Beyond a passive conduit: implications of lymphatic biology for kidney diseases

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ABSTRACT

The kidney contains a network of lymphatic vessels which clear fluid, small molecules and cells from the renal interstitium. Through modulating immune responses and *via* crosstalk with surrounding renal cells, lymphatic vessels have been implicated in the progression and maintenance of kidney disease. In this Review, we provide an overview of the development, structure and function of lymphatic vessels in the healthy adult kidney. We then highlight the contributions of lymphatic vessels to multiple forms of renal pathology, emphasizing chronic kidney disease, transplant rejection and polycystic kidney disease, and discuss strategies to target renal lymphatics using genetic and pharmacological approaches. Overall, we argue the case for lymphatics playing a fundamental role in renal physiology and pathology, and treatments modulating these vessels having therapeutic potential across the spectrum of kidney disease.

Lymphatic vessels serve as a conduit for the clearance of tissue fluid, cells and small molecules, within a protein rich fluid termed lymph, from the interstitial compartment of vertebrate organs. This fluid enters the lymphatic system via lymphatic capillaries within tissues, travelling down a hierarchical network of collecting vessels before reaching lymph nodes which drain to large ducts, eventually returning lymph to the venous circulation. Through the identification of molecular markers of lymphatics, advances in imaging technologies and novel genetic tools to visualize and manipulate their function, the roles of lymphatic vessels have expanded to include cholesterol transport[1], clearance of cerebrospinal fluid[2], electrolyte homeostasis[3] and regulating peripheral tolerance, innate and adaptive immunity[4,5].

The kidney possesses a lymphatic system that has been implicated in the progression and maintenance of kidney disease[6–8]. However, in spite of an increasing understanding of lymphatic biology in other organs (reviewed in[9–11]), the kidney lymphatics have received relatively little attention. In this Review, we first outline how lymphatics form during kidney development and describe their structure and function in adult kidneys. We then combine our current understanding of kidney lymphatics with parallels drawn from lymphatic biology in other organs to discuss their potential contribution to and therapeutic implications for several renal diseases.

DEVELOPMENT, ARCHITECTURE AND FUNCTION OF KIDNEY LYMPHATICS

Development

A combination of immunohistochemical studies[12–15] and more recently, three-dimensional (3D) imaging coupled with quantitative analysis have characterized lymphatic development in mouse and human embryonic kidneys (**Figure 1**)[16]. In mouse, the metanephros, the precursor to the adult kidney, begins to form around embryonic day (E)10.5[17]. At early stages of its development[18], the embryonic kidney is devoid of lymphatics. It is not until E14.5, after considerable development of the blood vasculature[19,20], that lymphatic endothelial cells (LECs) are detectable as a cellular plexus using immunohistochemistry for lymphatic markers (**Table 1**) such as Prospero homeobox protein 1 (PROX1) or vascular endothelial growth factor receptor (VEGFR)-3. The developing kidney lymphatics wrap around the base of the nascent kidney pelvis, and are suggested to be continuous with an extra-renal network supplying the ureter, adrenal gland and gonad[12,14]. Between E15.5 and E18.5, the hilar lymphatics rapidly remodel and expand, forming lumenized vessels that extend alongside arterioles into the renal cortex. During this time, a complex collecting duct network is established[21], fully differentiated cell types within the kidney emerge such as the mature glomerulus, segments of the nephron[22], perivascular and mesangial cells[23] and renal excretory function is initiated[17,24] . By the end of mouse gestation, lymphatics are present in both the hilum and the cortex. A similar pattern of lymphatic vessels is established by the end of the first trimester in humans[16].

Macroarchitecture

In the mature adult kidney, lymph drainage begins in the cortical interstitium, with blind ended lymphatic capillaries draining into arcades running with arcuate arteries at the corticomedullary junction (**Figure 1**)[25,26]. The cortical lymphatics then follow the interlobar blood vessels, descending towards the renal pelvis. Finally, the lymphatics drain out of the

kidney through hilar lymphatic vessels, located adjacent to the major renal arteries and veins as they enter and exit the kidney.

