Collectin-11 Promotes the Development of Renal Tubulointerstitial Fibrosis

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ABSTRACT

Collectin-11 is a recently described soluble C-type lectin, a pattern recognition molecule of the innate immune system that has distinct roles in host defense, embryonic development, and acute inflammation. However, little is known regarding the role of collectin-11 in tissue fibrosis. Here, we investigated collectin-11 in the context of renal ischemia-reperfusion injury. Compared with wild-type littermate controls, Collec11 deficient (CL-11−/−) mice had significantly reduced renal functional impairment, tubular injury, renal leukocyte infiltration, renal tissue inflammation/fibrogenesis, and collagen deposition in the kidneys after renal ischemia-reperfusion injury. In vitro, recombinant collectin-11 potently promoted leukocyte migration and renal fibroblast proliferation in a carbohydrate-dependent manner. Additionally, compared with wild-type kidney grafts, CL-11−/− mice kidney grafts displayed significantly reduced tubular injury and collagen deposition after syngeneic kidney transplant. Our findings demonstrate a pathogenic role for collectin-11 in the development of tubulointerstitial fibrosis and suggest that local collectin-11 promotes this fibrosis through effects on leukocyte chemotaxis and renal fibroblast proliferation. This insight into the pathogenesis of tubulointerstitial fibrosis may have implications for CKD mediated by other causes as well.


CKD is a progressive loss of kidney function over a period of time. CKD was ranked 19th in the list of causes for global deaths in 2013. Prevalence of CKD is estimated to be 8%–16% worldwide and expected to rise substantially in the coming decades.1 AKI is one of the main causes of CKD, among other causes (e.g., diabetes and hypertension, infectious GN, renal vasculitis).2 Renal fibrosis is the principal process underlying of the progression of CKD to ESRD. Progressive tubulointerstitial fibrosis is the final common pathway for all kidney diseases leading to ESRD. Because there are currently no...
specific treatments for tubulointerstitial fibrosis, a deeper understanding of the molecular and cellular basis of tubulointerstitial fibrosis will be beneficial to the development of effective strategies that diminish or even reverse tubulointerstitial fibrosis in CKD.

Renal tubulointerstitial fibrosis is characterized by progressive loss of renal function and renal histologic lesions mainly including inflammatory cell infiltration, tubule damage (e.g., tubular atrophy, tubule loss), and accumulation of extracellular matrix (ECM) (collagen deposition).\textsuperscript{4,5} The pathogenesis of tubulointerstitial fibrosis is complex, involving multiple cell types and molecular pathways.\textsuperscript{4,5} The major cell type involved in production of ECM is the myofibroblast which produces a large amount of collagen I (COL I) and fibronectin (FN).\textsuperscript{6,7} Myofibroblasts mainly differentiate from renal interstitial fibroblasts. Other types of cells in the kidney such as bone marrow–derived fibrocytes, stromal mesenchymal cells, and renal tubular epithelial cells have been reported to be able to transform into myofibroblasts.\textsuperscript{8} The inflammatory microenvironment of the kidney after renal injury is thought to play a key role in determining the dynamic balance between tissue destruction and repair, and ongoing inflammation including inflammatory cell infiltration and local production/release of proinflammatory and profibrogenic molecules drives the fibrotic process.\textsuperscript{9}

Collectins (soluble collagenous C-type lectins) are a part of the innate immune system.\textsuperscript{10,11} Well described collectins include mannose-binding lectin (MBL) and lung surfactant proteins (e.g., SP-A). They function as pattern recognition receptors that bind to carbohydrates or carbohydrate moieties on the surface of pathogens (e.g., monosaccharides, mannose-containing glycans, LPS, DNA, microorganisms) via interaction with the carbohydrate recognition domain.\textsuperscript{12}–\textsuperscript{14} Collectin-11 (CL-11; also known as CL-K1 and encoded by Collec11) is a recently described member of the collectin family and displays structural similarities with MBL, SP-A, and SP-D. CL-11 consists of a carbohydrate recognition domain, followed by a neck region and a collagen-like region, and is known to bind to various molecules/molecular patterns (e.g., monosaccharides, mannose-containing glycans, LPS, DNA, microorganisms) via interaction with the carbohydrate recognition domain.\textsuperscript{15–18} CL-11 has a wide tissue distribution; high-level expression was found in the kidney, liver, and adrenal gland.\textsuperscript{17} CL-11 is known to play important roles in embryonic development and host defense,\textsuperscript{19,20} whereas relatively little is currently known about the pathogenic roles of CL-11.

Our recent work in a murine model of renal ischemia/reperfusion (IR) injury demonstrated that CL-11 has a pathogenic role in AKI.\textsuperscript{21} However, the effect of CL-11 on chronic renal inflammation and tissue fibrosis is presently unknown. Such information will improve our understanding of diverse functions of CL-11 in the pathogenesis of renal injury. Previous studies have shown that many factors (e.g., Wnt pathway, Hedgehog pathway, Gremlin1, TGF-\textbeta) responsible for the control of embryonic deovlement are also key players in tissue repair and fibrosis.\textsuperscript{22–25} Given the role of CL-11 in embryonic development and its potential for regulation of diverse cellular processes, we hypothesized that CL-11 also plays important roles in renal fibrosis. In this study, we employed two models (i.e., bilateral renal IR injury and synegetic kidney transplantation) in Collec11 deficient (CL-11\textsuperscript{−/−}) mice to interrogate the role of CL-11 in the development of renal tubulointerstitial fibrosis. We also performed a series of \textit{ex vivo} analyses and \textit{in vitro} experiments (using primarily cultured murine renal fibroblasts and peritoneal exudate leukocytes) to explore the mechanisms by which CL-11 promotes renal fibrosis. Our data demonstrate a pathogenic role for CL-11 in promoting chronic renal inflammation and tubulointerstitial fibrosis. The mechanism by which CL-11 mediates profibrotic effects involves both the promotion of leukocyte migration and stimulation of renal fibroblast proliferation.

