Lymphangiogenesis in kidney and lymph node mediates renal inflammation and fibrosis

Guangchang Pei*, Ying Yao*, Qian Yang, Meng Wang, Yuxi Wang, Jianliang Wu, Pengge Wang, Yueqiang Li, Fengming Zhu, Juan Yang, Ying Zhang, Weiqi Yang, Xuan Deng, Zhi Zhao, Han Zhu, Shuwang Ge, Min Han, Rui Zeng†, Gang Xu†

Lymphangiogenesis is associated with chronic kidney disease (CKD) and occurs following kidney transplant. Here, we demonstrate that expanding lymphatic vessels (LVs) in kidneys and corresponding renal draining lymph nodes (RDLNs) play critical roles in promoting intrarenal inflammation and fibrosis following renal injury. Our studies show that lymphangiogenesis in the kidney and RDLNs is driven by proliferation of preexisting lymphatic endothelium expressing the essential C-C chemokine ligand 21 (CCL21). New injury-induced LVs also express CCL21, stimulating recruitment of more CCR7+ dendritic cells (DCs) and lymphocytes into both RDLNs and spleen, resulting in a systemic lymphocyte expansion. Injury-induced intrarenal inflammation and fibrosis could be attenuated by blocking the recruitment of CCR7+ cells into RDLN and spleen or inhibiting lymphangiogenesis. Elucidating the role of lymphangiogenesis in promoting intrarenal inflammation and fibrosis provides a key insight that can facilitate the development of novel therapeutic strategies to prevent progression of CKD-associated fibrosis.

INTRODUCTION

All forms of chronic kidney disease (CKD) present with renal fibrosis, a progressive and irreversible pathological feature. Human and animal studies have established that interstitial infiltration of activated leukocytes triggers renal fibrogenesis either by direct injury of renal parenchymal cells or by secretion of cytokines that promote myofibroblast activation (1, 2). Therefore, localized immune system components and inflammatory mediators in kidneys are potential targets of therapeutic interventions addressing renal fibrosis (3, 4). Additional studies are needed, however, to identify key target mechanisms or pathways underlying the pathology-related interactions between kidney tissues and immune system regulators that drive renal fibrosis.

The unique structural features and tissue microenvironment of the kidney are protected by a localized immune system characterized by distinct immune cell subsets and functional suites of signaling molecules. However, regulatory mechanisms governing dynamic interactions between the kidney and immune system are as yet poorly defined. Lymphatic vessels (LVs) link the kidney to the larger immune system, acting as an exclusive conduit for passage of soluble antigens and immune cells from the kidney to the renal draining lymph nodes (RDLNs). LVs therefore participate in shaping kidney immunity and potentially balancing physiological pathways that promote healthy organ maintenance and responses to injury (5).

Organ injury stimulates enhanced expression of the key C-C chemokine ligand 21 (CCL21). LVs provide the route whereby inflammatory cells, including activated dendritic cells (DCs) and lymphocytes expressing the CCL21 receptor CCR7, migrate toward the CCL21-expressing organ or to the corresponding lymph node (6). Mature DCs that reach the lymph node induce rapid and sustained congestion of lymphocyte traffic, and their number influences the proliferation of T lymphocytes and the resulting magnitude of the effector response (7). CCR7 and its ligand, CCL21, play an important role in directing effector memory T lymphocyte trafficking to the lungs via afferent LVs (8). Mice lacking CCR7 exhibit impaired DC and T lymphocyte trafficking to DLNs and consequently fail to mount an adaptive immune response, indicating that CCL21/CCR7 signaling is necessary for lymphatic system-depending cell trafficking from the periphery to the organ and its corresponding DLNs (9).

Peripheral solid organ lymphangiogenesis has been reported in association with various inflammatory conditions including transplant rejection, hypertension, matrix stiffness, myocardial infarction, and tumor metastasis (10–13). Vascular endothelial growth factor C (VEGF-C) and VEGF-D along with their receptor VEGF receptor 3 (VEGFR3) have been identified as key molecular regulators of LV growth and maintenance (14). Recent findings suggest that basic fibroblast growth factor–2 (FGF-2) also acts to promote lymphangiogenesis by interacting with the LV endothelial hyaluronan receptor 1 (LYVE-1) transmembrane domain, a process that is independent of the activation of VEGF3 signaling (15, 16).

Blocking VEGF-C/D binding to VEGF3 has been shown to depress allograft CCL21 production, down-regulate DC traffic to the spleen, and attenuate transplant rejection (17). Further, VEGF-C-dependent stimulation of lymphatic function promoted drainage and antigen clearance during skin inflammation (18). Thus, peripheral organ lymphangiogenesis can serve multiple roles in both induction and resolution of local immune responses in different organs and different immune microenvironments (5). Accumulating evidence now indicates that intrarenal lymphangiogenesis occurs at the site of interstitial lesions and strongly correlates with fibrosis in human CKD biopsies (19). Intrarenal lymphangiogenesis has been identified in interstitial fibrotic regions in a rat remnant kidney model and also in a proteinuria model in which increased VEGF-C and VEGF-D expression was detected in the kidney (20–23). In addition, potent fibrogenic cytokines including transforming growth factor β1 (TGFβ1) and connective tissue growth factor (CTGF) were found to participate in lymphangiogenesis via up-regulation of VEGF-C in a unilateral ureteral obstruction (UOU) model (24, 25). Blocking VEGF-C/D–VEGFR3 signaling decreased UOU-induced renal lymphangiogenesis (22).
To date, RDLN lymphangiogenesis has not, to our knowledge, been reported in the literature. The contributions of the normally existing intrarenal lymphatic network and injury-associated lymphangiogenesis to inflammation and the pathology of renal fibrosis remain unknown. We used several animal models to address this knowledge gap. We found that following renal injury, lymphangiogenesis occurs via local proliferation of existing endothelium in the kidney and corresponding RDLNs. Blocking recruitment of CCR7+ cells into RDLNs and the spleen through inhibition of lymphangiogenesis or administration of a CCR7-neutralizing antibody markedly attenuated intrarenal inflammation and fibrosis. These findings identify lymphangiogenesis as a novel target of therapeutic interventions designed to inhibit intrarenal inflammation and fibrosis associated with CKD.

**RESULTS**

**Intrarenal lymphangiogenesis is associated with renal interstitial inflammatory cell infiltration and fibrosis in CKD patients**

We established a cohort of 318 CKD renal tissues with eight healthy controls (table S1) and observed that intrarenal LVs were increased in CKD (Fig. 1A). Proliferation and expansion of renal lymphatic endothelium were evaluated by using D2-40/proliferating cell nuclear antigen (PCNA) double labeling (Fig. 1B). To confirm specificity of D2-40 labeling of human lymphatic endothelial cells (LECs), we tested two additional LEC markers, VEGFR3 and prospero homeobox 1 (PROX1) (fig. S1A). The results showed a positive relationship between LYVE-1–expressing vessels and LEC labeling in human LVs. On the basis of these observations, CKD patient specimens were divided into LVs low (n = 185) and LVs high (n = 93) groups. To examine whether renal microenvironmental factors were responsible for promoting the growth of LVs, we compared levels of VEGF-C, VEGF-D, and FGF-2, which are all reported to support LEC growth (14–16). All of these factors were present at higher levels in LVs high group specimens relative to the LVs low group (Fig. 1C).

We investigated the expression of CCL21 and cells expressing its receptor, CCR7. We detected the ligand expressed by LECs and found CCR7+ cells distributed within the lumen of intrarenal LVs and in adjacent areas (Fig. 1D). While we cannot distinguish whether these are de novo LVs or growth of preexisting LVs, our observations indicate that lymphangiogenesis in CKD is associated with recruitment of DCs and lymphocytes via CCL21/CCR7 signaling.

To understand immune cell trafficking during lymphangiogenesis in CKD, we further characterized CCR7+ immune cells. We observed that CD1c+ DCs, CD4+ T cells, CD8+ T cells, and CD20+ B cells expressed CCR7 in tissue biopsies (Fig. 1E). Furthermore, we found higher levels of infiltrating inflammatory and regulatory cells in the renal interstitium of LVs high group specimens compared to the LVs low group. These cells included CD68+ macrophages, CD1c+ DCs, CD209+ DCs, CD4+ T cells, CD8+ T cells, CD45RO+ activated T cells, and CD20+ B cells (Fig. 1F and fig. S1C).

CKD patients in which tissue samples exhibited higher densities of intrarenal LVs presented with severe proteinuria, lower estimated glomerular filtration rate (eGFR), greater intrarenal interstitial inflammation, and more severe renal fibrosis at the time of renal biopsy (Fig. 1, G and H). These findings indicate that intrarenal lymphangiogenesis is associated with intrarenal inflammation and fibrosis in CKD patients.

**Local proliferation of preexisting lymphatic endothelium largely accounts for post-injury intrarenal and RDLN lymphangiogenesis**

To further characterize lymphangiogenesis in kidney disease, we investigated intrarenal and RDLN lymphangiogenesis in different mouse models exhibiting renal fibrosis including UUO, ischemia reperfusion injury (IRI), folic acid–induced nephropathy (FAN), adriamycin nephropathy (AN), and nephrotoxic nephritis (NTN). Accompanied by renal fibrosis and atrophy (fig. S2, A and B), we found increased density of intrarenal LYVE-1+ LECs in the LVs of all disease model kidneys (Fig. 2A). We observed expansion of LVs in the corresponding RDLNs after various renal injuries (Fig. 2, A and B). Two additional LEC markers were tested, VEGFR3 and PROX1, that validated LYVE-1 as an LV LEC marker in mice (fig. S1B).

We used coimmunostaining of Ki67 and LYVE-1 to monitor proliferation. We observed increased intrarenal proliferation in LVs (LYVE-1+ and Ki67+ vessels) in UUO and IRI kidneys compared to sham–surgery control kidneys (Fig. 2, C and D). A similar degree of proliferation was observed in the corresponding RDLNs after UUO or IRI (Fig. 2, C and E). In addition, we found that lymphangiogenesis-related cytokines VEGF-C, VEGF-D, and FGF-2 were up-regulated in renal tubular epithelial cells (TECs), some renal interstitial cells, and RDLNs after renal injury (Fig. 2F).