Cellular architecture

Initially within organs, lymph enters lymphatic capillaries, which consist of a single, continuous layer of LECs. Unlike most blood vessels, lymphatic capillaries have a sparse, discontinuous basement membrane and lack supporting cells such as vascular smooth muscle cells, pericytes or fibroblasts[13]. Instead, LECs lining lymphatic capillaries overlap, held together by specialized button-like junctions[27] and physically connected to surrounding extracellular matrix (ECM) by fibrillin-rich anchoring proteins[28,29]. As fluid leaks across blood capillaries, the ECM in the interstitium expands causing anchoring proteins to pull on LECs. Consequently, button-like junctions between LECs open, allowing the constituents of lymph to enter lymphatics paracellularly (**Figure 1**)[30]. Solutes may alternatively enter lymphatics transcellularly, *via* vesicle formation and transcytosis across LECs[31]. Lymphatic capillaries drain lymph into functional units of pre-collecting and collecting vessels[13] known as lymphangions. Within these larger caliber vessels, LECs are lined by continuous zipper-like junctions, are supported by smooth muscle and mural cells and contain valves to facilitate unidirectional lymph flow[27,32].

Heterogeneity of lymphatics and 'lymphatic-like' vessels

Lymphatic vessels have a unique molecular signature distinguishable from that of blood endothelia[33]. Some markers are expressed in all lymphatic vessels, such as PROX1 and VEGFR-3. However, there is heterogeneity in the molecular profile between lymphatic capillaries, pre-collecting and collecting vessels[9]. Heterogeneity of the adult kidney's blood vascular system is well recognized; with blood endothelial cells molecularly distinguishable between cortex, medulla and glomerulus[34,35]. Whether similar molecular diversity exists within kidney lymphatics remains unexplored.

Populations of hybrid kidney blood vessels expressing both blood and lymphatic endothelial markers have been identified[36,37]. Peritubular capillaries; which facilitate the reabsorption of fluids and molecules from adjacent cortical tubular epithelium, express CD31 and VEGFR-3, but not other lymphatic markers PROX1, lymphatic vessel hyaluronan receptor (LYVE-1) or podoplanin (PDPN)[37]. Conversely in a recent study, ascending vasa recta (AVR), which maintain the medullary osmotic gradient critical for urinary concentrating ability, were found to express PROX1 and VEGFR-3 but not LYVE-1 or PDPN[36]. Unlike LECs, specialized functions of peritubular capillaries and AVR are facilitated by endothelial fenestrations; identifiable in electron micrographs as transcellular channels of ~70 nm in diameter[38]. Whether these vessels, which have been termed 'lymphatic-like', share other molecular or structural features with LECs is yet to be determined, but clearly multiple markers in parallel are required to reliably distinguish kidney lymphatics from other cell types in the kidney.

Function and composition of renal lymph

Based on their anatomical location[39] and uptake of radiolabeled albumin[40], kidney lymphatics are proposed to drain the interstitium of the renal cortex and hilum interstitium, but not the medulla. In the cortex, a mismatch between tubular reabsorption and the capacity for uptake by peritubular capillaries may raise cortical interstitial pressure[41] and facilitate lymphatic clearance. Early functional studies indicate a functional interplay between kidney lymphatic flow, venous pressure[42–45] and solute load[46,47] that warrants further investigation[48–50].