RESULTS

\textbf{CL-11 Mediates Tubule Damage and Renal Function Impairment in the Late Phase of Renal IR Injury}

To assess the effect of CL-11 on renal function and histologic injury during the late phase of renal IR injury, we induced

![Figure 1. Deficiency of CL-11 reduces tubule damage and renal function impairment in the late phase of renal IR. (A) BUN levels in CL-11\textsuperscript{+/+} and CL-11\textsuperscript{−/−} mice at day 2 and day 7 post-renal ischemia (30 min) reperfusion. Data were analyzed by two-way ANOVA. Dashed line represents the BUN level in normal mice. (B) Representative images of PAS staining on kidneys of CL-11\textsuperscript{+/+} and CL-11\textsuperscript{−/−} mice at day 7 post-renal IR, taken at the cortical-medullary junction. Arrows indicate injured tubules. Scale bars, 100 \mu m. (C) Histologic scores for renal tubular injury in the mice illustrated in (B). Data were analyzed by unpaired, two-tailed t test. ***P<0.001. (A and C) Each dot represents an individual mouse and a representative of two independent experiments is shown.}
bilateral renal ischemia (30 min) in CL-11+/+ and CL-11−/− mice, followed by reperfusion up to 7 days. Renal function was evaluated at 2 and 7 days postreperfusion by measuring BUN, and significantly lower BUN levels were observed in CL-11−/− mice at both time points, compared with CL-11+/+ mice (Figure 1A), indicating that there was less impairment of renal function in CL-11−/− mice. CL-11−/− mice also displayed less severe renal histologic lesions (i.e., proximal tubule brush border loss, tubule necrosis, tubule atrophy, protein casting, cellular infiltration in the cortical medullary junction) at 7 days postreperfusion when compared with CL-11+/+ controls (Figure 1B). Histopathologic scores also confirmed the attenuation of renal lesions in the CL-11−/− mice (Figure 1C). Collectively, these results demonstrate that CL-11 deficiency not only protects mice from AKI, but also reduces tubular damage and renal function impairment in the late phase of IR injury.

CL-11 Is Required for Accumulation of ECM in the Kidney after Renal IR

Accumulation of ECM (collagen deposition) in the kidney after renal IR injury (7 days postreperfusion) was initially assessed by Sirius red staining. Compared with CL-11+/+ mice, CL-11−/− mice displayed a significant reduction of Sirius red staining in the tubular interstitium (Figure 2, A and B). Accumulation of ECM was further analyzed by immunohistochemistry for detection of ECM proteins (COL I, FN) and cytoskeletal protein (vimentin). Compared with CL-11+/+ mice, CL-11−/− mice exhibited markedly reduced COL I and FN deposition and vimentin expression in the tubular interstitium, as well as a better preservation of proximal tubular epithelial cells (assessed by lotus tetragonolobus lectin [LTL] staining) (Figure 2C). Intrarenal expression of COL I and FN was also analyzed by reverse-transcription quantitative PCR (RT-qPCR). Significantly lower mRNA levels of these molecules were observed in the kidneys from CL-11−/− mice, compared with CL-11+/+ controls (Figure 2D). Taken together, these observations demonstrate that CL-11 deficiency reduces accumulation of ECM and proximal tubule damage, which corresponds well with a reduction in renal function impairment and histologic scores described in Figure 1.

CL-11 Is Required for Renal Inflammatory Cell Infiltration, Tissue Inflammation, and Fibrogenesis after Renal IR

An influx of inflammatory cells is a hallmark of tubulointerstitial fibrosis. We therefore examined inflammatory cell infiltration in the kidneys of CL-11+/+ and CL-11−/− mice after the induction of renal IR injury using flow cytometry and immunohistochemistry. Flow cytometry analysis of renal cell suspensions showed that, under normal conditions, there was no significant difference in basal levels of leukocytes between CL-11+/+ and CL-11−/− mice (data not shown). After induction of renal IR injury, leukocyte infiltration increased in both groups of mice. However, CL-11−/− mice had significantly lower numbers of CD45+ (total leukocytes) in the kidneys at 2 and 7 days postreperfusion, when compared with CL-11+/+ controls (Figure 3, A and B). Because the

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Deficiency of CL-11 reduces accumulation of ECM in the kidney after renal IR. (A) Representative images of Sirius red staining on kidneys of CL-11+/+ and CL-11−/− mice at day 7 post-IR, taken at the cortical-medullary junction. Scale bars, 100 μm. Arrows indicate positive-stained areas. (B) Quantification of Sirius red-stained areas corresponding to the CL-11+/+ and CL-11−/− mice in (A). Data were analyzed by unpaired, two-tailed t test (n=35–50 viewing fields from 7 to 9 mice/group). ***P<0.001. (C) ECM protein deposition in kidneys. Left panels, representative fluorescence microscope images of COL I (red), FN (red), vimentin (red), lotus tetragonolobus lectin (LTL) (a proximal tubular marker) (green), and DAPI (blue) staining on kidneys of CL-11+/+ and CL-11−/− mice at day 7 post-IR, taken at the cortical-medullary junction. Arrows indicate positive-stained areas. Scale bars, 20 μm. Right panels, quantification of positively stained areas of COL I, FN, and vimentin, corresponding to the CL-11+/+ and CL-11−/− mice in left panels. Data were analyzed by unpaired, two-tailed t test (n=6 viewing fields from 2 mice/group). ***P<0.001. (D) Relative mRNA levels of COL I and FN in kidneys of CL-11+/+ and CL-11−/− mice at day 7 post-IR, determined by RT-qPCR. Data were analyzed by unpaired, two-tailed t test (n=6–7 mice per group). Each dot represents an individual mouse. *P<0.05; **P<0.01. SR, Sirius red.
infiltrating leukocytes (CD45+) in the kidney postreperfusion mainly comprised Ly6G+ (neutrophil) and Ly6G−F4/80+ (monocyte/macrophage [MO/MΦ]), we further analyzed the infiltration of these two types of cells. CL-11−/− mice had lower numbers of both Ly6G+ (neutrophil) and Ly6G−F4/80+ (monocyte/macrophage [MO/MΦ]) in the injured kidneys, which was more clearly demonstrated at day 2 for neutrophil and day 7 for MO/MΦ (Figure 3, A and B). Immunohistochemistry also demonstrated significantly lower numbers of CD45+, F4/80+ cells, and Ly6G+ cells in CL-11−/− kidneys at 7 days postreperfusion compared with CL-11+/+ controls (Figure 3C), in agreement with flow cytometry data. In addition, we assessed the effect of CL-11 on renal tissue inflammation and fibrogenesis after renal IR injury. Intrarenal gene expression of proinflammatory cytokines (TNF-α, IL-1β, IL-6), chemokines (CXCL1, CCL2), and profibrotic factors (TGF-β, PDGF) was significantly lower in CL-11−/− kidneys compared with CL-11+/+ controls at 7 days postreperfusion (Figure 4). These results together demonstrate that CL-11 is required for renal inflammatory cell infiltration, tissue inflammation, and fibrogenesis after renal IR injury.