To further analyze direct contributions of bone marrow (BM)–derived cells to lymphangiogenesis in the UUO model, we used two different approaches, parabiosis and BM chimeras. Parabiosis surgery was performed between wild-type mice and green fluorescent protein (GFP) transgenic mice. Chimeras were generated by GFP strain BM transplantation into wild-type mice (fig. S2, C and D). We observed only low levels of LYVE-1 and GFP colocalization in UUO kidneys following parabiosis and in BM chimeras (Fig. 2, G and H), indicating that LEC generation during intrarenal and RDLN lymphangiogenesis is largely the result of proliferation of preexisting lymphatic endothelium rather than direct transdifferentiation of BM-derived cells. This discovery—that preexisting LECs are the source of lymphatic expansion—has important consequences for further investigations of pathological lymphangiogenesis in CKD.

**Intrarenal and RDLN lymphangiogenesis accompanied by expansion of lymphocytes in the kidney, RDLN, and spleen**

We examined whether lymphangiogenesis in the kidney and RDLN could influence immune organ expansion. We observed perirenal lymphadenecrosis and splenomegaly in UUO and IRI mice. The RDLNs and spleens were increased in size and weight compared to sham–surgery control mice (Fig. 3, A and B). Immunofluorescence analysis revealed more I-A.D+ DCs, CD3+ T lymphocytes, and B220+ B lymphocytes infiltrating UUO mouse kidneys, RDLNs, and spleens than observed in control animals (Fig. 3, C and D). Flow cytometry confirmed that the numbers of DCs, CD4+ T cells, CD8+ T cells, and CD19+ B cells were increased in kidneys, RDLNs, and spleens from UUO mice (Fig. 3, E to H; flow cytometry gating strategies are indicated in fig. S3, A to D). The proportion of CD11b+CD11c+I-A.D+CD86+ DCs in the kidney was increased after UUO (fig. S3E, left graph). Populations of CD11b+CD11c+I-A.D+ DCs (fig. S3E, right graph) and CD44high effector-memory CD4+ T and CD8+ T cells (fig. S4F) also showed expansion in UUO mouse RDLNs and spleens. Tamm–Horsfall protein (THP), a kidney-restricted antigen, was reported in the renal interstitial tissue of UUO kidneys (26). We confirmed this finding (fig. S4A) and further detected CD11c+ DCs containing THP.
Fig. 1. Intrarenal lymphangiogenesis in patients with CKD accompanied by intrarenal inflammatory cells and renal fibrosis. (A) Intrarenal LVs in kidneys of various pathological types and healthy control kidneys immunolabeled with D2-40. MCD, minimal change disease; MN, membranous nephropathy; IgAN, IgA nephropathy; LN, lupus nephritis; TIN, tubulointerstitial nephritis; ON, obstructive nephropathy. (B) D2-40 (red) and PCNA (green) dual immunolabeling to identify proliferating intrarenal lymphatic endothelium in control (n = 8) and CKD patients (n = 20). (C) Representative images (40×) showing intrarenal pro-lymphangiogenesis cytokines in control (n = 8) and CKD patient (n = 20) samples. Left: Immunohistochemical staining. Right: Area positive for pro-lymphangiogenesis cytokines counted in high-power field (HPF). (D) Dual immunostaining of CKD renal biopsy specimens: D2-40 (red), CCL21 (green), and CCR7 (green). (E) Representative immunofluorescence images showing CD1c+ DCs, CD4+ T lymphocytes, CD8+ T lymphocytes, and CD20+ B lymphocytes expressing CCR7 in renal biopsies of CKD patients. (F) Interstitial inflammatory cells in LVs low group (n = 185) and LVs high group (n = 93) CKD patient renal biopsy specimens. Proteinuria grading: I, 0 to 1 g/24 hours; II, 1 to 2 g/24 hours; III, above 2 g/24 hours. eGFR grading: I, above 90 ml/min; II, 60 to 90 ml/min; III, below 60 ml/min. Inflammatory cells and interstitial fibrosis grading: I, mild; II, moderate; III, severe. Color-coded bars indicate different grades. (H) Representative images (40×) showing intrarenal fibrosis [Masson-trichrome staining (MTS) and fibronectin] of LVs low and LVs high groups. Fifteen patients from each group are represented. **P < 0.01, ***P < 0.001. Values are mean ± SEM.
Fig. 2. Intrarenal and RDLN lymphangiogenesis after renal injury resulting from local proliferation of preexisting lymphatic endothelium. (A) Intrarenal LVs (80×) and RDLN LVs (10×) immunostained for LYVE-1 in different renal fibrosis models. (B) Changes in LV numbers in UUO kidney and LV area in the RDLN following IRI. (C) LYVE-1 (red) and Ki67 (green) dual immunolabeling of proliferative LECs in kidneys and RDLNs at day 3 (D3) after UUO or IRI. White arrow indicates dual-labeled cell. (D and E) Changes in Ki67+ LV numbers in UUO kidney and Ki67+LYVE-1+ cells in the RDLN after IRI. (F) Upper panel: Representative immunohistochemical images (40×) showing pro-lymphangiogenesis cytokines VEGF-C, VEGF-D, and FGF-2 on kidney (left) and RDLN (right) from UUO C57BL/6J mice at day 7 after surgery. Lower left panel: qPCR analysis of cytokine expression. Lower right panel: Immunohistochemical quantification. (G and H) LYVE-1 (red) and GFP (green) dual fluorescence imaging to indicate origin of newly formed LVs at day 3 after UUO. n = 5 mice per group. Statistical analysis was performed using the Mann-Whitney U test. *P < 0.05, **P < 0.01, ***P < 0.001. Values are mean ± SEM.
Fig. 3. Intrarenal and RDLN lymphangiogenesis accompanied by lymphocyte expansion in the kidney, RDLN, and spleen. (A) Morphological changes in mouse kidney (periodic acid–Schiff (PAS) staining), RDLN, and spleen following UUO or IRI. (B) Change in weight of RDLN (left) and spleen (right) following UUO or IRI (primary axis, RDLN; secondary axis, spleen) relative to mouse body weight (BW). (C) Representative immunofluorescence images (40×) showing I-A<sup>+</sup> cells in the kidney, RDLN, and spleen of UUO or IRI model mice at day 3 after surgery. (D) Representative immunofluorescence images (10×) showing B220<sup>+</sup> (green) B and CD3<sup>+</sup> (red) T lymphocytes in kidneys, RDLN, and spleen of UUO or IRI model mice at day 3 after surgery. (E) CD45<sup>+</sup>CD11c<sup>+</sup> cell numbers in the RDLN and spleen of UUO model mice analyzed by flow cytometry. (F to H) CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> cells, CD45<sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup> cells, and CD45<sup>+</sup>CD19<sup>+</sup>CD3<sup>−</sup> cells analyzed by flow cytometry: (F) whole kidney, (G) RDLN, and (H) spleen. (I) LYVE-1<sup>+</sup> LV (green) and CCL21 (red) colocalization in the kidney and RDLN of normal control, UUO (day 3 after surgery), and IRI (day 3 after surgery) mice. (J) Representative immunofluorescence images showing LYVE-1 and CCR7 staining in the kidney and RDLN of normal control, UUO (day 3 after surgery), and IRI (day 3 after surgery) mice. (K) Percentage of CCR7<sup>+</sup> cells in the kidney, RDLN, and spleen at different time points after UUO analyzed by flow cytometry. (L and M) I-A<sup>+</sup> splenocytes (1 × 10<sup>5</sup>) of GFP mice were sorted and injected under the renal capsule of UUO (day 3 after surgery) mice. Tissue was collected 6 hours later. (L) Co-identification of extrinsic GFP-positive cells with LVs (red) showed GFP<sup>+</sup> cells in the lumen of LVs (white arrows) in kidneys and RDLN. (M) Colocalization of extrinsic GFP and CCR7 (red). n = 5 mice per group. Statistical analysis was performed using the Mann-Whitney U test. n.s. > 0.05, *P < 0.05, **P < 0.01. Values are mean ± SEM.
in corresponding RDLN from UUO mice (fig. S4B). Our observations indicate tissue-specific activation of the adaptive immune response following renal injury. Injury induced intrarenal and RDLN lymphangiogenesis accompanied by splenomegaly and perirenal lymphadenectasis.

**Kidney and RDLN LVs express CCL21 and contain luminal CCR7+ cells**

To examine whether post-injury intrarenal and RDLN lymphangiogenesis is responsible for recruitment of DCs and lymphocyte expansion, we analyzed chemokines expressed by LVs. In our UUO model, LVs did not express chemokines, including CXCL12 and CX3CL1, as previously reported in lymphangiogenesis in other organs (fig. S4, C and D). We found that most of the kidney and RDLN LVs expressed CCL21 (fig. 3I and fig. S4, E and F), and these LVs were infiltrated by CCR7+ cells (fig. 3J). The I-A<sup>D<sub>III</sub></sup> DCs were CCR7 positive and localized either around or within the lumen of LVs in kidneys and RDLN of UUO mice (fig. S4, G and H). We used flow cytometry to determine the percentage of CCR7+ cells in the kidney, RDLN, and spleen at different time points after UUO surgery. CCR7+ DCs and CCR7+ lymphocyte populations were expanded in the kidney, RDLN, and spleen (fig. 3K). The trafficking of DCs into the lumen of LVs in UUO mice was examined by injection of GFP<sup>+</sup> 1-A<sub>III</sub> splenocytes under the renal capsule. GFP<sup>+</sup> 1-A<sub>III</sub> cells detected in the lumen of LVs within the kidney and RDLN were CCR7 positive (fig. 3, L and M). These data indicate that expansion of CCR7+ cells is associated with activation of the adaptive immune response following UUO. Expanding LVs expressing CCL21 may contribute to CCR7+ cell expansion by promoting their trafficking into RDLNs.

**Conditional knockdown of LYVE-1-expressing LVs attenuated lymphocyte expansion, perirenal lymphadenectasis, and splenomegaly**

To analyze whether increased renal and RDLN LVs play a role in promoting or maintaining expansion of intrarenal, RDLN, and spleen lymphocytes in the UUO model, we generated a LYVE-1-Cre/iDTR double-transgenic mouse (Fig. 4A) in which LYVE-1–expressing LVs could be ablated in a diphtheria toxin (DT)–inducible manner based on selective expression of LYVE-1 in most LVs. DT receptor (DTR) was specifically expressed by LECs in LVs (fig. S4B). DT induced a significant (26%) reduction in LVs 24 hours after administration of 1.25 ng of DT per gram of body weight. Meanwhile, the density of CD31+ blood vessels in LYVE-1-Cre/iDTR mice did not change (fig. S5, A to C). To identify any confounding off-target effects induced by DT, we administered DT to wild-type control mice. No renal injuries were detected until 20 days after DT administration (fig. SSD). Furthermore, no kidney morphology changes or effects on renal tubule survival [monitored by lotus tetragonolobus lectin (LTL) and dolichos biflorus agglutinin (DBA) staining] were observed in non-surgery LYVE-1-Cre/iDTR mice (fig. SSE). Because LYVE-1 is expressed in the liver blood sinusoid endothelium of healthy livers (27), we assessed liver LYVE-1 expression and liver injury and found no overt change after DT administration (fig. SSF). While DT did not result in damage to intestine or skin, RDLNs did appear reduced in size (fig. SSG). The number of LYVE-1+ LVs was markedly reduced in RDLNs, and similar reductions in LVs were observed in other organs (fig. SSH).