Rich in immunoglobulins and albumin[40], lymph, like plasma, also contains complement cascade components, coagulation factors, ECM proteases and their inhibitors and enzymes involved in cellular metabolism. Lymph is also enriched in nuclear histones, cytosolic enzymes, transcription factors and ribosomal components[51], likely derived from cellular apoptosis[52]. Few studies have examined the composition of renal lymph. Sodium, chloride, potassium and calcium content of lymph draining from the kidneys may have physiological relevance[43,47]. Analysis of rodent models of renal ischaemia perfusion injury have identified

renal lymph to contain cytokines such as interleukin (IL)-1β and IL-6, tumor necrosis factor (TNF)-α and monocyte chemoattractant protein 1 (MCP-1)[53], albeit low in quantities compared to blood draining from the kidney. Renal draining lymph nodes receive dendritic cells (DC), T and B lymphocytes from afferent renal lymphatics[54]. These studies advocate roles for kidney lymphatics in the maintenance of peripheral tolerance and clearance of cellular debris in adult renal physiology. Renal lymph can also drain renin and angiotensin II[53,55], but the physiological relevance of this route to the systematic circulation is unclear.

ROLES OF LYMPHATICS IN KIDNEY DISEASES

Lymphangiogenesis and its role in chronic renal injury and fibrosis

Structural changes to the vasculature are a prominent feature of chronic kidney disease (CKD). Whereas peritubular capillaries undergo rarefaction, potentially triggering interstitial hypoxia and generating a profibrogenic environment within diseased kidney[59], renal lymphatics proliferate and sprout, giving rise to new vessels in a process termed lymphangiogenesis[60]. In biopsies of immunoglobulin A nephropathy, focal glomerulosclerosis, lupus, anti-neutrophil cytoplasmic antibody-related glomerulonephritis, diabetic kidney disease (DKD) and chronic interstitial nephritis acquired from patients ranging from moderate CKD to end-stage kidney disease (ESKD), the cross sectional area of lymphatics is significantly greater than in non-diseased kidneys[26,61]; a finding replicated in multiple murine models of CKD[54,62–66].

Lymphangiogenesis facilitates the clearance of inflammatory cells from the damaged tissue environment; vital steps in the resolution of inflammation and prevention of fibrotic remodeling. This function is illustrated by ligating lymphatics exiting the kidney in rats, leading to loss of renal function with tubulointerstitial fibrosis and mesangial expansion[67,68]. However, the accumulation of DCs, T and B lymphocytes and fibroblasts and accompanying

tubulointerstitial fibrosis in rodent or human CKD still occurs despite a lymphangiogenic response[26,54,69]. An explanation for this may come from studies from other organs which indicate that lymphangiogenesis, when occurring as a response to chronic inflammation, results in leaky vessels with a reduced capacity for clearance[70]. Thus, therapies to target lymphangiogenic pathways hold potential for restoring clearance function, modulating the inflammatory environment and preventing fibrotic remodeling in CKD (**Figure 2**). Whether enhancing lymphangiogenesis may also exert beneficial effects through clearance of interstitial edema or inflammatory macromolecules within the kidney is not known.

Cellular and molecular mechanisms of lymphangiogenesis in the diseased kidney

Studies of lymphangiogenesis in development[71] or pathology[72] have identified a plethora of growth factors that promote or inhibit lymphatic vessel growth. Of those studied in the kidney, vascular endothelial growth factor (VEGF)-C and VEGF-D are central for lymphangiogenesis in renal disease. These growth factors predominantly trigger lymphangiogenesis by activation of VEGFR-3, but VEGF-C can also act via VEGFR-2 to stimulate LEC and blood vessel proliferation and migration[73]. In the rat remnant kidney[63], murine unilateral ureteral obstruction (UUO)[74], human biopsies of IgAN, DKD[26] and chronic allograft rejection[75] VEGF-C is highly expressed by macrophages. These macrophages, which are likely derived from bone marrow and infiltrate the diseased kidney from the vasculature[76], may be modulated by transforming growth factor (TGF)-β1 and TNF-α release[62,77] from damaged renal cells[78,79], or from hypoxia-inducible-factor 1 activation[80] due to local renal hypoxia[59,81]. Proximal tubular and collecting duct epithelium are also potential sources of VEGF-C upon renal injury[69,77], although the relative contributions of individual cell-types to lymphangiogenesis are unknown. Expression of VEGF-D is increased in kidney lysates from a mouse model of UUO[62], and immunostaining demonstrates injured tubular epithelium as a potential cellular source in cisplatin-induced nephrotoxicity and ischemia-reperfusion injury (IRI) in mice[66]. Induction of VEGF-D in the tubular epithelium of otherwise healthy adult mice, resulted in a four-fold expansion of the mean cross-sectional area of

kidney lymphatics, demonstrating the potent effect of VEGF-D on renal lymphangiogenesis[82].