CL-11 Promotes Leukocyte Migration In Vitro

The association of CL-11 with renal inflammatory cell infiltration raises the question of whether CL-11 has direct effect on leukocyte migration. It has been reported that several plant lectins such as concanavalin A (Con A) and peanut agglutinin can promote human neutrophil migration,26 in a carbohydrate-dependent manner. We speculated that CL-11 may have such function. We therefore performed a chemotaxis assay to evaluate the effects of CL-11 on leukocyte migration using murine peritoneal exudate cells (>80% neutrophil/MO/MΦ) and recombinant CL-11 (rCL-11). We first characterized the rCL-11 by western blot. Under a reducing condition, rCL-11 displayed a (predicted) band with 34 kD molecular mass, reflecting a monomeric unit of CL-11. This band was also detected in normal mouse serum (Supplemental Figure 1). We also checked and confirmed that the endotoxin levels in the working concentrations of rCL-11 used in our study are far below the reported lowest concentration of endotoxin (0.5 ng/ml) that showed effects in cell culture experiments, according to the company datasheet. Our chemotaxis results showed that rCL-11 strongly induced leukocyte migration in an rCL-11 dose–dependent manner (150–1200 ng/ml) (Figure 5A). Migration induced by rCL-11 (600 ng/ml) was comparable to that induced by N-formyl-met-leu-phe (fMLP), a well described chemotactant (100 nM)
Figure 5B, indicating the potency of CL-11 in chemotaxis. To assess the possibility that CL-11 mediates leukocyte migration through complement activation, we performed the chemotaxis assay using peritoneal exudate cells from C3^{-/-} mice. Results showed that CL-11 significantly increased the migration of leukocytes of C3^{-/-} mice leukocytes; the effect was comparable to that on WT leukocytes (Figure 5C). We next explored the ligands which could be responsible for CL-11-mediated leukocyte migration. We initially examined the specificity and intensity of carbohydrate moieties in our murine leukocyte preparations by lectin-binding assay using several fluorescein-labeled lectins: galanthus nivalis lectin (GNL) (for detection of terminal α(1,3) linked mannose residues), lens culinaris agglutinin (LCA) (for detection of branched fucose/α-linked mannose residues), LTL (for detection of terminal α-L-fucose and fucose alone), and ulex europaeus I (UEA I) (for detection of α(1,2) linked fucose residues). Flow cytometry analysis showed that GNL, LCA, LTL, and UEA I were able to bind to murine leukocytes; markedly high binding intensities were observed with GNL and LCA, indicating predominant presence of mannosyl residues (Man) on the cell surface (Supplemental Figure 2A). We then assessed whether blocking carbohydrate recognition of CL-11 by using its preferential monosaccharide ligands (L-fucose, D-mannose) could inhibit chemotactic effect of CL-11 on leukocytes. Preincubation of rCL-11 with D-mannose effectively reduced CL-11-mediated leukocyte migration. Preincubation with L-fucose led to a small reduction of the migration, but preincubation with a less preferred monosaccharide ligand (D-galactose) had

**Figure 3.** Deficiency of CL-11 reduces renal leukocyte infiltration after renal IR. (A and B) Renal inflammatory cell infiltration in CL-11^{+/+} and CL-11^{-/-} mice at day 2 and day 7 post-renal IR, determined by flow cytometry. (A) Stepwise gating strategy used in flow cytometric analysis of leukocytes (CD45^+), neutrophils (Ly6G^+), and macrophages (F4/80^+). (B) Quantification of leukocytes (CD45^+), neutrophils (CD45^+Ly6G^+), and macrophages (CD45^+F4/80^+). Data were analyzed by unpaired, two-tailed t test (n=4 mice per group). *P<0.05; **P<0.01. Each dot represents an individual mouse. (C) Renal inflammatory cell infiltration in CL-11^{+/+} and CL-11^{-/-} mice at day 7 post-renal IR, determined by immunohistochemistry. Left panels, representative images of immunohistochemical staining for CD45, Ly6G, and F4/80 in kidneys of CL-11^{+/+} and CL-11^{-/-} mice. Scale bars, 50 μm. Right panels, quantifications of CD45^+ cells, Ly6G^+ cells, and F4/80^+ cells in kidneys of CL-11^{+/+} and CL-11^{-/-} mice. Data are shown as mean±SEM (n=31–45 viewing fields from 7 to 9 mice/group) and were analyzed by unpaired, two-tailed t test. ***P<0.001. (A–C) A representative of two independent experiments is shown. SSC: side scatter.
no effect on CL-11–mediated leukocyte migration (Figure 5D). These results demonstrate that CL-11 has a chemotactic effect on murine leukocytes, and the effect is carbohydrate-dependent.