We applied the UUO model in LYVE-1-Cre/iDTR transgenic mice and administered DT to reduce LVs. We observed less reduction of both intrarenal and RDLN LVs at day 3 (fig. 4, C to E). Strikingly, perirenal lymphadenectomy and splenomegaly were attenuated after DT administration (Fig. 4, F and G), and this was also seen in the IRI model (fig. S7, A and B). DCs, T cells, B cells, and percentages of CCR7+ DCs in the RDLN and CCR7+CD4+ T cells, CCR7+CD8+ T cells, and CCR7+CD19+ B cells in the RDLN and spleen were decreased in DT-treated UUO mice compared to untreated controls (Fig. 4, F and G). The percentage of CD44<sup>high</sup> effector-memory T cells was decreased in the RDLN after DT treatment (fig. S6, A and B). This was also observed in spleen, with the exception of CD4+ CD44<sup>high</sup> effector-memory T cells (fig. S6B). Lymphocyte proliferation, assessed by Ki67 labeling, was also suppressed, while DC proliferation was unchanged (fig. S6, A and B). Real-time polymerase chain reaction (PCR) analysis revealed that mRNA levels of inflammation-related genes including CCL21a, CCR7, CD86, STMN1, Ki67, T-BET, GATA3, and FOXP3 were also down-regulated in the spleen. By contrast, interleukin-10 (IL-10) expression was increased after DT administration (fig. S6C). Thus, conditional LV knockdown (KD) suppressed lymphocyte expansion in the RDLN and spleen in both UUO and IRI mouse models (fig. S7).

**Conditional KD of LVs attenuated UUO-induced intrarenal inflammatory infiltration and renal fibrosis**

Proliferating LVs decreased in kidneys following DT administration (fig. S6D). Flow cytometry revealed reduced numbers of infiltrating cells including CD45<sup>+</sup> leukocytes, CD11c<sup>+</sup> DCs, CD11b<sup>+</sup> CD11c<sup>+</sup> I-AD<sup>+</sup>CD86<sup>+</sup> DCs, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, CD19<sup>+</sup> B cells, macrophages, neutrophils, and natural killer cells in kidneys of DT-treated mice (fig. 4H). Kidney injury molecule 1 (KIM-1) expression was also decreased in renal TECs at day 3 following UUO (fig. S6E). This was accompanied by decreased expression of LV markers (PROX1, LYVE-1, and CCR7), inflammation markers [IKKB, IL-17, IFN-γ (interferon-γ), TNF-α (tumor necrosis factor-α), CD11c, and CD86], and injury-related renal tubule marker (RIP3), as determined by quantitative PCR (qPCR). By contrast, renal tubule survival (determined using SLC22A6) was enhanced at day 3 after UUO (fig. S6F). To ascertain that LYVE-1<sup>+</sup> macrophages were not eliminated, we calculated the proportion of intrarenal CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>LYVE-1<sup>+</sup> cells among CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells and detected no difference between treated and untreated UUO mice, indicating that DT administration did not deplete LYVE-1<sup>+</sup> macrophages (fig. S6G). Flow cytometry analysis furthermore indicated that CD45<sup>+</sup> total leukocytes were lower in kidneys of DT-treated compared to untreated mice at day 7 after UUO (fig. S6H). Immunofluorescence analysis showed reduced renal infiltration of CD3<sup>+</sup>, B220<sup>+</sup>, and F4/80<sup>+</sup> cells in DT-treated mice (fig. S6I). Real-time PCR revealed down-regulated kidney expression of inflammatory cytokine genes including IL-2, IL-6, IL-1β, IL-4, IL-10, CCL21b, CXCL12, CXCCL1, macrophage migration inhibitory factor (MIF), CXCL10, and CCL3; renal fibrosis–related genes including TGFβ1, CTGF, platelet derived growth factor beta (PDGFRβ), alpha smooth muscle actin (α-SMA), platelet-derived growth factor receptor beta (PDGFRβ), and fibronectin; and pro-lymphangiogenesis factors VEGF-C and VEGF-D following DT treatment at day 7 (fig. 4, I and J, and fig. S6J).

We also observed that UUO-induced CD31<sup>+</sup> peritubular capillary rarefaction was improved by administration of DT and was accompanied by higher expression of VEGF164 (an angiogenic isolement VEGF-A) (fig. S6, J and K). In our unilateral surgery models, the concentration of serum creatinine was not significantly increased due to compensation by the normal contralateral kidney (fig. S6L) (28).
Fig. 4. Conditional KD of LVs in LYVE-1-Cre/iDTR mice attenuated UUO-induced lymphocyte expansion in both RDLN and spleen, intrarenal inflammatory infiltration, and renal fibrosis. (A) Conditional LYVE-1 Cre KD cells generated from the progeny of LYVE-1-Cre and Rosa26-DTR mice. (B) LYVE-1 (green) and DTR (red) colocalization in kidneys of LYVE-1-Cre and LYVE-1-Cre/iDTR mice. (C) Scheme of conditional LV knockout in LYVE-1-Cre/iDTR UUO mice by injection of DT. Control mice received phosphate-buffered saline (PBS) injection. ip, intraperitoneally. (D and E) LYVE-1 staining of intrarenal and RDLN LVs. (F and G) Changes in the RDLN and spleen weight. Immune cell numbers and percentage of CCR7-positive cells analyzed by flow cytometry in the RDLN and spleen. DT or PBS was administered to UUO mice on day 3 after surgery. (H) Flow cytometry analysis of renal inflammatory cell infiltration after LV knockout in UUO mice (day 3 after surgery). (I) qPCR analysis of renal inflammation–related cytokine expression in UUO mice (day 7 after surgery). (J) qPCR analysis of renal fibrosis–related cytokine expression in UUO mice (day 7 after surgery). (K) PAS staining of UUO kidney sections at days 7 and 14 after surgery. (L) Weight of left (affected) kidney of UUO mice at days 7 and 14 after surgery. (M) Total kidney collagen levels (adjusted for tissue weight) in UUO mice measured using hydroxyproline assay (days 7 and 14 after surgery). (N) Representative images (40×) showing remaining renal tubules labeled by AQP-1 and degree of interstitial fibrosis indicated by Sirius red staining, α-SMA, and fibronectin labeling. PH3 and Ki67 dual staining shows level of G2-M arrest in TECs. Positive staining counted in HPF. n = 5 mice per group. Statistical analysis was performed using the Mann-Whitney U test. n.s. > 0.05, *P < 0.05, **P < 0.01. Values are mean ± SEM.
The previously reported TEC cell cycle arrest mediating kidney fibrosis was reduced as a result of DT treatment at day 7 after UUO, as shown by double staining of TECs arrested at the G2/M phase with Ki67 and PH3. This suggested that TEC-mediated fibrosis was decreased after LV KD. Aquaporin 1 (AQP-1) levels of the remaining renal tubules were found to exceed those of controls (Fig. 4K). Fibronectin, α-SMA, and Sirius red staining analysis revealed that renal fibrosis was also reduced in DT-treated mice compared to untreated controls at day 7 after UUO (Fig. 4K). Renal atrophy (a result of renal fibrosis) was significantly reduced (Fig. 4, L and M), and total kidney collagen levels (measured by hydroxyproline assay) were mitigated by DT treatment (Fig. 4N). Thus, LV KD in our UUO model resulted in suppression of renal inflammation and fibrosis. This result was also observed in a UUO model treated with DT at 24 hours after surgery (fig. S6, M to P) and in an IRI model (fig. S7, A to G).

Inhibition of lymphangiogenesis by soluble VEGFR3 or LYVE-1 fusion constructs attenuated CCR7+ cell expansion in the kidney, RDLN, and spleen and ameliorated renal inflammation and fibrosis

Soluble VEGFR3 and LYVE-1 can suppress lymphangiogenesis by inhibiting the VEGF-C/VEGF-D–VEGFR3 and FGF-2–LYVE-1 signaling pathways, respectively (14–16). In vitro, although soluble VEGFR3-Fc fusion protein (sVEGFR3-FC) sequestered VEGF-C and VEGF-D and soluble LYVE-1-Fc (sLYVE-1-FC) sequestered FGF-2, both fusion proteins induced proliferation of SVEC4-10 LECs and neither induced apoptosis (fig. S8, A to C). In in vivo studies, we treated C57BL/6j mice with sVEGFR3-FC or sLYVE-1-FC 1 day and 2 hours before UUO surgery and 1, 3, and 5 days after surgery. Soluble Fc (sFc) protein was used as control (Fig. 5A). Seven days after UUO surgery, we observed that LV density in the kidney and RDLN of sVEGFR3-FC– and sLYVE-1-FC–treated mice was reduced compared to controls (Fig. 5, B and D). LV density in control non-UUO kidneys was unchanged by sVEGFR3-FC or sLYVE-1-FC administration (fig. S9A). The effects of sVEGFR3-FC or sLYVE-1-FC on renal lymphangiogenesis after UUO could be attributed to inhibition of intrarenal lymphatic endothelial proliferation (fig. S9B). While kidney size was increased in sVEGFR3-FC– and sLYVE-1-FC–treated mice, the RDLN and spleen were smaller than those in sFc-treated controls (Fig. 5, C, E, and F). Flow cytometry showed that the numbers of CD11c+ DCs, CD4+ T cells, and CD8+ T cells and the percentage of CCR7+ T cells were reduced in the RDLN, spleen, and kidney in sVEGFR3-FC– and sLYVE-1-FC–treated mice relative to sFc-treated controls (Fig. 5, E to G). Real-time PCR revealed down-regulated kidney mRNA levels of LV markers (PROX1, LYVE-1, CCL21b, and CCR7), pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), and pro-fibrogenic factors (TGFβ1, CTGF, and PDGFBB) in sVEGFR3-FC– and sLYVE-1-FC–treated mice (Fig. 5H and fig. S9C). sVEGFR3-FC and sLYVE-1-FC treatment led to higher levels of AQP-1 labeling the remaining renal tubules (fig. S9D), and Sirius red, fibronectin, and α-SMA analysis showed lower levels of renal fibrosis relative to controls (Fig. 5, I and J, and fig. S9D). The total collagen levels in the kidney were also lower after sVEGFR3-FC or sLYVE-1-FC administration (Fig. 5K). Thus, inhibition of lymphangiogenesis in the kidney and RDLN by sVEGFR3-FC or sLYVE-1-FC was associated with reduced intrarenal inflammation and fibrosis induced by UUO. Similar results were obtained in IRI model studies (fig. S9, E and F).