Connective tissue growth factor (CTGF), an ECM-associated heparin-binding protein, has been identified as a contributor to renal lymphangiogenesis. CTGF is highly expressed by damaged tubular epithelium and interstitial cells (likely macrophages[83] or fibroblasts[84]) in human kidneys with urinary obstruction or DKD[85]. Following total knockout of CTGF in adult mice, UUO resulted in reduced lymphangiogenesis and VEGF-C mRNA levels compared with wildtype obstructed kidneys. In culture, CTGF induces VEGF-C production in immortalized mouse and human proximal tubular epithelial cell lines and binds directly to VEGF-C in a dose-dependent manner[85]. Whether and how CTGF exhibits activity directly upon LECs has not yet been examined. Inflammatory mediators, secreted by a variety of cell types upon tissue injury, also have roles in lymphangiogenesis. Upon stimulation of LECs by the inflammatory milieu, PROX1 is activated and VEGFR-3 upregulated downstream of nuclear factor-kappa B, increasing the responsiveness of lymphatics to VEGF-C and VEGF-D[86]. One inflammatory mediator which stimulates renal lymphangiogenesis is lymphotoxin-α (LTα), with overexpression in mice proximal tubules leading to expansion of cortical lymphatics accompanied by T and B lymphocyte-rich infiltrates[87]. However, it was not determined whether LTα stimulates LECs directly, or acts indirectly through other renal cell-types.

Lymphvasculogenesis in renal injury

In addition to lymphangiogenesis, some evidence suggests that a small proportion of LECs arise from differentiation of tissue-resident or circulating progenitors in a process termed lymphvasculogenesis[9–11]. In gender-mismatched renal transplants, in which male recipients received female donor kidneys, immunohistochemical analysis demonstrated that 4.5% of $PROX1^{+}$ $PDPN^{+}$ lymphatics contained a single Y chromosome indicating a host-derived contribution to graft lymphatics[88]. From these experiments, it was proposed that bone

marrow-derived macrophages can transdifferentiate into lymphatic endothelium in inflammatory contexts[89].

In contrast, a study performing parabiosis between GFP transgenic and wildtype mice or adoptive transfer of GFP-expressing bone marrow in murine UUO[54] showed low levels of co-expression of GFP and LYVE-1$^+$ lymphatics occurred, although this was not quantified. It is not clear whether the efficiency of GFP or timepoint of the experiment contributed to the difference between the above studies. More extensive lineage tracing is required to validate these findings, but until then, a myeloid origin of LECs during renal injury cannot be ruled out.

Targeting lymphangiogenesis in chronic renal injury

Several strategies have been implemented to augment renal lymphangiogenesis in preclinical studies (**Table 2**). Daily intraperitoneal administration of a recombinant isoform of VEGF-C protein (VEGF-C156S), which binds preferentially to VEGFR-3 over VEGFR-2[90], led to an expansion of the peri-arterial renal lymphatic network, but not blood microvasculature in murine UUO. VEGF-C156S also attenuated collagen I/III deposition, reduced pro-inflammatory macrophage number and lowered total TGF-β1 in UUO kidneys compared with untreated controls[91]. To what extent VEGF-C156S may exert these beneficial effects through VEGFR-3$^+$ blood endothelial cells in the kidney[36,37] is unclear.