**CL-11 Stimulates Renal Fibroblast Proliferation In Vitro**

Renal interstitial fibroblasts are the key effector cells in the development of renal fibrosis. Plant lectins such as Con A are known to have stimulatory effects on hamster renal fibroblast proliferation. We therefore sought to investigate the possibility that CL-11 can stimulate renal fibroblast proliferation. To this end, we cultured primary renal fibroblasts from mice and confirmed that >95% were positive for vimentin (Supplemental Figure 3). Proliferation assay was performed on the fibroblasts treated with rCL-11 and controls for 24 hours using Click-iT EdU Alexa Fluor 488 Imaging Kit. Initial experiments showed that treatment of fibroblasts with different concentrations of rCL-11 (in the range of 300–1200 ng/ml) significantly increased EdU-labeled cell numbers, with a maximal stimulation at 600 ng/ml (Figure 6A). The stimulatory effect of CL-11 on fibroblast proliferation was further confirmed at the concentration of 600 ng/ml, which is comparable to that stimulated by Con A at 1 μg/ml concentration (Figure 6, B and C). These results clearly demonstrate that CL-11 has stimulatory effects on renal fibroblasts.

It has been shown that CL-11 preferentially binds to mannose-containing glycans. We therefore sought to investigate whether the effect of CL-11 on renal fibroblast proliferation is dependent on interaction with Man on renal fibroblast surface. We first examined the specificity and intensity of carbohydrate moieties on renal fibroblasts by using fluorescein-labeled lectins. Flow cytometry revealed the high binding intensities of GNL and LCA and the low binding intensities of UEA I and LTL, indicating predominant presence of Man on renal fibroblast surface (Supplemental Figure 2B). Fluorescence microscopy confirmed this by showing positive staining of GNL and LCA in renal fibroblasts (Figure 6D). Using confocal microscopy, presence of Man on the cell surface also corresponded with CL-11 binding (Figure 6E). We then assessed whether blocking CL-11–Man interaction can inhibit the effect of CL-11 on renal fibroblast proliferation. Proliferation assay showed that prior treatment of renal fibroblasts with α-mannosidase significantly inhibited CL-11–mediated fibroblast proliferation, when compared with control treatment; but prior treatment with β-galactosidase did not affect CL-11–mediated fibroblast proliferation (Figure 6F). These observations support the concept that Man on the surface of renal fibroblasts is involved in CL-11–mediated fibroblast proliferation.

**Local Production of CL-11 in the Kidney Plays an Important Role in the Development of Renal Tubulointerstitial Fibrosis**

CL-11 has relatively low serum concentrations (approximately 300 ng/ml), compared with MBL (approximately 2 μg/ml), and usually exists in the circulation as a dimeric or trimeric form (>200 kD). When complexed with CL-10 in the circulation, it forms a large molecule (up to 800 kD). These large molecules may not be able to penetrate into the interstitial space of organs, including the kidney. Therefore, we hypothesized that local production of CL-11 within the kidney is important for the development of renal tubulointerstitial fibrosis. To test this, we performed syngeneic mouse kidney transplantation in the following combinations: (1) CL-11+/+ (donor) to CL-11+/+ (recipient), and (2) CL-11+/+ (donor) to CL-11−/− (recipient), and assessed donor renal tubular damage (by periodic acid–Schiff [PAS] staining) and collagen deposition (by Sirius red staining) at 7 days post-transplantation. CL-11−/− isografts displayed much less tubular damage and collagen deposition than CL-11+/+ controls (Figure 7). The extent of reduction in tubular damage and collagen deposition in CL-11−/− isografts was comparable to that observed in the kidneys of

![Figure 3. Continued.](image-url)
CL-11−/− mice (have a generalized CL-11 deficiency) after renal IR injury, indicating a predominant role for local production of CL-11 in this model.

DISCUSSION

Our recent work in a murine model of acute renal IR injury has shown that AKI in the early phase is dependent on CL-11.21 It was proposed that CL-11 detects stress-induced L-fucose pattern on renal tubules to trigger complement activation that mediates renal tubular epithelial injury. In this study, we extended our observations to the late phase of renal IR injury and examined the effect of CL-11 on renal chronic inflammation and fibrosis after renal IR. Overall, our data clearly demonstrate a pathogenic role for CL-11 in the progression of renal tubulointerstitial fibrosis, and suggest novel mechanisms for the profibrotic effects of CL-11, namely the promotion of leukocyte migration and stimulation of renal fibroblast proliferation.

A key observation from this study is that renal chronic inflammation and tubulointerstitial fibrosis are dependent on CL-11. We have shown that CL-11−/− mice are protected from deterioration of renal function, with attenuated renal chronic tissue inflammation (i.e., inflammatory cell infiltration, tubule injury, intrarenal synthesis of proinflammatory and profibrotic molecules) and attenuated ECM accumulation in the tubulointerstitial space. In renal isografts, attenuated tubule damage and collagen deposition were only observed in CL-11−/− deficient kidneys. This observation gives rise to an intriguing question of how CL-11 contributes to chronic renal inflammation and tubulointerstitial fibrosis.

In terms of the cellular mechanisms underlying this novel pathogenic role of CL-11 as a mediator of chronic renal inflammation and tubulointerstitial fibrosis, we have made two important in vitro observations. First, a potent effect of CL-11 on leukocyte (mainly neutrophil/MO/MΦ) migration was observed, indicating that CL-11 has chemotactic activity. Given that our leukocyte migration assays were performed in a serum-free condition and CL-11 had clear effects on leukocyte