CCR7 neutralizing antibody inhibited CCR7+ cell trafficking into RDLN and spleen and attenuated renal inflammation and fibrosis

To further demonstrate that inhibition of CCR7+ cell expansion attenuated intrarenal inflammation, we administrated CCR7 neutralizing antibody to UUO model mice (Fig. 6A). We observed decreased numbers of immune cells and reduced tissue volume for both RDLN and spleen, corresponding to decreased percentages of CCR7+ DCs and CCR7+ lymphocytes in the anti-CCR7 treatment group of mice (Fig. 6, B and C). Intrarenal infiltration of inflammatory cells, expression of pro-inflammatory markers (IKKβ, IFN-γ, TNF-α, CX3CL1, CXCL10, CCL2, and CXCL12), and markers of renal fibrosis (TGFβ1, CTGF, PDGFβB, α-SMA, PDGFRβ, and fibronectin) were all reduced by anti-CCR7 antibody (Fig. 6, D, F, and G). In addition, the degree of renal atrophy was decreased (Fig. 6E), and total kidney collagen levels were reduced (Fig. 6H). Staining with Sirius red and fibronectin analysis showed that renal fibrosis was reduced in the antibody treatment group (Fig. 6I). These results suggest that systemic CCR7+ cell expansion in the RDLN and spleen following renal injury acts to promote renal inflammation and fibrosis. Figure 6J summarizes the role of lymphangiogenesis within the kidney and RDLNs in mediating inflammation and fibrosis.

DISCUSSION

The physiological functions of LVs and their contributions to pathological mechanisms during chronic disease conditions have not been extensively investigated. We have addressed this gap in the context of CKD by comprehensively investigating the role of lymphangiogenesis in kidneys and RDLNs. We describe a previously unrecognized function of lymphangiogenesis as a key process in the kidney and RDLNs mediating intrarenal inflammation and progressive fibrosis. Although it has been previously reported that lymphangiogenesis is a common feature in the progression of renal fibrosis (19), the origin and function of intrarenal LVs in CKD remained undefined. Here, we observed extensive LEC proliferation in a cohort of 289 CKD patients that was not evident in healthy controls. CKD patients with higher density intrarenal LVs presented with more severe proteinuria and renal fibrosis and decreased eGFR. Our mouse model experiments revealed localized LEC proliferation as the cellular origin of lymphangiogenesis in injured kidney and corresponding RDLN. Newly generated LVs, like preexisting LVs, expressed the essential CCL21. We conclude that the expanded LV system played an important role in recruiting CCR7+ DCs and lymphocytes into RDLN and spleen, thereby inducing systemic CCR7+ cell recruitment and accelerating intrarenal inflammation. Suppression of lymphangiogenesis by genetic techniques or administration of blocking biological reagents inhibited systemic CCR7+ cell recruitment and CKD progression.

Human renal transplant studies have suggested that lymphatic progenitor cells are derived from the circulation and then incorporated into growing LVs (29). Other studies conclude that newly formed LVs “sprout” from the preexisting local lymphatic network with zero or minimal contributions from BM-derived cells (6, 15, 30). Our findings support the latter studies. We found that mouse BM-derived cells were distributed in adjacent areas rather than incorporated into expanding LVs in injured kidney and RDLN. We further observed that lymphangiogenesis-related factors were locally up-regulated. Although VEGF-A derived from B lymphocytes has been confirmed
Fig. 5. Suppression of lymphangiogenesis in C57BL/6J mice by sVEGFR3-FC or sLYVE-1-FC attenuated CCR7⁺ cell expansion in the kidney, RDLNs, and spleen and ameliorated intrarenal inflammation and fibrosis. (A) Schematic showing intervention in UUO mice with the extracellular domain of VEGFR3 [sVEGFR3-FC (sR3)], LYVE-1 [sLYVE-1-FC (sLY)], or sFC (control). WT, wild-type; iv, intravenously. (B and D) LYVE-1 staining of intrarenal and RDLN LVs after sVEGFR3-FC or sLYVE-1-FC treatment of UUO mice (day 7 after surgery). (C) PAS staining of kidney section and kidney weight after sVEGFR3-FC or sLYVE-1-FC treatment of UUO mice (day 7 after surgery). (E to G) Representative RDLN or spleen and weight of RDLN or spleen after sVEGFR3-FC or sLYVE-1-FC treatment of UUO mice (day 7 after surgery). Numbers of CD11c⁺ DCs, CD4⁺ T cells, and CD8⁺ T cells and percentage of CCR7⁺ T cells in the RDLN, spleen, and kidney in sVEGFR3-FC– or sLYVE-1-FC–treated mice determined by flow cytometry. (H) qPCR analysis of profibrogenic cytokines in UUO mice (day 7 after surgery). (I) Sirius red staining to analyze interstitial fibrosis in UUO mice (day 7 after surgery). Area of positive staining (40×) was determined in HPF. (J) Fibronectin staining to analyze interstitial fibrosis in UUO mice (day 7 after surgery). Area of positive staining (40×) was determined in HPF. (K) Total kidney collagen levels (adjusted for tissue weight) determined by hydroxyproline assay in UUO mice (day 7 after surgery). n = 5 mice per group. Statistical analysis was performed using the Mann-Whitney U test. n.s. > 0.05, *P < 0.05, **P < 0.01. Values are mean ± SEM.
Fig. 6. CCR7 neutralizing antibody inhibited CCR7+ cell expansion in the kidney, RDLN, and spleen and attenuated renal inflammation and fibrosis. (A) Rat anti-mouse CCR7 monoclonal neutralizing antibody intervention scheme in C57BL/6J UUO mouse model. Intervention control: rat anti-mouse IgG. (B) Representative RDLNs and RDLN weight of UUO mice (day 7 after surgery) following CCR7 antibody (Ab) treatment. Percentage of RDLN CCR7-positive cells after CCR7 antibody treatment determined by flow cytometry. (C) Representative spleens and spleen weight of UUO mice (day 7 after surgery) following CCR7 antibody treatment. The numbers of spleen immune cells were determined by flow cytometry. (D) Percentage of CCR7-positive cells and numbers of immune cells of UUO mice (day 7 after surgery) analyzed by flow cytometry. (E) PAS-stained kidney sections and weights of left (affected) kidneys of UUO mice (day 7 after surgery). (F and G) Intrarenal inflammation and fibrosis-related markers in UUO mice (day 7 after surgery) analyzed by qPCR. (H) Total kidney collagen levels (adjusted for tissue weight) in UUO mice (day 7 after surgery) determined by hydroxyproline assay. (I) Interstitial fibrosis determination by Sirius red staining and fibronectin detection. Area of positive staining was counted in HPF. n = 5 mice per group. Statistics were analyzed using the Mann-Whitney U test. n.s. > 0.05, *P < 0.05, **P < 0.01. Values are mean ± SEM. (J) Schematic summarizing the role of kidney and DLN lymphangiogenesis in inflammation and fibrosis.
to participate in lymph node lymphangiogenesis (31, 32), we found that expression of lymphangiogenesis-related factors VEGF-C, VEGF-D, and FGF-2 was increased in RDLNs following renal injury. This suggests that renal injury–associated kidney and RDLN lymphangiogenesis arises from preexisting LEC proliferation activated by local lymphangiogenesis-related factors, rather than BM-derived cell transdifferentiation. BM-derived cells may indirectly participate in lymphangiogenesis, however, by differentiating into macrophages and secreting pro-lymphangiogenesis factors (33).

Although it is difficult to delineate the distinct roles of adaptive and innate immunity in renal fibrosis, growing evidence points to adaptive immune activation as a dominant factor promoting the formation of fibrotic lesions in CKDs of diverse etiologies (34, 35). Kerjaschki et al. (36) reported that lymphangiogenesis following human kidney transplants was associated with immunologically active lymphocytic infiltrates. In our cohort of 289 CKD patients, we found that higher numbers of intrarenal LVs were associated with more severe DC and lymphocyte infiltration, which we also observed in mouse UUO- and IRI-induced fibrosis. Our studies further showed that lymphangiogenesis in the RDLN and spleen was accompanied by lymphangiogenesis in the kidney and corresponding RDLN. The observed increase in both weight and the percentage of CD11c⁺I-Α⁺ DCs and CD44high T lymphocytes implies that a robust adaptive immune response occurred in the lymph nodes and the spleen along with renal injury–induced kidney and RDLN lymphangiogenesis.

While we observed adaptive immunity–linked inflammation and fibrosis after UUO, previous reports have suggested that adaptive immunity does not play a direct role in UUO-induced fibrosis. In vivo and ex vivo model studies showed that, following UUO, intrarenal DCs differentiated into mature cells exhibiting enhanced antigen-presenting capacity and that antigen-specific T cell proliferation was increased (37, 38). Other studies support our conclusion that adaptive immunity is linked to fibrosis. Lymphopenic RAG⁻/⁻ mice displayed significantly less interstitial expansion and collagen deposition compared to wild-type controls following UUO. Reconstitution of lymphopenic RAG knockout mice with purified CD4⁺ cells before UUO resulted in significantly increased interstitial expansion and collagen deposition (39). The immunosuppressive agents azathioprine, mycophenolate mofetil, and rituximab that inhibit lymphocyte proliferation have also been reported to prevent fibrosis in the UUO model (40, 41). We also showed that DCs in the renal lymph node increased following UUO and that a fraction of this cell population expressed the “kidney-restricted” antigen THP, indicating recognition of the antigen in the tubulointerstitium and trafficking of kidney-specific antigens to the RDLN (42). To confirm this, we injected GFP⁺ DCs beneath the renal capsule and found GFP⁺ DCs trafficked to the RDLN through LVs. As antigen presentation is vital to initiate an effective adaptive immune response, our finding that renal DCs present renal antigens to RDLN after UUO suggests that adaptive immunity does play a role in UUO-induced renal fibrosis. Our results suggest that expanded LVs play a role in promoting DC recruitment into the RDLN.