Another strategy to augment lymphangiogenesis in CKD has been the ectopic expression of pro-lymphangiogenic growth factors in transgenic mice. In adult mice with either salt-sensitive or nitro-l-arginine methyl ester (L-NAME)-induced hypertension, tubular VEGF-D overexpression increased cortical lymphatic density whilst reducing renal macrophage and T lymphocyte or DC accumulation. This lead to an decrease in systolic blood pressure in both models[92], but the effect of VEGF-D overexpression on fibrotic remodeling in the hypertensive kidney was not explored. Moreover, a servo-control technique to maintain renal

perfusion pressure was not applied, so it is not clear whether the mechanism of injury arises as a direct consequence of L-NAME on the kidney or indirectly from hypertension. Another study used mice overexpressing VEGF-C from podocytes in streptozocin-induced DKD. Podocyte VEGF-C overexpression significantly reduced the hallmarks of early DKD, including albuminuria, mesangial expansion and decreased glomerular collagen deposition[93]. This effect was attributed to restoration of glomerular endothelial barrier function, but the authors did not examine enhanced renal lymphangiogenesis as a potential cause.

Two other rodent studies explored the hypothesis that inhibition of lymphangiogenesis might be beneficial in CKD. Rats, intravenously delivered adriamycin to trigger proteinuria, were treated with a monoclonal anti-VEGFR3 antibody (IMC-3C5)[94] from six weeks after induction of nephropathy. At 12 weeks of follow-up, treatment with IMC-3C5 significantly reduced the mean cortical lymphatic vessel number in both healthy and adriamycin-treated kidneys, without altering leukocyte count, collagen deposition or interstitial fibrosis in the injured kidneys. Though the authors concluded tubulointerstitial inflammation and fibrosis to be independent of lymphangiogenesis in adriamycin nephropathy, the late onset of treatment may influence the efficacy of IMC-3C5. Nevertheless, these results, when interpreted in light of above studies, suggest that augmentation, rather than inhibition, of lymphangiogenesis is beneficial in CKD.

In another study[54], the kidneys of mice with LYVE-1$^+$ cells ablated and then subjected to either UUO or IRI harbored lower numbers of infiltrating DCs, T and B lymphocytes, macrophages, neutrophils, natural killer cells, and genes encoding for either for inflammatory cytokines or associated with renal fibrosis seven days after injury. Reduction of the inflammatory milieu was also observed in UUO and IRI mice administered soluble LYVE-1 or VEGFR-3 fusion proteins. The anti-inflammatory and anti-fibrotic effects of LYVE-1$^+$ cell-ablation and fusion protein delivery were attributed to inhibition of lymphangiogenesis, as

both strategies reduced the density and decreased proliferation of LYVE-1$^+$ lymphatics in diseased kidneys. To what extent these strategies target non-LECs or cells outside the kidney to exert their beneficial effects is unclear.

Contribution of lymphatics to adaptive immunity in renal transplant rejection

A lymphangiogenic response has been documented in rejected human renal allografts[75,88,95–97] and rodent models of transplant nephropathy[98–100]. In this context, lymphangiogenesis is associated with lymphocyte-rich infiltrates and correlates with allograft fibrosis and impaired graft function[75]. A body of evidence points towards a fundamental role for lymphatics in the generation and maintenance of adaptive immune responses in renal transplants. Across organs, LECs maintain peripheral tolerance by actively modulating immune cell function, including T and B lymphocytes and DCs, by activating or inhibiting their maturation, driving the proliferation or apoptosis of these cells, generating chemokine gradients for their chemotaxis or controlling their trafficking and efflux through lymph[4]. In different contexts, it has emerged that LECs may themselves archive soluble antigen for presentation to DCs[101,102].

Injured cells within the tubulointerstitium in renal allograft rejection may guide the sprouting of lymphatic endothelium. One potential candidate is C-X-C motif chemokine receptor (CXCR)7, which is upregulated on kidney lymphatic vessels in acute allograft rejection in humans[95]. Abundance of CXCR7$^+$ lymphatics correlate with the serum creatinine level in these patients. At the same time, the mRNA level of the CXCR7 ligands, C-X-C motif chemokine ligand type (CXCL)11 and CXCL12, was significantly higher in the tubulointerstitium of human renal allografts with borderline lesions or undergoing acute rejection. The cellular sources of CXCL12 and CXCL11 were not identified in this study, but in renal ischemic injury, injured tubular epithelium was shown to secrete CXCL12[103]. CXCL12 results in dose-dependent increases in migration of murine and human LECs *in vitro* and promotes tube formation in the latter[104]. Thus, chemokines such as CXCL12,

generated by inflamed regions, may guide lymphangiogenesis towards sites of injury in rejecting allografts (**Figure 3**).