Figure 4. Deficiency of CL-11 reduces renal tissue inflammation and fibrogenesis after renal IR. Relative mRNA levels of proinflammatory (A) and profibrogenic factors (B) in the injured kidneys of CL-11+/+ and CL-11−/− mice at day 7 post-renal IR, determined by RT-qPCR. Data were analyzed by unpaired, two-tailed t test, except for the PDGF that was analyzed by Mann–Whitney test (n=8 mice/group). Each dot represents an individual mouse. *P<0.05; ***P<0.001.
Figure 5. CL-11 promotes leukocyte migration in vitro. (A) Cell migration in response to medium (Ctrl), and medium containing different concentrations of rCL-11. (B) Cell migration in response to medium (Ctrl), medium containing rCL-11 (600 ng/ml), or N-formyl-met-leu-phe (fMLP) (100 nM). (A and B) Data were analyzed by one-way ANOVA with Tukey’s post-test (n=5 per group). (C) Cell migration in response to medium (Ctrl) and medium containing rCL-11 (600 ng/ml) in C3Δ−/− and WT leukocytes. (D) Cell migration in response to rCL-11 (600 ng/ml) (rCL-11) or rCL-11 (600 ng/ml) that had been preincubated with D-(+)-mannose (Man), L-(&#234;)-fucose (Fuc), or D-(+)-galactose (Gal) (all at 2.5 mM). (C and D) Data were analyzed by one-way ANOVA with Tukey post-test (n=5–19). (A–D) Representative of at least three independent experiments. *P<0.05; **P<0.01; ***P<0.001.
have shown that neutrophil chemotaxis mediated by several plant lectins was effectively inhibited by preincubation of the lectins with their carbohydrate ligands.26 Furthermore, our lectin screening results revealed predominant expression of Man on leukocyte cell surface, supporting that CL-11–mediated leukocyte migration is mainly through a Man recognition mechanism. With regard to the molecule(s) responsible for the action of CL-11 on renal fibroblast proliferation, our study suggests that Man is also involved in CL-11–mediated cell proliferation. This is supported by our findings that CL-11 binds to Man on the fibroblast cell surface and removal of mannose (using α-mannosidase), but not galactose (using β-galactosidase), abrogates the effects of CL-11 on the fibroblast proliferation.

Predominant presence of Man on the cell surface of leukocytes and renal fibroblasts raises the possibility that other collectins (e.g., MBL, SP-A) could play similar roles as CL-11 in these cellular processes, thus participating in the pathogenesis of renal fibrosis.

Renal inflammation is an initial protective response to kidney injury. However, excessive inflammatory responses in the kidney upon injury or unresolved renal inflammation are thought to be important drivers of the process of fibrosis.9 This notion is further supported by our in vitro observations that supernatants, resulting from cocultures of inflammatory cells (murine peritoneal exudate cells) with tubular debris, contained high levels of proinflammatory cytokines (TNF-α, IL-1β, and TGF-β) and were able to stimulate renal fibroblast proliferation (Supplemental Figure 6). Therefore, CL-11–mediated AKI (tubule damage, cellular infiltration), as a consequence of complement activation, would also stimulate the process of tubulointerstitial fibrosis. In agreement with this, C3−/− mice which have intact CL-11 also displayed less collagen deposition in the peritubular interstitium and cellular infiltration in the kidney on day 7 postreperfusion (Supplemental Figure 7). Thus, there is a possibility that CL-11 may be causing fibrosis in vivo, at least partly, through the earlier activation of complement.

On the basis of our findings in this study and our published data in the study of acute

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**Figure 6.** CL-11 stimulates renal fibroblast proliferation and ECM production. (A) Proliferation rate of renal fibroblasts without or with different concentrations of CL-11. Data were analyzed by one-way ANOVA (n=6 viewing fields from 2 cell samples/group). (B) Representative fluorescent images of EdU-labeled renal fibroblasts without or with rCL-11 (600 ng/ml) and Con A (1 μg/ml) treatment. Scale bars, 50 μm. (C) Quantification of EdU-labeled renal fibroblasts, corresponding to the fibroblasts in (B) and expressed as proliferation rate. Data were analyzed by one-way ANOVA with Tukey’s post-test (n=8 viewing fields from 2 cover glass/group). *P<0.05; **P<0.01. (B and C) A representative of three independent experiments is shown. (D) Fluorescence microscopy images of LCA (green), GNL (green), and DAPI (blue) staining in renal fibroblasts. Scale bars, 10 μm. (E) Representative confocal image of two independent experiments showing renal fibroblasts (from CL-11−/− mice) that had been incubated with rCL-11 (600 ng/ml) for 24 hours and subjected to staining for CL-11 (red), LCA (green), and nuclear marker DAPI (blue). Scale bars, 10 μm. (F) Proliferation rate of renal fibroblasts that had been pretreated with buffer alone (Ctrl) or buffer containing α-mannosidase (5 mM) or β-galactosidase (5 mM), followed by incubation with rCL-11 (600 ng/ml). Data were analyzed by one-way ANOVA with Tukey post-test (n=8 viewing fields from 2 cover glass/group). **P<0.01.
injury, we propose that CL-11 contributes to chronic renal inflammation and tubulointerstitial fibrosis through three pathways: (1) by stimulating renal fibroblast proliferation, (2) by mediating influx of inflammatory cells, and (3) by triggering complement activation that increases renal tissue inflammation and fibrogenesis (Figure 8). In conclusion, this study is the first to demonstrate a pathogenic role for CL-11 in the development of kidney fibrosis, and describes novel cellular and molecular mechanisms for CL-11 that contribute to progression of tubulointerstitial fibrosis, thus opening new avenues for studying the roles of CL-11 in renal fibrosis mediated by other causes and supporting therapeutic blocking of the CL-11–ligand interaction in tubulointerstitial fibrosis.

CONCISE METHODS

Reagents

The following reagents were used in this study: Infinity Urea (655870; Fisher Diagnosis, VI); rat monoclonal anti-F4/80 Ab (ab16911), rabbit polyclonal anti-COL I Ab (ab34710), rabbit polyclonal anti-FN Ab (ab2413), rabbit monoclonal anti-vimentin Ab (EPR3776) (ab92547) (all are from Abcam, Cambridge, UK); TRITC-conjugated goat anti-rabbit IgG Ab (T6778; Sigma, MO); rat monoclonal anti-CD45 Ab (MCD4500; Caltag Laboratories, CA); rat monoclonal anti-Ly-6B.2 Ab (MCA771G; Bio-Rad antibodies, Oxfordshire, UK); HRP-conjugated rabbit anti-rat Ig Ab (P0450; DAKO); APC rat anti-mouse CD45 Ab (103112), PE rat anti-mouse Ly-6G Ab (127607), FITC rat anti-mouse