LVs can potentially promote both recruitment and transport of CCR7⁺ cells. CCL21, the ligand for CCR7, is expressed by LVs in human kidney transplants (36). We analyzed our CKD biopsy tissue samples and found that CCL21 was specifically expressed at high levels by LVs. DCs and lymphocytes expressing CCR7 were distributed around the renal LVs. In animal models, intrarenal LVs were similarly observed to regionally express the highest levels of CCL21. Subcapsular injection experiments showed that GFP⁺ DCs in injured kidneys expressed CCR7 and were transported through LVs into RDLNs. We found that increased CCR7⁺ DC and lymphocyte cell populations were associated with kidney and RDLN lymphangiogenesis. This increase was reversed by the introduction of CCR7 neutralizing antibody. This finding suggests that trafficking of DCs and lymphocytes into RDLN and spleen is dependent upon the CCL21–CCR7 interaction and therefore that CCR7 is necessary for lymphocyte expansion (43, 44). Following renal injury, kidney and RDLN lymphangiogenesis and the resulting overall increased expression of CCL21 may promote CCR7⁺ DC and lymphocyte cell trafficking into the RDLN.

To clarify the role of kidney and RDLN lymphangiogenesis in CCR7⁺ cell trafficking, we used LYVE-1-Cre/iDTR mice to conditionally knock down LVs. LYVE-1 is a relatively specific and widely used marker for LECs (45). Jang et al. (46) reported that the LYVE-1-Cre/iDTR system allowed ablation of LYVE-1 LECs in a DT-dependent manner. However, they found that LYVE-1-Cre/iDTR mice died of sepsis without visible edema between 24 and 60 hours after high-dose DT administration (46). To avoid this problem, we administered low-dose DT (1.25 ng/g body weight) to LYVE-1-Cre/iDTR mice. In our study, mice survived without significant lesions in vital organs (including liver, skin, and intestine) being observed before or after UUO or IRI surgery. We found that DT administration led to suppression of UUO- and IRI-induced lymphangiogenesis in the kidney and RDLN. We observed contraction of RDLN and spleen, reduced percentages of CCR7⁺ DCs and lymphocytes in the RDLN and spleen, and a lower percentage of CD44high proliferating lymphocytes. Together, these observations are compatible with DT administration eliciting attenuation of response in immune organs linked to suppressed lymphangiogenesis. This was accompanied by lessening of renal tubular injury and fibrosis. Unexpectedly, suppression of lymphangiogenesis resulted in reversal of UUO-induced CD31⁺ peritubular capillary rarefaction. We think that this was a secondary consequence of suppressed lymphangiogenesis and reduced renal inflammation, which allowed regeneration of renal tubules and increased expression of VEGF164 (an angiogenic isomer of VEGF-A) (47, 48).

LYVE-1 is also expressed on the surface of macrophages (49). No difference in the abundance of kidney LYVE-1⁺ macrophages was observed between DT-treated and non-treated LYVE-1-Cre/iDTR mice. Therefore, the intervention targeting LYVE-1 specifically affected LYVE-1⁺ LECs without affecting LYVE-1⁺ macrophages (50). Consistent with our findings, Jang et al. (46) transplanted BM of LYVE-1-Cre/iDTR mice into irradiated mice and found that the population of LYVE-1⁺ macrophages in the recipient mice did not differ from controls after administration of DT at a dose of 100 ng per mouse. Furthermore, ablation of macrophage-lineage cells required continuous DT injections for more than 3 days (51). That low-dose DT used in the present study differentially affected LECs but failed to ablate LYVE-1⁺ macrophages may be due to newly generated LECs expressing much higher levels of LYVE-1 compared to macrophages.

VEGF-C/D–VEGFR3 signaling is critical for lymphangiogenesis, and VEGFR3 is a potential therapeutic target for suppression of lymphangiogenesis (9, 52, 53). Note that independent of VEGFR3 activation, FGF-2 also induces lymphangiogenesis through the FGF-2/LYVE-1 pathway (15, 16). Our study examined the effects of targeting
extracellular domains of VEGFR3 and LYVE-1, observing in both cases suppression of renal and RDLN lymphangiogenesis, including similar decreases in CCR7+ cells and down-regulation of the immune response in the RDLN and spleen. Suppression of lymphangiogenesis using biopeptides also attenuated UUO- and IRI-induced renal inflammatory infiltration and fibrosis. Our work builds upon that of Yazdani et al. (54), who reported that proteinuric rats treated with the VEGFR3 antibody IMC-3C5 showed a significant reduction in both ED1-positive macrophages and collagen III (α1) mRNA levels. Their study did not reveal an IMC-3C5-mediated effect on renal fibrosis, which we believe may reflect two main differences between the present study and that of Yazdani et al. First, our study used sVEGFR3-FC that inhibits both VEGF-C– and VEGF-D–mediated VEGFR3 signaling and might therefore exhibit biological effects distinct from those elicited by the VEGFR3 antibody. Second, we initiated intervention at an earlier time point. Either one or both of these factors may explain why targeting VEGFR3 positively affected renal fibrosis in our study.

We also found that sLYVE-1-FC inhibited LEC proliferation and lymphangiogenesis after UUO. This is consistent with the work done by Johnson et al. (55) who showed that targeted elimination of LYVE-1, either through antibody blockade or depletion of the DC hyaluronan coat, delayed lymphatic trafficking of dermal DCs and also blunted their capacity to prime CD8+ T cell responses in skin-draining LNs.

This is furthermore consistent with our observed suppression of lymphangiogenesis by sLYVE-1-FC decreasing recruitment of CCR7+ DCs and expansion of lymphocytes in the RDLN and spleen.

We have discovered a novel link between lymphangiogenesis and immunomodulation following renal injury. Although our LV knockout interventions were not in themselves specifically targeted to renal LVs, we found no evidence in mouse models that other organs were significantly affected. While the precise mechanisms underlying splenic changes during lymphangiogenesis remain undefined, our studies strongly suggest that before the development of renal fibrosis, lymphangiogenesis in the kidney and RDLN promotes local proliferation (“sprouting”) of preexisting LVs. Furthermore, we found that this process enhances the trafficking of DCs and lymphocytes and is directly responsible for the resulting inflammatory expansion observed in the RDLN and spleen. It is this expansion of the inflammatory response that then leads to intrarenal inflammation and eventually renal fibrosis. We found that genetic or biological suppression of lymphangiogenesis resulted in a weakened inflammatory response in the RDLN and spleen and subsequently downregulated renal inflammatory infiltration and ultimately attenuated renal fibrosis. Thus, our results provide evidence of LV inhibition as a potential targeted immunomodulatory therapeutic strategy for patients suffering from progressive renal fibrosis.

In summary, our studies investigating the consequence of LV suppression via genetic and biopharmacologic approaches identify a previously unrecognized immunoregulatory effect of lymphangiogenesis on inflammatory activation in the RDLN, spleen, and kidney. This effect was observed in the tissue microenvironment of UUO- and IRI-induced renal injury. We found that continuous and irreversible injury inflicted by UUO or IRI led to LV expansion with up-regulated CCL21 expression and recruitment of CCR7+ DCs and lymphocytes into the RDLN and spleen. This ultimately led to exacerbation of renal inflammation and fibrosis, rather than inflammation resolution, an outcome consistent with recent studies (56). Lund et al. (57) found that K14-VEGFR3-Ig mice, which lack dermal LVs, exhibited drastically reduced leukocyte infiltration and cytokine expression around tumors compared to wild-type mice. Muchowicz et al. (58) reported that sustained inhibition of lymphangiogenesis by sVEGFR3 also inhibited the ability of intratumorally inoculated DCs to translocate to local lymph nodes and resulted in fewer tumor-infiltrating IFN-γ–secreting or tumor antigen–specific CD8+ T cells. Thus, our insights into previously unknown contributions of lymphangiogenesis to renal inflammatory activation can potentially be widely applied to diseases (beyond CKD) in which pathological conditions lead to aberrant generation of LVs.

**METHODS**

**Study approval**

Animal care and experimental procedures were approved by the Experimental Animal Ethics Committee of Huazhong University of Science and Technology. The use of human tissue samples was approved by the Ethics Committee for Clinical Studies of Huazhong University of Science and Technology. Human tissue samples were obtained from patients who provided informed consent.

**Renal biopsy specimens**

The demographic and clinical characteristics of the patient cohort are listed in table S1. Tissue sections were derived from kidney biopsies from CKD patients or from trauma-induced nephrectomy. Samples were provided by the Department of Pathology, Tongji Hospital of Huazhong University of Science and Technology. Patients with hepatitis B virus (HBV) infection or who had received glucocorticoids or immunosuppressants before biopsy were excluded. Renal pathologists blinded with regard to corresponding patient clinical data evaluated interstitial inflammation and interstitial fibrosis (mild, moderate, or severe) according to established pathology guidelines (59).

**Mice**

LYVE-1-Cre mice, iDTR mice, and GFP mice were purchased from The Jackson Laboratory. C57BL/6 mice (age, 8 to 10 weeks; weight, ~22 to 25 g) were purchased from Hua Fukang (Beijing, China). The LYVE1-Cre mouse was chosen for its selective enhanced GFP expression in the nuclei of cells expressing LYVE-1. To ablate LYVE1-expressing LVs in a DT-dependent manner, LYVE-1-Cre mice were crossed with iDTR mice (LYVE-1-Cre/iDTR mice). LVs were ablated by administering DT (1.25 ng/g body weight, dissolved in Hanks’ balanced salt solution; Sigma-Aldrich) to LYVE-1-Cre/iDTR mice intravenously unless otherwise indicated. All mice were provided with a standard diet and access to water. Mice were anesthetized and euthanized with 1% sodium pentobarbital solution (0.009 ml/g, Sigma, USA) by intraperitoneal injection. Animals were bred and maintained at Tongji Medical College of Huazhong University of Science and Technology.

**Chimeric mice**

Eight- to 10-week-old wild-type recipient mice received 9.5 Gy of gamma irradiation. Three hours after irradiation, BM cells (1 × 10^7) from donor mice were injected into tail veins of recipient mice. Six weeks later, the BM-transplanted recipient mice underwent UUO or IRI surgery.