Increasing the efflux of immune cells from renal allografts is associated with poor graft function in rat renal transplant[105]. One factor responsible for immune cell mobilization is $CD9^+$ $CD63^+$ exosome-rich endothelial vesicles, which are identified surrounding lymphatic vessels in chronic allograft nephropathy. Exposure of cultured LECs to TNF-α; which is released from damaged renal cells[78,79], increases the chemokine and growth factor content of EEVs. These EEVs promote the transmigration of human DCs across LECs. A key chemokine supporting this process, released by LECs stimulation by TNF-α or DCs is chemokine (C-C motif) ligand (CCL)21[106,107]. CCL21 encourages chemotaxis of DCs towards lymphatic vessels[108], their transmigration across LECs[106] and the egress of DCs through lymphatics and towards lymph nodes[109]. In human chronic renal allograft rejection, CCL21-secreting LECs are found in close association with nodular infiltrates[75] containing multiple leukocyte subtypes expressing the CCL21 receptor, chemokine (C-C motif) receptor (CCR)7[110], including T- and B-lymphocytes and DCs. Increased expression of CCL21 is associated with recurrent nephropathy in renal transplant patients independent of their age, gender or expression levels CCR7 within the transplanted kidney[111]. Thus, LEC-generated EEVs and chemokine gradients likely modulate immune tolerance in renal transplantation by mobilizing antigen-presenting cells through lymphatics and towards lymph nodes (**Figure 3**).

Therapies to inhibit lymphangiogenesis may represent a strategy to improve the outcome and survival of renal allografts. To our knowledge, the targeting of VEGFR-3/VEGF-C or other canonical lymphangiogenic pathways have not been tested in animal models of renal transplant, though has shown to be beneficial in cardiac graft survival in rat[112]. It is emerging that existing clinical therapies that promote renal transplant survival, such as sirolimus, an inhibitor of mammalian target of rapamycin (mTOR), may exert their effects directly on LECs by inhibiting lymphangiogenesis[113,114]. Another therapeutic strategy tested in rat renal

allografts is inhibition of Rho-associated protein kinase (ROCK). By treating transplant-recipient rats with lysozymes conjugated to a ROCK inhibitor (Y27632), glomerular and tubulointerstitial macrophage influx was decreased at one- and four-days post-transplantation respectively, accompanied by a significant decrease in lymphatic vessel abundance. However, blood pressure and proteins associated with fibrosis, including vimentin and procollagen-1$^{\alpha}$1, did not significantly change upon Y27632 treatment[98]. Whether inhibition of mTOR or ROCK directly exert anti-lymphangiogenic effects, or whether lymphatic abundance decreases as a secondary consequence of immunosuppression, is not clear.