Figure 7. Kidney-specific deficiency of CL-11 protects against the development of tubulointerstitial fibrosis. Histologic injury and renal fibrosis are shown for CL-11+/+ mice transplanted with kidneys from CL-11+/+ or CL-11−/− littermates. Kidney grafts were collected 7 days after transplantation. Pictures were taken at the cortical-medullary junction. (A) Left panel, representative images of PAS staining. Arrows illustrate injured tubules. Scale bars, 50 µm. Right panel, histologic scores for renal tubular injury in the mice. Data were analyzed by unpaired, two-tailed t test (n=49–60 viewing fields from 7 to 8 mice/group; 0.49 mm² per viewing field). ***P<0.001. (B) Left panel, representative images of Sirius red staining. Arrows show collagen deposition. Scale bars, 50 µm. Right panel, quantification of Sirius red–stained areas. Data were analyzed by unpaired, two-tailed t test (n=22–32 viewing fields from 3 to 4 mice/group). *P<0.05.
Figure 8. Schematic diagram of proposed mechanism by which CL-11 contribute to renal tubulointerstitial fibrosis. On the basis of our findings in this study and our published data in the study of acute injury, we propose that local production of CL-11 (by renal tubular epithelial cells and possibly by other cells) is upregulated by renal IR. Locally produced CL-11: (1) stimulates renal interstitial fibroblasts and causes cell proliferation and ECM production; (2) mediates influx of inflammatory cells into the kidney—these inflammatory cells produce proinflammatory and profibrotic factors in response to debris stimulation, which increase tissue inflammation and fibrogenesis; and (3) detects stress-induced L-fucose pattern on renal tubules to trigger complement activation that mediates renal tubular epithelial injury and production of proinflammatory and profibrotic factors by tubular epithelial cells, which increase renal tissue inflammation and fibrogenesis. Mo, monocyte; Neu, neutrophil.

**Induction of Renal IR Injury**
Renal IR injury was induced as we previously described with some modifications. In brief, mice were anesthetized by isoflurane and kept warm on a heated pad. A midline abdominal incision was made. The renal arteries and veins were isolated and bilaterally occluded for 30 minutes with micro aneurysm clamps. After removal of the clamps, 0.5 ml of warm saline was put in the abdomen and the incision was sutured. Blood samples were taken at day 2 (tail bleeding) and day 7 (cardiac puncture) after reperfusion for renal function assessment. Kidneys were harvested at day 7 for histopathology, flow cytometry, and RT-qPCR.

**Assessment of Renal Function, Pathology, and Fibrosis**
Renal function was assessed by measuring the BUN in the serum using a standard urease kit called Infinity Urea. Renal histopathologic changes and fibrosis were assessed as described previously. In brief, kidneys were fixed with 4% formaldehyde in PBS for 48 hours and embedded in paraffin. Paraffin sections (2–4 μm) were stained with PAS stain or Sirius red. Stained kidney sections were scanned with a Hamamatsu Nanozoomer 2.0 HT slide scanner (Hamamatsu Photonics, Hamamatsu, Japan) and viewed using NDP2 software. Renal histopathologic changes and fibrosis were assessed as described previously. The assessment was on the basis of histopathologic changes (i.e., cellular infiltration, loss of proximal tubule brush border, tubule necrosis, tubule atrophy) that were mainly located at the cortical medullary junction area. Five viewing fields (0.49 mm²/field) from each kidney were examined. Renal fibrosis was assessed on Sirius red–stained sections. The positively stained areas were quantified by imaging analysis (ImageJ software; National Institutes of Health, Bethesda, MD). Briefly, six to eight viewing fields from the cortical medullary junction of each kidney were examined. Positively stained areas were expressed as a percentage of the whole field area (0.49 mm²). All of the aforementioned quantitative analyses were performed in a blinded fashion by two experienced persons.

**Mice**
Homozygous CL-11−/− mice on a C57BL/6 background were purchased from Mutant Mouse Resource and Research Centers (UC Davis, Davis, CA) and have been back-crossed onto the C57BL/6 strain for six generations. WT littermates (CL-11+/+) were used as controls. Male mice (8–12 weeks) were used in all of the experiments, unless specified. Animal procedures adhered to the Animals (Scientific Procedures) Act of 1986.

**Immunohistochemistry**
Immunohistochemistry was performed on frozen sections (approximately 4 μm) of kidneys. For the detection of inflammatory cells, kidney sections were fixed in acetone and blocked by 10% rabbit
serum, then incubated with rat anti-mouse CD45, Ly-6B.2, and F4/80 antibodies, respectively, at 4°C overnight and followed by HRP-conjugated rabbit anti-rabbit polyclonal antibody. The sections were scanned with a Hamamatsu Nanozoomer 2.0 HT slide scanner. Four fields (0.12 mm² per field) in the cortical medullary junction of each kidney were randomly examined and the positively stained cells were counted. The quantitative analysis was performed in a blinded fashion by two experienced persons. For the detection of cell proliferation rate was calculated using the following formula: proliferating cell number (representing the total cell number) and EdU-positive nuclei (representing proliferation) were counted using Image J. The proliferating cell number) were counted using Image J. The proliferating cell number was determined using the following formula: proliferation rate=(proliferating cell number/total cell number)*100%.

Assessment of Inflammatory Cell Infiltration in the Kidney
Single renal cell suspensions were prepared using a method described previously. Kidneys were weighed, minced, and incubated with collagenase D (0.75 mg/ml) for 10 minutes at 37°C with gentle agitation. The collagenase was inactivated with an equal volume of DMEM-F12 containing 10% FCS. The digested tissue mixture was then passed through a 40-μm nylon sieve to remove tissue debris. The cell segments were collected and treated with red cell lysis buffer to remove remaining RBC. The cell pellet was washed and resuspended in PBS containing 1% BSA followed by flow cytometric analysis. The cells were preincubated with FcR blocking antibody (CD16/32), and then stained with rat anti-mouse APC-conjugated CD45, PE-conjugated Ly6G, and FITC-conjugated F4/80 antibodies, or the appropriate isotype control antibodies at 4°C for 20 minutes. In order to quantify absolute cell counts in kidney tissue, we used CountBright absolute counting beads in our flow cytometry assays, according to the manufacturer’s instructions. All flow cytometric analyses were performed using Calibur Flow Cytometer (BD Biosciences) and Flowjo software (Tree Star, OR).