**Parabiosis**

Animal pairs underwent parabiosis based on a modification of the technique described by Bunster and Meyer (60). Following anesthesia,
I-A^D+ cells were sorted from spleens of UUO GFP mice and pooled. Injection under the renal capsule

Toxic serum.

and were euthanized 7 days after injection of nephro-

models

The UUO model was generated by ligation of the left ureter. Ischemia (unilateral IRI) was induced by the retroperitoneal approach in the left kidney for 30 min at 37°C. Mice were anesthetized with 1% sodium pentobarbital solution (0.009 ml/g, Sigma, USA) by intraperitoneal injection. The left renal pedicle was clamped with an atraumatic vascular clip for 30 min (Roboz Surgical Instrument Co., Germany) through a flank incision. The left kidney turned purple subsequent to clamping. Clamps were removed after 30 min to start reperfusion, and the left kidney reverted to red within approximately 10 s. The body temperature was maintained between 36.6° and 37.2°C throughout the procedure, monitored using a rectal probe (FHC, USA). The muscle layer and skin were closed with 4-0 nylon sutures. Body temperature was maintained at 36.8° to 37.2°C by a 0-silk suture followed by skin closure using interrupted 4-0 nylon sutures. Sham-surgery control mice underwent a similar surgical procedure without clamping of the left kidney pedicle. FAN entailed the creation of a 0.5-cm free skin flap. Forelimb and hindlimb were joined from the olecranon to the knee. Subcutaneous tissue was dissected to form a 0.5-cm free skin flap. Forelimb and hindlimb were joined from the olecranon to the knee. Subcutaneous tissue was dissected to create a 0.5-cm free skin flap. Forelimb and hindlimb were joined by a 0-silk suture followed by skin closure using interrupted 4-0 nylon sutures. Body temperature was maintained at 36.8° to 37.2°C throughout the procedure. Postoperatively, animals recovered in a heated cage, and each parabiont was given 1 ml of warm (37°C) saline. Analgesia was given twice a day for 2 days. Sulfatram water was provided for 28 days.

Injection under the renal capsule

I-A^D+ cells were sorted from spleens of UUO GFP mice and pooled into a single-cell suspension. GFP^+I-A^D+ splenocytes (1 × 10^6) were injected under the renal capsule, into the upper pole in kidneys (C57BL/6J mice) on day 3 after UUO surgery. Mice were euthanized 6 hours after injection. Renal tissue samples were fixed in 4% paraformaldehyde (PFA).

Renal histopathology

Kidneys were fixed in 4% PFA for 24 hours and embedded in paraffin. Periodic acid–Schiff (PAS) staining was performed to evaluate pathological injury. Masson and Sirius red staining were performed to estimate renal fibrosis. Two blinded renal pathologists quantified staining in eight randomly selected fields. Data were analyzed using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA).

Immunohistochemistry and immunofluorescence

Antigens were recovered by treatment of samples at 98°C for 10 min in 10 mM citrate buffer (pH 6.0). For immunohistochemical analysis of human specimens, endogenous peroxidase was blocked with 10% H_2O_2 for 20 min and nonspecific antigens were blocked with serum for 30 min at room temperature. The slides were then incubated with specific primary antibodies at 4°C for 12 hours. Antibodies used were as follows: D2-40 (1:50, dianaminobenzidine (DAKO)), CD4 (1:50, DAKO), CD8 (1:50, DAKO), CD20 (1:50, DAKO), CD68 (1:50, DAKO), DC-SIGN (1:20, Santa Cruz Biotechnology), CD1c (1:50, Abcam), VEGF-C (1:50, Santa Cruz Biotechnology), VEGF-D (1:100, R&D Systems), VEGF-A (1:50, Abcam), and FGF-2 (1:100, R&D Systems). Slides were then treated with horseradish peroxidase–conjugated secondary antibodies. For immunofluorescence analysis, nonspecific antigens were blocked with serum for 30 min at room temperature. The slides were then incubated with specific primary antibodies at 4°C. Antibodies used were as follows: LYVE-1 (1:100, R&D Systems or 1:100, AngioBio, USA), VEGF3 (1:100, R&D Systems), PROX1 (1:50, Abcam), D2-40 (1:50, DAKO), CD31 (1:100, Abcam), podoplanin (1:100, R&D Systems), CCL21 (1:100, R&D Systems for mouse and 1:100, Abcam for human), CXCL12 (1:100, R&D Systems), CX3CL1 (1:100, R&D Systems), CCR7 (1:50, R&D Systems for mouse and 1:100, Abcam for human), CD3 (1:50, Abcam), F4/80 (1:50, Abcam), B220 (1:100, BD Biosciences), Ly6G (1:100, Abcam), I-A^D (1:100, Abcam), KIM-1 (1:500, R&D Systems), Ki67 (1:200, Abcam), GFP (1:50, Promoter), CD11c (1:50, Abcam), THP (1:100, Millipore), PCNA (1:50, Santa Cruz Biotechnology), α-SMA (1:100, Abcam), fibronectin (1:50, Abcam), and AQP-1 (1:200, Abcam). The slides were then incubated with fluorescence–labeled secondary antibodies (Jackson ImmunoResearch). Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) (Roche, Switzerland).

Representative images were acquired using an Olympus microscope and DP73 camera. TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) staining was performed using a kit (Roche, Switzerland) according to the manufacturer's instructions. VEGF-C, VEGF-D, FGF-2, fibronectin, and α-SMA staining were carefully quantified by two blinded renal pathologists reading eight randomly chosen fields in high-power field (HPF) on each slide. The data were analyzed using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA). The numbers of CD31-positive peritubular capillaries and AQP-1–positive renal tubules were counted in HPF, as described above.

Soluble mouse LYVE-1 and VEGF3 fusion construct cloning, purification of Fc fusion proteins, and intervention procedure

Segments of murine LYVE-1 complementary DNA (cDNA) (position 241–924 in GenBank, clone AJ311501) encoding the extracellular domain of LYVE-1 and VEGF3 cDNA (position 26–2363 in GenBank, clone L07296) encoding the extracellular domain of VEGF3 were separately fused to Fc [mouse IgG2a (immunglobulin G2a)] in the pcDNA3.1(+)/Fc (mouse IgG2a) vector (Futaibio, Jiangsu, China). The pcDNA3.1 (+)/Fc (mouse IgG2a) vector was used as control. Constructs were transfected into 293T cells using calcium phosphate. Transfected cells were grown in UltraCHO medium (Lonza) for 3 days before harvesting culture supernant. After adjustment of supernatant pH [using 2 M tris-HCl buffer (pH 8.0)], the fusion proteins were purified by affinity chromatography on a column (1-ml bed volume) of protein A–Sepharose (Sigma) eluted with 1 M glycine-HCl buffer (pH 3.0). Fractions containing Fc fusion proteins were neutralized by the addition of 0.05 volume of 2 M tris-HCl buffer (pH 8.0), and the purity was confirmed by SDS–polyacrylamide gel electrophoresis (63–65). sVEGF3-FC or sLYVE-1-FC was administered to wild-type mice by tail vein injection (0.5 µg/g) 24 hours before UUO or IRI surgery. sFc was injected as a control. UUO mice were administered VEGF3-FC, sLYVE-1-FC, or sFc (0.5 µg/g) on days 0, 1, 3, and 5 and euthanized on day 7 after
UO. IRI mice were administered sVEGFR3-FC, sLYVE-1-FC, or sFC (0.5 μg/g) on days 0, 1, 3, 7, 10, and 13 and euthanized on day 14 after IRI.

**CCR7 intervention**

Monoclonal rat anti-mouse CCR7 antibody IgG2A (clone no. 4B12, R&D Systems) was injected intravenously into wild-type mice (dose, 0.5 μg/g) 24 hours before UUO. Rat IgG2A isotype (R&D Systems) was injected as control. Equivalent doses of CCR7 antibody or rat IgG2A isotype were administered on days 0, 1, 3, and 5, and mice were euthanized on day 7 following UUO.

**LV and inflammatory cell counting**

For human renal biopsy specimens, D2-40–stained sections were scanned at low power to identify three LV “hotspots,” which were then examined at 40×. D2-40–positive vessels with a clearly defined linear vessel shape, but not single endothelial cells, were used for counting. Inflammatory cell density was also determined in the three areas of high-density infiltration using a method analogous to that for LV enumeration (66). In mouse models, LV-1 staining in renal sections was imaged at 40×. LV-1–positive vessels with a clearly defined linear or well-defined linear vessel shape, but not single endothelial cells, were used for counting. The objectivity of LV counting was improved by imaging the LV in the longitudinal section of the murine kidney and subsequently calculating the numbers of LVs using Image-Pro Plus software. The number of LVs in each field was ranked from high to low. The top 20 fields were regarded as hotspots. The average value represented the density of the entire kidney. When assessing LVs in the longitudinal section of the murine kidney and subse-

**Quantitative real-time PCR**

Total RNA was extracted from renal and splenic tissues using TRIzol (Guangzhou, China). One microgram of RNA was reverse-transcribed using the extraction kit manufacturer’s instructions (Invitrogen, USA). Monoclonal rat anti-mouse CCR7 antibody IgG2A (clone no. 4B12, R&D Systems) was injected intravenously into wild-type mice (dose, 0.5 μg/g) 24 hours before UUO. Rat IgG2A isotype (R&D Systems) was injected as control. Equivalent doses of CCR7 antibody or rat IgG2A isotype were administered on days 0, 1, 3, and 5, and mice were euthanized on day 7 following UUO.

**Biochemical analysis and hydroxyproline assays**

Serum creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and albumin levels were measured using an automatic

---

*Pei et al., Sci. Adv. 2019; 5 : eaaw5075     26 June 2019*
biochemical analyzer (Roche, Germany). Kidney hydroxyproline levels were measured using a commercial kit according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). $A_{550}$ (absorbance at 550 nm) was determined using a microplate reader (BioTek, USA).