Polycystic kidney disease

The hallmark of polycystic kidney diseases (PKD), the most common of which is autosomal dominant (AD)PKD caused by mutations in *PKD1* or *PKD2*, is the formation and growth of multiple epithelial fluid-filled cysts within the kidney, driving inflammation, fibrosis and resulting in a progressive decline in renal function. Our understanding of the progression of PKD has largely focused on renal epithelial cell metabolism, fluid transport, survival and differentiation or molecular crosstalk[115]. However, polycystin 1 and 2, encoded by *PKD1* and *PKD2* respectively, are also expressed within lymphatics[116]. Zebrafish with a loss-of-function mutation in the *pkd1a* gene (*lyc1)*, a duplicate gene encoding polycystin 1, have lymphatic defects with the main axial lymphatic vessel failing to form during development. In mice, knockout of *Pkd1* or *Pkd2* results in blood-filled lymph sacs, severe edema in the absence of structural heart defects, hemorrhaging, cutaneous lymphatic vessel defects and early lethality[116]. The lymphatic defects in the skin were replicated in mice with conditional knockout of *Pkd1* using an endothelial *Sox18-CreER*^{T2} mouse line[117]. Small interfering RNA-mediated knockdown of *PKD1* or *PKD2* in human LECs results in loss of cell number, filopodial abnormalities, disorganized adherins junctional complexes and impairs capillary network formation and cell migration in wound-healing scratch assays. In this model and also in *Pkd1*-null mice, the orientation of the Golgi apparatus in individual LECs was found to

be randomized. Together, these findings suggest that the polycystins cell-autonomously regulate LEC orientation and migration and are required for normal lymphatic development.

Using wholemount immunofluorescence, optical clearing and high-resolution 3D imaging[16,118] we examined the kidney lymphatics in mice homozygous for a p.R3277C allele (*Pkd1*^{RC}), a slow progressing model of ADPKD. We found that lymphatic vessels and corticomedullary cysts in this model sat in close proximity, suggestive of fluid transport[31] or molecular crosstalk between LECs and cyst epithelium. We found complex lymphatic defects in homozygous *Pkd1*$^{RC/RC}$ mice including a stunting of the lymphatic network relative to the volume of the kidney and a significant decrease in the diameter of large hilar lymphatics. The presence of these defects at E18.5, an early timepoint of cyst progression in this mouse model, suggests that these defects could arise directly due to loss of *Pkd1* in LECs, rather than secondarily from structural changes to the kidney due to cyst expansion and compression. In either case, defective lymphatic function may contribute to cyst expansion through impaired clearance of cells or tissue fluid (**Figure 3**).

In a preclinical study, we targeted the lymphatics in PKD by delivering recombinant VEGF-C intraperitoneally in two rapidly progressing mouse models of PKD. In *Pkd1*$^{nl/nl}$ mice, which have hypomorphic *Pkd1* alleles and renal vascular malformation[119], VEGF-C administration lead to an expansion of renal lymphatics[37]. Additionally, VEGF-C restored the defective architecture of VEGFR-3^{+} peritubular capillaries observed in *Pkd1*$^{nl/nl}$ *mice*. These changes were accompanied by reduced inflammation, decrease in cyst size and normalization of kidney to body weight ratio. Our findings suggest targeting VEGFR-3^{+} endothelium, including lymphatics and lymphatic-like vessels, could reduce disease severity and progressive decline in renal function observed in cystic renal disease.

FUTURE DIRECTIONS

There is a rapidly increasing body of evidence supporting a role for lymphatic vessels across multiple forms of renal disease. However, several questions remain. Structural changes to renal lymphatics are not evident in murine or human AKI[26,66]. However, the possibility that renal lymphatics are altered at the molecular or functional level in acute nephropathy, and whether these can be exploited to prevent the transition from AKI to CKD, warrants further study. The mechanisms of action of pro- or anti-lymphangiogenic factors to prevent fibrotic remodeling in preclinical models of CKD, whether through clearing inflammatory cells, modulating factors secreted by LECs, effects on the blood vasculature through VEGFR-2 activation or consequences on other cells in the fibrotic environment are yet to be elucidated. Strategies to target lymphatics or modulate lymph egress[120,121] could be delivered in synergy with pre-existing or emerging approaches, such as anti-fibrotic drugs in CKD, immunosuppressive agents in renal transplant or epithelial-centric medication in PKD.