RT-qPCR
Total RNA extraction from kidney tissue and cells and reverse transcription reaction were performed as previously described. qPCR was performed with a DyNAmo HS SYBR Green qPCR kit and an MJ Research PTC-200 Peltier Thermal Cycler (Bio-Rad) according to the manufacturer’s instructions. Each sample was amplified in duplicate. The relative gene expression was analyzed using the $2^{-ΔΔCt}$ method and expressed as $2^{-ΔΔCt}$, where Ct is cycle threshold, $ΔΔCt$=testing samples $Δ$(Ct)−control samples $Δ$(Ct); $Δ$(Ct)=testing gene (Cl)−18s (Ct). The control samples were normal CL-11+/+ kidneys and the testing samples were injured kidneys. The primer sequences and size are shown in the Supplemental Table 1.

Chemotaxis Assay
Chemotaxis assay was performed with murine peritoneal exudate cells (>80% neutrophil/MO/MΦ) which were prepared from peritoneal lavage of C3 -/- or WT mice 1 day after intraperitoneal injection of 1 ml 3% thioglycollate using a disposable 96-well cell migration system (106–3; Neuro Probe). Thirty microliters of cell culture medium (RPMI 1640 with 1% BSA) (negative control) or the medium containing rCL-11 (150–1200 ng/ml) or fMLP (100 nM) (positive control) were added to each chamber well. The framed filters with 3 μm pore size were placed to chamber wells and 50 μl of cell suspension (2×10⁶ cells in RPMI 1640 with 1% BSA) was added to the top of each filter. The chamber was incubated for 1.5 hours at 37°C with 5% CO₂. The cells that migrated to the bottom of each well were collected and stained with trypan blue and counted under a microscope. In some experiments, rCL-11 (600 ng/ml) was preincubated with 2.5 mM D-mannose, L-fucose, or D-galactose for 1 hour at room temperature in the presence of 2 mM Ca²⁺ and then added to the lower chamber wells.

Mouse Renal Fibroblast Culture
Primary renal fibroblast culture was prepared from kidneys of CL-11+/+ mice as described previously with modification in the culture medium. In brief, the renal capsule was peeled off and a longitudinal incision was made on the kidney. The sections of cortex were carefully cut off and diced into mince. They were digested with 0.1% collagenase II in DMEM/F-12 medium for 20 minutes and passed through 250-, 106-, and 75-μm metal sieves and a 40-μm nylon sieve. The unfiltered tubular debris on the nylon sieve was collected and treated macrophages for the study of cytokine expression described below. The filtered cells were cultured in DMEM/F-12 medium containing 10% FCS and 100 U/ml penicillin and 100 μg/ml streptomycin. They experienced at least three passages to eliminate contaminated epithelial cells. Finally, the pure fibroblasts exhibited an elongated, spindle-shaped morphology with vimentin-positive staining in immunohistochemistry.

Proliferation Assay
Mouse renal fibroblasts were seeded on 1% gelatin-coated 8 mm cover glass in a 48-well plate with 3×10⁵ cells per well. They came to 70%–80% confluence after 2 days of culture. The cells were incubated in the medium only (DMEM/F-12 with 5% FCS) or the medium containing different concentrations of rCL-11 (300–1200 ng/ml) or Con A (1 μg/ml) for 24 hours. In some experiments, cells were preincubated with buffer alone or buffer containing a-mannosidase (5 mM) or b-galactosidase (5 mM) for 1 hour at 37°C. After three washes in PBS, the cells were treated with rCL-11 (600 ng/ml) for 24 hours. At the end of rCL-11 treatment, cells were incubated with serum-free DEME/F-12 containing 10 mM EdU for 2 hours and fixed in 3.7% formaldehyde. Incorporated EdU was detected by a Click-it EdU Alexa Fluor 488 Imaging kit according to manufacturer’s instructions. Pictures were taken by an Olympus BX51 microscope. The DAPI-positive nuclei (representing the total cell number) and EdU-positive nuclei (representing the proliferating cell number) were counted using Image J. The proliferation rate was calculated using the following formula: proliferation rate=(EdU-positive nuclear number/DAPI-positive nuclear number)×100%.

Detection of Man in Renal Fibroblasts
The mouse renal fibroblasts were fixed in 4% PFA for 15 minutes (without permeabilization) and stained with DAPI and fluorescein-labeled GNL and LCA for 1 hour at room temperature. Pictures were then taken with an A1R point scanning confocal microscope.
Detection of CL-11 Binding to Renal Fibroblasts

The renal fibroblasts cultured from CL-11−/− mice were incubated with rCL-11 (600 ng/ml) for 24 hours and fixed in 4% PFA for 15 minutes. Without cell membrane permeabilization, the cells were blocked with 10% donkey serum and incubated with rabbit anti-human CL-11 antibody overnight at 4°C, followed by the secondary donkey anti-rabbit Alexa Fluor 594 antibody, fluorescein-labeled GNL or LCA, and DAPI for 1 hour at room temperature. The staining was examined via an A1R point scanning confocal microscope.

Mouse Kidney Transplantation

Kidney transplants were performed in mice as previously described. Briefly, mice were anesthetized with isoflurane (Abbott Laboratories). Donor kidney along with the ureter was harvested, including the renal artery with a small aortic cuff and the renal vein. These vascular cuffs were anastomosed to the recipient abdominal aorta and vena cava, respectively, below the level of the native renal vessels. Total ischemic time was about 35 minutes. The donor ureter was connected to the recipient bladder. The right native kidney was removed before the vascular anastomosis. The transplanted kidney was collected 7 days after surgery. PAS and Sirius red staining were performed to assess tissue damage and renal fibrosis.

Statistical Analyses

Data are shown either as mean±SEM or the readout for individual mice. An unpaired, two-tailed t test was used to compare two groups. A P value <0.05 was considered significant. One-way ANOVA was used for comparisons between three or more groups. All analyses were performed using Graph Pad Prism Version 5 (Graph Pad Software).