**Flow cytometry**

To characterize immune infiltration, the entire UUO kidney and its corresponding contralateral control were minced and then digested in 2 ml of collagenase (400 U of type I collagenase) in Dulbecco’s modified Eagle’s medium at 37°C for 25 min. Spleen and lymph node cells were ground into single-cell suspensions and filtered through 100-nm mesh before immunostaining cells with a fixable viability dye. For fluorescence-activated cell sorting (FACS) analysis, phycoerythrin (PE)–conjugated anti–LYVE-1 antibody, fluorescein isothiocyanate (FITC)–conjugated anti-mouse/human CD3 antibody, BV510-conjugated CD45 antibody, peridinin chlorophyll protein (PerCP)/Cy5.5-conjugated anti-mouse CD8 antibody, APC-conjugated anti-mouse Ly6G antibody, FITC-conjugated anti-mouse F4/80 antibody, PE-conjugated anti-mouse Ly6G antibody, PE-conjugated anti-CD11c antibody, APC-conjugated anti-mouse CD3 antibody, PerCP/Cy5.5-conjugated anti-mouse/human CD3 antibody, PerCP/Cy5.5-conjugated anti-mouse I-A$^D$ antibody, APC-conjugated anti-mouse CD4 antibody, Pacific blue–conjugated anti-CD19 antibody, PE-conjugated anti-mouse NK1.1 antibody, FITC-conjugated anti-mouse CD44 antibody, and PE-conjugated anti-mouse LY6C. Proliferating cells were analyzed by flow cytometry using an anti-Ki67 antibody (clone SolA15, eBioscience) with the Transcription Factor Staining Buffer Set (00-5523-00, eBioscience). The dead cells were excluded by staining with APC/Cy7-conjugated Zoom. APC/Cy7-conjugated isotype, FITC-conjugated isotype, PerCP/Cy5.5-conjugated isotype, PE-conjugated isotype, APC-conjugated isotype, Alexa Fluor 488–conjugated isotype, and BV421-conjugated isotype were used as isotype controls. Positive cells were sorted using a BD FACs, and the data were analyzed using FlowJo v7.6.3 software.

**In vitro cell culture assays**

SVEC4-10 cells (American Type Culture Collection, USA) were used for LEC in vitro experiments (7). Cell Counting Kit-8 (CCK-8) assay: VEGF-C or VEGF-D (0, 1, 10, and 100 ng/µl) and FGF-2 (0, 1, 10, and 20 ng/µl) were added to promote cell proliferation. sVEGF3-FC (100 ng/µl) or sLYVE-1-FC (250 ng/µl) was added to inhibit proliferation. sFC at the same concentration was used as control. Forty-eight hours after stimulation, SVEC4-10 cells in 96-well plates were incubated with 100 µl of 50 µmol EdU for 2 hours and then immobilized with 4% polyoxymethylene. EdU staining was imaged with Cell-Light EdU Apollo 567 In Vitro Imaging Kit (C10310-1, RiboBio Co., Guangzhou, China) according to the manufacturer’s instructions. Nuclei were stained with Hoechst 33342.

**Apoptosis assay:** 24 hours following stimulation with either sVEGF3-FC (100 ng/µl) or sLYVE-1-FC (250 ng/µl), an Annexin V–FITC (AV)/propidium iodide (PI) assay was performed to analyze apoptosis (BD Biosciences, USA). Flow cytometry was then used to detect AV- and PI-positive cells.

**Statistical analysis**

The data were first analyzed for normal distribution. The density of LVs and inflammatory cells in human samples showed positively skewed distribution. CKD patients were divided into LVs$^{\text{low}}$ and LVs$^{\text{high}}$ groups by intrarenal LV numbers graded from the second lowest terrace versus the highest terrace. Box plots were created using SigmaPlot 10.0. Values greater than 95% or less than 5% were shown as a dot, while values less than 95% or greater than 5% were hidden in positive and negative error line. Values less than 75% or greater than 25% were hidden in bars. Data conforming to normal distribution were presented as mean ± SEM and were prepared using GraphPad Prism software version 5.0. We used the nonparametric two-tailed unpaired or paired Mann–Whitney U test to evaluate P values. n.s. > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/6/eaaw5075/DC1

**Fig. S1.** LVs markers and intrarenal interstitial inflammatory cells of the LVs$^{\text{low}}$ and LVs$^{\text{high}}$ groups in kidneys of CKD patients.

**Fig. S2.** Renal fibrosis is accompanied by atrophy.

**Fig. S3.** Flow cytometry gating strategies.

**Fig. S4.** THP protein in RDLNs.

**Fig. S5.** Conditional KD of LVs elicited by DT injection in mice that did not undergo surgery.

**Fig. S6.** Conditional KD of LVs in LYVE-1-Cre/iDTR mice attenuated UUO-induced lymphocyte expansion in both RDLN and spleen, intrarenal inflammatory infiltration, and loss of capillaries.

**Fig. S7.** Conditional KD of LVs in LYVE-1-Cre/iDTR mice attenuated IRI-induced splenomegaly, perirenal lymphadenectomy, intrarenal inflammation, and fibrosis at day 14.

**Fig. S8.** sVEGF3-FC or sLYVE-1-FC attenuated lymphatic endothelial proliferation but did not induce apoptosis in vitro.

**Fig. S9.** sVEGF3-FC or sLYVE-1-FC attenuated intrarenal lymphatic endothelium proliferation, renal inflammation, and fibrosis.

**Table S1.** Patient clinical and demographic characteristics.

**REFERENCES AND NOTES**

1. M. D. Breyer, K. Susztak, The next generation of therapeutics for chronic kidney disease. *Nat. Rev. Drug Discov.* 15, 568–588 (2016).

2. G. Pei, R. Zeng, M. Han, P. Liao, X. Zhou, Y. Li, C. Zhang, L. Liu, Y. Yao, G. Xu, Renal interstitial infiltration and tertiary lymphoid organ neogenesis in IgA nephropathy. *Clin. Am. Soc. Nephrol.* 9, 255–264 (2014).

3. H. J. Anders, B. Suarez-Alvarez, M. Grigorescu, O. Foresto-Neto, S. Steiger, J. Desai, J. A. Marschner, M. Monarpisheh, C. Shi, J. Jordan, L. Müller, N. Burzlaff, B. Täuber, S. R. Mullay, The macrophage phenotype and inflammasome component NLRP3 contributes to nephrocalcinosis-related chronic kidney disease independent from IL-1-mediated tissue injury. *Kidney Int.* 93, 656–669 (2018).

4. M. A. Alikhán, M. Huynh, A. R. Kitching, J. D. Ooi, Regulatory T cells in renal disease. *Clin. Transl. Immunol.* 7, e1004 (2018).

5. G. J. Randolph, S. Ivanov, B. H. Zinselmeyer, J. P. Scallan, The lymphatic system: Integral roles in immunity. *Annu. Rev. Immunol.* 35, 31–52 (2017).

6. B. Vigil, D. Aebischer, M. Nitschke, M. Ilevya, T. Röthlin, O. Antsiferova, C. Hahn, Tissue inflammation modulates gene expression of lymphatic endothelial cells and dendritic cell migration in a stimulus-dependent manner. *Blood* 118, 205–215 (2011).

7. A. Martin-Fonteche, S. Sebastiani, U. E. Höpken, M. Ugoccioni, M. Lipp, A. LanzaVecchia, F. Sallust, Regulation of dendritic cell migration to the draining lymph node: Impact on lymphocyte traffic and priming. *J. Exp. Med.* 198, 615–621 (2003).

8. S. K. Bromley, S. Y. Thomas, A. D. Luster, Chemokine receptorCCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nat. Immunol.* 6, 895–901 (2005).

9. R. Förster, A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Müller, E. Wolf, M. Lipp, CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 99, 23–33 (1999).
11. M. Frye, A. Taddei, C. Dierkes, I. Martinez-Corral, M. Fielden, H. Ortsäter, J. Kazenwadel, Pei et al., Sci. Adv. 2019; 5 : eaaw5075     26 June 2019

14. K. Vaahtomeri, S. Karaman, T. Mäkinen, K. Alitalo, Lymphangiogenesis guidance by paracrine and pericellular factors. Genes Dev. 31, 1615–1634 (2017).

15. N. Platonova, G. Miquel, B. Regenfuss, S. Taouji, C. Curisien, E. Chevet, A. Bikfalvi, Evidence for the interaction of fibroblast growth factor-2 with the lymphatic endothelial cell marker LYVE-1. Blood 121, 1229–1237 (2013).

16. H. L. Wong, G. Jin, R. Cao, S. Zhang, Y. Cao, Z. Zhou, MT1-MMP sheds LYVE-1 on lymphatic endothelial cells and suppresses VEGF-C production to inhibit lymphangiogenesis. Nat. Commun. 7, 10824 (2016).

17. I. A. Nykanen, H. Sandelin, R. Krebs, M. A. I. Keränen, R. Tuuminen, T. Kärpanen, Y. Wu, B. Pytowski, P. K. Koskinen, S. Ylä-Herttuala, K. Alitalo, K. B. Lemström, Targeting lymphatic vessel activation and CCL21 production by vascular endothelial growth factor receptor-3 inhibition has a pivotal immunomodulatory and antiangiogenic effect in cardiac allografts. Circulation 121, 1413–1422 (2010).

18. R. Huggenberger, S. Ullmann, S. T. Proulx, B. Pytowski, K. Alitalo, M. Detmar, Stimulation of lymphangiogenesis through C-C motif chemokine receptor 2-dependent cross talk by inhibiting VEGF receptor signaling attenuates kidney microvascular rarefaction and fibrosis. Proc. Natl. Acad. Sci. U. S. A. 104, 12080–12085 (2007).

19. T. M. El-Achkar, R. McCracken, Y. Liu, M. R. Heitmeier, S. Bourgeois, J. Ryerse, X.-R. Wu, L. Yang, T. Y. Besschetnova, C. R. Brooks, J. V. Shah, J. V. Bonventre, Epithelial cell cycle arrest in G2/M mediates kidney fibrosis after injury. Nat. Med. 16, 535–543 (2010).

20. D. Kerjaschki, N. Huttary, I. Raab, H. Regele, K. Bajzak, F. Bartel, S. M. Kröber, E. Brakenhielm, Selective stimulation of cardiac lymphangiogenesis reduces myocardial ischemia-reperfusion injury. Transplantation 101, 1033–1042 (2013).

21. R. K. Jain, LYVE-1 is not restricted to the lymph vessels: Expression in normal liver blood and lymphatic vessel activation and CCL21 production by vascular endothelial growth factor receptor-3 inhibition has a pivotal immunomodulatory and antiangiogenic effect in cardiac allografts. Circulation 121, 1413–1422 (2010).

22. R. Huggenberger, S. Ullmann, S. T. Proulx, B. Pytowski, K. Alitalo, M. Detmar, Stimulation of lymphangiogenesis through C-C motif chemokine receptor 2-dependent cross talk by inhibiting VEGF receptor signaling attenuates kidney microvascular rarefaction and fibrosis. Proc. Natl. Acad. Sci. U. S. A. 104, 12080–12085 (2007).

23. A. Braun, T. Worbs, G. L. Moschovakis, S. Halle, K. Hoffmann, J. Börger, M. Förster, Different lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. Nat. Immunol. 12, 879–887 (2011).

24. H. Han, J. Zhu, Y. Wang, Z. Zhu, Y. Chen, L. Lu, W. Jin, X. Yan, R. Zhang, Renal recruitment of B lymphocytes exacerbates tubulointerstitial fibrosis by promoting monocyte mobilization and infiltration after unilateral ureteral obstruction. J. Pathol. 241, 80–90 (2017).

