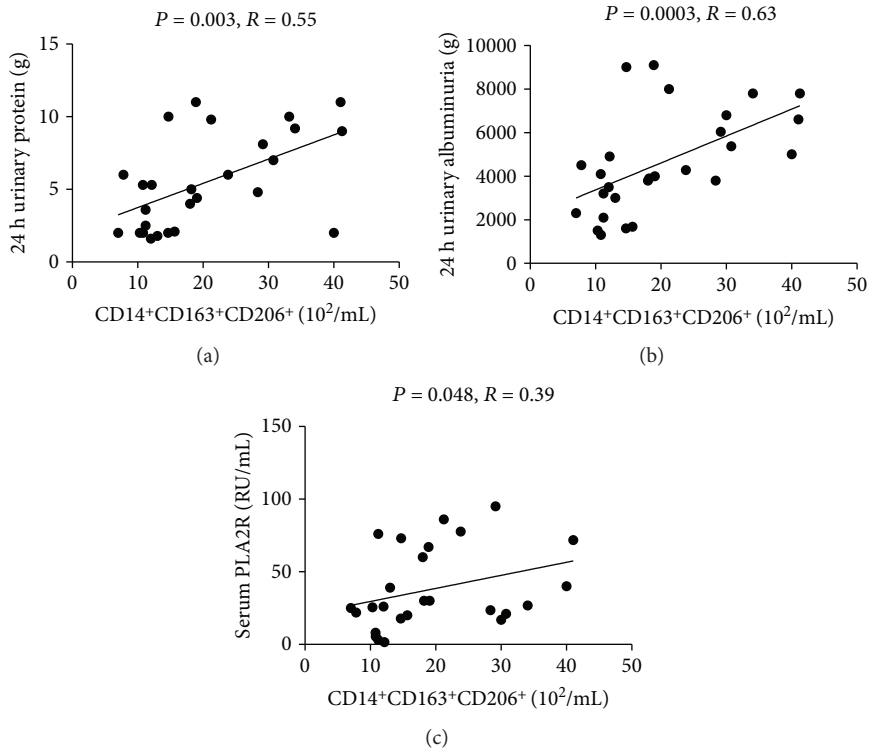

Figure 4: Correlation analysis. The potential correlations between the numbers of monocyte subsets and the values of clinical features in IMN patients were analyzed. The numbers of circulating $CD14^+CD163^+CD206^+$ M2 cells were positively correlated with the values of 24 h urinary protein (a), 24 h urinary albumin (b), and serum PLA2R (c), respectively. Each point represents an individual subject.

Table 2: Effect of treatment on the values of clinical measures in follow-up IMN patients.

	Before treatment	After treatment
Age, years	39 (28–58)	
Female/male	3/3	
Monocytes (10^9/L)	0.3 (0.2–0.4)	0.6 (0.4–0.8)*
Serum uric acid (μmol/L)	300 (166–450)	320 (180–420)
Triglycerides (mmol/L)	2.5 (0.7–3.6)	1.9 (0.97–4)
Total cholesterol (mmol/L)	6.5 (5.8–10)	6 (4.3–7.8)
Serum albumin (g/L)	25 (18–28)	30 (26–35)*
Urinary proteins (g/24 h)	7.8 (2.5–10)	1.6 (0.5–3)*
eGFR (CKD-EPI) (mL/min/1.73 m^2)	110 (95–120)	118 (106–125)

Data are presented as median range. *$P < 0.05$ versus the values before treatment.

Similar to macrophages, monocytes can express surface markers with different functions at different stages of renal diseases [13, 23]. Previous observations have indicated that alternatively activated M2 cells are involved in the pathogenic mechanism of some renal diseases by inhibiting inflammatory activity and promoting fibrosis [13, 24, 25]. However, only a few studies have been performed on the association of monocyte phenotypes in the development of IMN.

The current study investigated the numbers of circulating monocyte subpopulations and their potential association with disease severity in patients with early-stage IMN. In the study, the patients had higher $CD14^+CD163^+$ M2 monocyte counts than the HCs. Furthermore, the numbers of $CD14^+CD163^+CD206^+$ M2 monocyte not only increased in the three subgroups of IMN but also shared the same changes in trend with disease progression. In other words, the $CD206^+$ M2 cells were positively correlated with the degree of proteinuria. Similarly, a previous study has shown that $CD206^+$ monocytes are associated with proteinuria in acute tubulointerstitial nephritis [18]. Therefore, our findings suggested that the increased $CD14^+CD163^+CD206^+$ M2-like monocytes may contribute to the pathogenesis of incipient IMN in adults and could serve as a sensitive indicator for evaluating IMN severity. The mechanism may be as follows: macrophage infiltration in tubulointerstitial lesion is a common case in the pathological process in incipient IMN. And the extent of proteinuria is a major determinant of tubulointerstitial damage. We hypothesized that such damage could trigger peripheral $CD14^+CD163^+CD206^+$ M2-like monocytes, which may be stimulated by IL-4 and IL-13 from Th2-type immune responses in IMN [26, 27], into renal tissues and contribute to tissue repair. Although having protective effect on renal injury, M2 monocytes can secrete fibronectin involved in renal fibrosis [13]. IMN can develop into chronic kidney disease characterized by progressive renal fibrosis. It is possible that increased $CD14^+CD163^+CD206^+$ monocytes have profibrotic effect while they