ACKNOWLEDGMENTS

This work was supported by the Medical Research Council of the United Kingdom (MR/L020254/1 to W.Z. and S.S.S.), the National Natural Science Foundation of China (NSFC 81170644 to K.L.), and the Visiting Scholarship of Xi’an Jiaotong University to King’s College London.

DISCLOSURES

None.

REFERENCES


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2017050544/-/DCSupplemental.
Figure 1. Western blot analysis of rCL-11 and CL-11 WT mouse serum.

rCL-11 (0.1 µg and CL-11 WT mouse serum (80 µg) were run in SDS-PAGE under a reducing condition, followed with western blot by using polyclonal goat anti-human COLEC11 Ab (1:300, Sant Cruz) and rabbit anti-goat HRP Ab (1:1000, Dako).
Figure 2. Leukocytes and renal fibroblasts express a great amount of mannose moieties on the cell surface.

Peritoneal exudate cells (collected 24h after intraperitoneal injection of 1 ml of 3% thioglycollate) and renal fibroblasts were incubated without or with fluorescein labelled lectins, including Ulex Europaeus I (UEA I), Lotus Tetragonolobus Lectin (LTL), Galanthus Nivalis Lectin (GNL) and Lens Culinaris Agglutinin (LCA), for 30 min and processed for flow cytometry. (a) histogram and quantification (shown as geometric mean of fluorescence intensity, GMFI) of the four lectins binding to peritoneal exudate cells. (b) histogram and quantification of the four lectins binding to renal fibroblasts.

Figure 2. Leukocytes and renal fibroblasts express a great amount of mannosyl residues on the cell surface. Peritoneal exudate cells (collected 24h after intraperitoneal injection of 1 ml of 3% thioglycollate) and renal fibroblasts were incubated without or with fluorescein labelled lectins, including Ulex Europaeus I (UEA I), Lotus Tetragonolobus Lectin (LTL), Galanthus Nivalis Lectin (GNL) and Lens Culinaris Agglutinin (LCA), for 30 min and processed for flow cytometry. (a) histogram and quantification (shown as geometric mean of fluorescence intensity, GMFI) of the four lectins binding to peritoneal exudate cells. (b) histogram and quantification of the four lectins binding to renal fibroblasts.
Figure 3. Characterisation of cultured renal fibroblasts by vimentin staining.

Representative fluorescence microscopy images of CL-11⁻/⁻ renal fibroblast cells (of 2 independent experiments) that had been stained for vimentin (green) and nuclear marker DAPI (blue). Negative control was performed using the 2nd antibody alone. Scale bars: 20 μm.
Figure 4. Deficiency of CL-11 does not influence C3d deposition in day 7 post-ischemic kidneys.

(a) Representative images of immunofluorescence staining of C3d in kidneys of normal CL-11+/+ mice and ischemic CL-11+/+ and CL-11−/− mice at day 7 post renal ischemia reperfusion. (b) Quantifications of C3d in kidneys corresponding to the mice represented in a. Data are shown as mean ± SEM and were analysed by One-way ANOVA with Tukey’s post-test (n=20 viewing fields from 4 mice/group). *, P<0.05; **, P<0.001; ns, no significant. Scale bars: 20 μm.
Figure 5. Detection of C3d deposition in renal fibroblast cultures

Figure 5. Detection of C3d deposition in renal fibroblast cultures. Fluorescence microscopy images of immunochemical staining of C3d (green) and DAPI (blue) in renal fibroblasts cultured from CL-11/- mice. Cells were incubated with recombinant CL-11 (rCL-11, 600ng/ml) for 48 h or 20% CL-11+/+ mouse serum for 30 min, followed by fixation with 4% paraformaldehyde. They were then stained with rabbit polyclonal anti-human C3d and goat anti-rabbit Alexa Fluor 488. (a) Renal fibroblasts treated with rCL-11. (b) Renal fibroblasts treated with 20% CL-11+/+ mouse serum. Scale bars: 20 μm.
Figure 6. Tubular debris stimulates macrophages to secrete pro-inflammatory factors, which increase fibroblast proliferation. Peritoneal exudate cells, mainly macrophages, were collected from *CL-11*/*+* mice 3 days after intraperitoneal injection of thioglycollate. They were incubated without or with tubular debris (200 ng/ml or 1000 ng/ml) for 24 h. Half of supernatants were used to measure TNF-α and IL-1β concentrations by ELISA. The other half was incubated with renal fibroblasts for 2 days and followed by EdU labelling. (a, b) TNF-α and IL-1β levels. Data are shown as mean ± SEM and were analysed by One-way ANOVA (n=4 per group), by comparing the control and renal tubular debris stimulated. (c) Counting of EdU-positive cells. Data are shown as mean ± SEM and were analysed by Unpaired two-tailed Student’s *t* test (n=4 per group). ***, *P*<0.0001. (a-c) A representative of 2 individual experiments is shown.
Figure 7: Renal fibrosis and tubule damage in C3−/− and C3+/+ mice following IR injury.

Representative images of Sirius red (SR) and Periodic Acid–Schiff (PAS) staining in C3+/+ and C3−/− mouse kidneys at day 7 post renal ischemia (30 min) reperfusion injury, taken at the cortical medullary junction. Arrows indicate positive stained areas and injured tubules. Scale bars: 100 μm.
**Supplementary Table 1**  
PCR primer sequences and product sizes

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* Primer-1 is identical to the coding strand; primer-2 is complementary to the coding strand.
SIGNIFICANCE STATEMENT

Collectin-11 (CL-11) is known to play important roles in embryonic development and host defense, as well as acute renal injury. However, the effect of CL-11 on chronic inflammation and tissue fibrosis is presently unknown. This manuscript reports a previously unknown pathogenic role for CL-11 in the development of tubulointerstitial fibrosis. It also defines two novel cellular mechanisms by which CL-11 promotes inflammatory cell migration and stimulates renal fibroblast proliferation that contribute to the development of tubulointerstitial fibrosis. This study provides new insight into the pathogenesis of tubulointerstitial fibrosis and opens new avenues for studying the roles of CL-11 in renal fibrosis mediated by other causes and tissue fibrosis in other organs.