25. R. Micano, S. Khan, D. Janesovíc, M. E. Lee, T. Hato, E. F. Simon, W. J. Whitt, G. T. S. E. Row, T. M. El-Achkar, Tamm-Horsfall protein regulates mononuclear phagocytes in the kidney. J. Am. Soc. Nephrol. 29, 841–856 (2018).

26. K. Vaahtomeri, S. Karaman, T. Mäkinen, K. Alitalo, Lymphangiogenesis guidance by paracrine and pericellular factors. Genes Dev. 31, 1615–1634 (2017).

27. N. Platonova, G. Miquel, B. Regenfuss, S. Taouji, C. Curisien, E. Chevet, A. Bikfalvi, Evidence for the interaction of fibroblast growth factor-2 with the lymphatic endothelial cell marker LYVE-1. Blood 121, 1229–1237 (2013).

28. R. Huggenberger, S. Ullmann, S. T. Proulx, B. Pytowski, K. Alitalo, M. Detmar, Stimulation of lymphangiogenesis through C-C motif chemokine receptor 2-dependent cross talk by inhibiting VEGF receptor signaling attenuates kidney microvascular rarefaction and fibrosis. Proc. Natl. Acad. Sci. U. S. A. 104, 12080–12085 (2007).

29. A. Braun, T. Worbs, G. L. Moschovakis, S. Halle, K. Hoffmann, J. Börger, M. Förster, Different lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. Nat. Immunol. 12, 879–887 (2011).

30. H. Han, J. Zhu, Y. Wang, Z. Zhu, Y. Chen, L. Lu, W. Jin, X. Yan, R. Zhang, Renal recruitment of B lymphocytes exacerbates tubulointerstitial fibrosis by promoting monocyte mobilization and infiltration after unilateral ureteral obstruction. J. Pathol. 241, 80–90 (2017).

31. R. Micano, S. Khan, D. Janesovíc, M. E. Lee, T. Hato, E. F. Simon, W. J. Whitt, G. T. S. E. Row, T. M. El-Achkar, Tamm-Horsfall protein regulates mononuclear phagocytes in the kidney. J. Am. Soc. Nephrol. 29, 841–856 (2018).

32. A. Braun, T. Worbs, G. L. Moschovakis, S. Halle, K. Hoffmann, J. Börger, M. Förster, Different lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. Nat. Immunol. 12, 879–887 (2011).

33. N. Sharma, A. P. Bezchitnik, L. Lefrançois, K. M. Khanna, CDT T cells enter the splenic T cell zones independently of CCR7, but the subsequent expansion and trafficking patterns of effector T cells after infection are dysregulated in the absence of CC(R)7 migratory cues. J. Immunol. 195, 2522–2536 (2015).

34. S. Banerji, J. Ni, S. X. Wang, S. Clasper, J. Su, R. Tammi, M. Jones, D. G. Jackson, LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J. Cell Biol. 164, 789–801 (1999).

35. J. Y. Jiang, J. Y. Koh, S.-H. Lee, J. Lee, K. H. Kim, D. Kim, G. Y. Koh, O. J. Yoo, Conditional ablation of LYVE-1+ cells unveils defensive roles of lymphatic microvessels in tissue injury and lymph node development. Nat. Immunol. 13, 2151–2161 (2012).

36. H. Dimke, M. A. Sparks, B. R. Thomson, S. Frische, T. M. Coffman, S. E. Quagg, Tubulovascular cross-talk by vascular endothelial growth factor maintains peritubular microvasculature in kidney. J. Am. Soc. Nephrol. 26, 1027–1038 (2015).

37. S.-L. Lin, F.-C. Chang, C. Schirmpf, Y.-T. Chen, C.-F. Wu, V.-C. Wu, W.-C. Chiang, F. Kühnert, C.-J. Kuo, Y.-M. Chen, K.-D. Wu, T.-J. Tsai, J. S. Duffield, Targeting endothelin-precysc chemokine by inhibiting VEGF receptor signaling attenuates kidney microvascular rarefaction and fibrosis. Am. J. Pathol. 178, 911–923 (2011).
49. K. Schledzewski, M. Falkowski, G. Molderhauer, P. Metharam, J. Kzhzychkowska, R. Ganss, A. Demory, B. Falkowska-Hansen, H. Kurzen, S. Ugreul, G. Geginat, B. Arnold, S. Goedt, Lymphatic endothelium-specific hyaluronan receptor LYVE-1 is expressed by stabilin-1+, F4/80+, CD11b+ macrophages in malignant tumours and wound healing tissue in vivo and in bone marrow cultures in vitro: Implications for the assessment of lymphangiogenesis. *J. Pathol.* **209**, 67–77 (2006).

50. H.-W. Lee, Y.-X. Qin, Y.-M. Kim, E.-Y. Park, J.-S. Hwang, G.-H. Huo, C.-W. Yang, W.-Y. Kim, J. Kim, Expression of lymphatic endothelium-specific hyaluronan receptor LYVE-1 in the developing mouse kidney. *Cell Tissue Res.* **343**, 429–444 (2011).

51. I. Goren, N. Allmann, N. Yogev, C. Schürmann, A. Linke, M. Holdener, A. Waisman, Ischemia-reperfusion injury enhances lymphatic endothelial VEGFR3 and rejection in cardiac allografts. *Am. J. Transplant.* **16**, 1160–1172 (2016).

52. A. Dashkevich, A. Raissadati, S. O. Syrjälä, G. Zarkada, M. A. I. Keränen, R. Tuuminen, Effects of diphtheria toxin-driven lysozyme M-specific cell lineage ablation on wound inflammatory, angiogenic, and contractive processes. *Am. J. Pathol.* **181**, 132–147 (2009).

53. S. Mertlitz, Y. Shi, M. Kalupa, C. Grötzinger, J. van Dorn, Targeting tubulointerstitial remodeling in proteinuric nephropathy in rats. *Dis. Model. Mech.* **8**, 919–930 (2015).

54. L. A. Johnson, S. Banerji, W. Lawrance, U. Gileadi, G. Prota, K. A. Holder, Y. M. Roshorm, J. Kim, Expression of lymphatic endothelium-specific hyaluronan receptor LYVE-1 in inflammatory, angiogenic, and contractive processes.

55. A. W. Lund, M. Wagner, M. Fankhauser, E. S. Steinskog, M. A. Broggi, S. Spranger, Effects of diphtheria toxin-driven lysozyme M-specific cell lineage ablation on wound inflammatory, angiogenic, and contractive processes. *Am. J. Pathol.* **181**, 132–147 (2009).

56. E. Gousopoulos, S. T. Proulx, J. Scholl, M. Uecker, M. Detmar, Prominent lymphatic vessel hyperplasia with progressive dysfunction and distinct immune cell infiltration in lymphpheda. *Am. J. Pathol.* **186**, 2193–2203 (2016).

57. A. W. Lund, M. Wagner, M. Fankhauser, E. S. Steinskog, M. A. Broggi, S. Spranger, T. F. Gajewski, K. Alitalo, H. P. Eikesdal, H. Wiig, M. A. Swartz, Lymphatic vessels regulate immune microenvironments in human and murine melanoma. *J. Clin. Invest.* **132**, 3389–3402 (2016).

58. A. Muchowicz, M. Wachowska, J. Stachura, K. Tonecka, M. Gabrysiak, D. Wołosz, Z. Pilch, W. W. Kilarski, L. Boon, T. J. Klaus, J. Golab, Inhibition of lymphangiogenesis impairs antitumour effects of photodynamic therapy and checkpoint inhibitors in mice. *Eur. J. Cancer* **83**, 19–27 (2017).

59. R. Ikei, S. Kobayashi, T. Niki, T. Namikoshi, T. Imakiire, Y. Kikuchi, T. Hanke, V. Cerundolo, N. W. Gale, D. G. Jackson, Dendritic cells enter lymph vessels by lymphangiogenesis. *Blood* **116**, 195–206 (2005).

60. C. S. M. Williams, R. D. Leek, A. M. Robson, S. Banerji, R. Prevo, A. L. Harris, D. G. Jackson, Absence of lymphangiogenesis and intratumoral lymph vessels in human metastatic breast cancer. *J. Pathol.* **2**, 195–206 (2003).

Acknowledgments
Funding: This work was supported by the Major Research Plan of the National Natural Science Foundation of China (grant no. 91742204), International (regional) Cooperation and Exchange Projects (NSFC-DFG, grant no. 81270770, 8147948, 81670633, 81570669, 81570615, 81770681, and 81770686), the National Natural Science Foundation of China for Young Scholars (grant no. 81600556), the National Key Research and Development Program (grant no. 2016YFC0906103), and the National Key Technology R&D Program (grant nos. 2013BAI09B06 and 2015BAI12B07). Author contributions: R.Z., G.P., G.X., and Y.Y. designed the study. G.P., Y.W., J.W., Q.Y., P.W., W.Y., Y.Z., X.D., Y.L., J.Y., F.Z., M.H., H.Z., and R.Z., G.P., G.X., and R.Z. conceived the project and supervised and coordinated all the work. R.Z., and G.X. analyzed the data and provided critical input. G.P., Y.Y., Y.W., J.W., S.G., M.H., M.W. performed the experiments, analyzed the data, and prepared the figures and tables. G.P., Y.Y., Y.W., J.W., R.Z., and G.X. contributed analytical tools. G.P., Y.W., J.W., Q.Y., P.W., W.Y., Y.Z., X.D., Y.L., J.Y., F.Z., M.H., H.Z., and M.W. performed the experiments, analyzed the data, and prepared the figures and tables. G.P., Y.Y., Y.W., J.W., R.Z., and G.X. contributed analytical tools. G.P., Y.Y., Y.W., J.W., M.H., R.Z., and G.X. analyzed the data and provided critical input. G.P., Y.Y., J.W., G.X. conceived the project and supervised and coordinated all the work.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 28 December 2018
Accepted 22 May 2019
Published 26 June 2019
10.1126/sciadv.aaw5075

Citation: G. Pei, Y. Yao, Q. Yang, M. Wang, Y. Wang, J. Wu, P. Wang, Y. Li, F. Zhu, J. Yang, Y. Zhang, W. Yang, X. Zhao, H. Zhu, S. Ge, M. Han, R. Zeng, G. Xu, Lymphangiogenesis in kidney and lymph node mediates renal inflammation and fibrosis. *Sci. Adv.* **5**, eaaw5075 (2019).