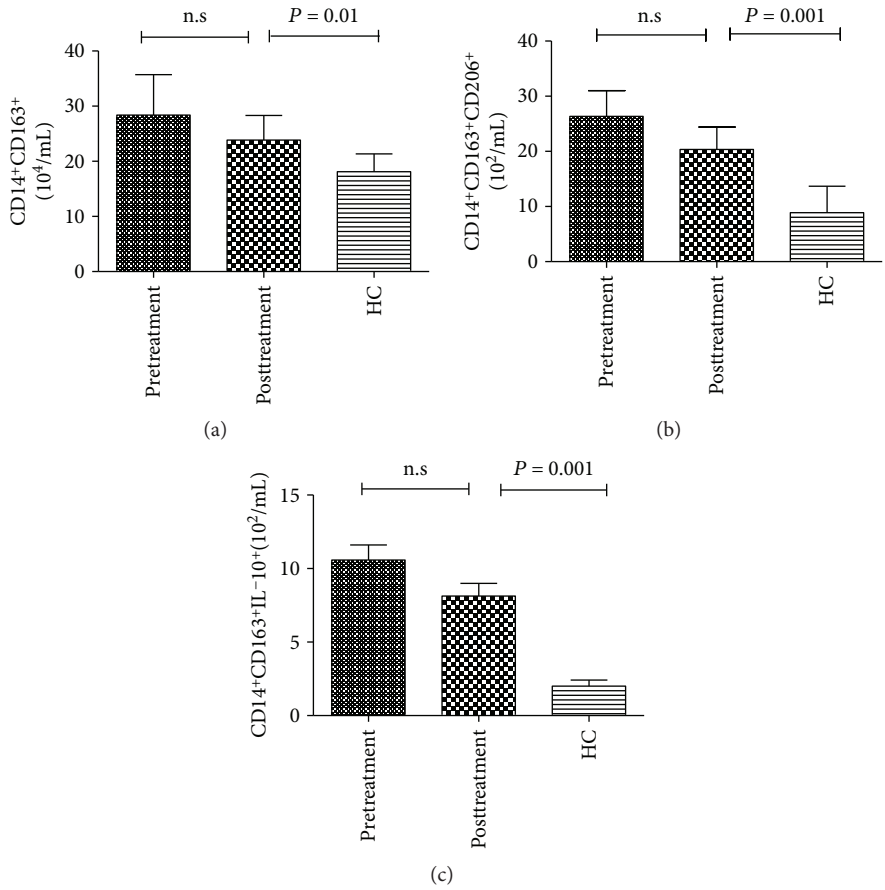

Figure 5: Changes in the counts of M2 monocyte subsets after drug treatment. The counts of different subsets of M2 cells were compared in patients before and after treatment and HCs.

ameliorate proteinuria in IMN. However, the hypothesis should be investigated in detail in future studies.

IL-10 is an important cytokine with respect to its effect on decreasing inflammation and triggering immunosuppressive activities [28]. Our observations have revealed upregulated concentrations of serum IL-10 in early-stage IMN patients, consistent with the previous reports [29, 30]. Furthermore, intracellular IL-10 concentrations in $CD14^+CD163^+$ M2 monocytes were found to be positively correlated with protein progression, but the serum IL-10 concentrations did not seem to be significantly associated with it. The positive relationship may reflect a functional response corresponding to increased numbers of $CD14^+CD163^+CD206^+$ M2-like monocytes. This result likely reflects the fact that serum IL-10 is also released by other immunocytes, such as Th2, regulatory T cells, and T follicular helper cells [29, 30], besides M2 monocytes. Above all, $IL\text{-}10^+$ M2 cells might be another useful indicator for evaluating the severity of incipient IMN.

The PLA2R levels have been clinically used as good biomarkers for evaluating IMN development, progression, and recurrence. Therefore, the association between monocyte subsets and clinical features in patients with incipient IMN was analyzed. The $CD14^+CD163^+CD206^+$ M2-like monocyte counts in the patients with IMN were positively correlated with the 24 h urine albumin and serum PLA2R levels, further supporting that the $CD14^+CD163^+CD206^+$ M2-like monocytes may play a potential role in the pathogenesis of IMN in early stage. And these subsets could provide sensitive markers for disease severity of IMN.

After 12 weeks of follow-up, the therapy resulted in partial remission in six patients with IMN based on the levels of 24 h urine protein. Although there were no differences in the numbers of M2 subsets before and after treatment, circulating $CD14^+CD163^+CD206^+$ M2-like cells in the patients after treatment were significantly greater than those in HCs, indicating that $CD14^+CD163^+CD206^+$ M2-like cells may be involved in the pathogenic process of IMN. The lack of statistical significance may stem from the small group of patients and/or the short follow-up period. Therefore, we will further study these effects with more patients and over a long-term follow-up period beyond 6 months. We also recognized other limitations of this preliminary study, such as the lack of functional analysis of $CD206^+$ M2-like cells. Accordingly, we are planning to conduct more detailed analyses of the role of M2 monocytes using a cell culture model and murine model of IMN.

In conclusion, the present findings demonstrate that the immune status of patients with early-stage IMN is closely associated with $CD14^+CD163^+CD206^+$ M2-like

monocytes. Thus, $CD14^+CD163^+CD206^+$ M2-like monocytes may serve as sensitive indicators for evaluating the severity of IMN. Nevertheless, the mechanism underlying the effect of $CD206^+$ M2 monocytes in the pathogenesis of IMN requires further study.

Data Availability

The data used to support the findings of this study have not been made available due to patient privacy.

Conflicts of Interest

The authors declare no financial or commercial conflicts of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. 30972610, 81273240), Jilin Province Science and Technology Agency (no. 20160101037JC, 20170622009JC, 2017C021, and 2017J039), Norman Bethune Program of Jilin University (2012206), and the fund of the State Key Laboratory of Kidney Diseases in PLA General Hospital.

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