New advances are rapidly transforming our understanding of lymphatics in health and disease. Amongst these, the concept of lymphatic heterogeneity; that every organ possesses a unique lymphatic vascular bed with organ-specific functions, has not been approached in the kidney. We used 3D imaging to identify a population of highly dynamic lymphatic endothelial cell clusters present during mammalian renal development[16]. In other organs, these clusters represent tissue-specific progenitors, which may impart molecular and functional heterogeneity in the adult lymphatic vasculature[9–11]. Another emerging area, demonstrated by recent studies in animal models and humans, is that lymphatics in skin and muscle may be key players in the regulation of tissue fluid and sodium homeostasis[122,123], revealing an unprecedented relationship between extra-renal lymphatics and kidney disease or salt-sensitive hypertension. Ultimately, for the renal lymphatic field to move forward, novel technologies to visualise[124,125], ablate[126–128] and genetically manipulate[129] lymphatic

endothelium need to be translated to renal research to advance our understanding of this understudied system in renal development, physiology and disease.

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TABLES

Table 1. Molecular markers of lymphatic endothelium in the adult kidney

LEC marker	Molecular function	Non-LEC expression in the kidney*
Prospero homeobox protein 1 (PROX1)	Transcription factor involved in the maintenance of lymphatic identity[130]	Thick ascending limbs of the loop of Henle restricted to the inner medulla[131] and reported in ascending vasa recta[36]
Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1)	Membrane glycoprotein and receptor for hyaluronan facilitating dendritic cell entry into lymphatic vessels[132]	Some endothelial cells in the glomerulus[12]
Vascular endothelial growth factor receptor 3 (VEGFR-3)	Receptor tyrosine kinase required for LEC tip function and vessel sprouting[133]	Ascending vasa recta[36] and cortical peritubular capillaries[37]
Podoplanin (PDPN)	Membrane glycoprotein maintaining separation between blood and lymphatic endothelium[134]	Podocytes[135]
Neuropilin 2 (NRP2)	Transmembrane protein interacting with VEGFR-3 promoting VEGF-C-mediated lymphatic sprouting[136]	Blood endothelial cells[137†]

* Determined from studies of mouse or rat adult kidney

† Derived from single-cell RNA sequencing as no published immunostaining for NRP2.

Table 2. Preclinical strategies targeting lymphangiogenesis in chronic renal injury

Strategy	Mechanism of action and model	Effect on renal lymphatics	Effect of strategy on model of CKD
VEGFC-156S	Mutated form of VEGF-C and selective agonist of VEGFR-3 to promote lymphangiogenesis[90]. 10µg per day delivered IP over 14 days in murine UUO[91]	Increase in cross-sectional area of LYVE-1$^+$ total and perivascular renal lymphatics compared to control	Reduction in fibrotic remodelling (Sirius red stain) Reduction in collagen I deposition (Western blot) Reduction in infiltrating macrophages (IHC) Reduction in TGF-β1 expression (Western blot)
Ksp-rtTA;TRE-Vegfd mice	Transgenic mice with doxycycline-dependent overexpression of VEGF-D from tubular epithelium to promote lymphangiogenesis. Tested in L-NAME-dependent hypertensive nephropathy for two weeks with or without three-week high salt diet[92]	Increase in cross-sectional area of LYVE-1$^+$ cortical renal lymphatics and branches per artery compared to control	Reduction in infiltrating macrophage and T-lymphocytes with high-salt diet (flow cytometry) Reduction in infiltrating macrophage and DCs without high-salt diet (flow cytometry)
Pod-rtTA;TRE-Vegfc mice	Transgenic mice with doxycycline -dependent overexpression of VEGF-C from podocytes. Diabetic nephropathy induced using 50mg/kg STZ per day for five days)[93]. Doxycycline either given before or four weeks after STZ injection	Effects attributed to glomerular VEGF activity, so renal lymphatics were not investigated	Reduction in urinary albumin to creatinine ratio (ELISA) Reduction in mesangial matrix expansion (histology) Reduction in collagen I (Sirius red stain)
IMC-3C5	Anti-VEGFR-3 antibody delivered to inhibit lymphangiogenesis in rats with adriamycin nephropathy[94]. Six weeks after adriamycin treatment, 40mg/kg bodyweight of IMC-C35 delivered three times per week IP	Reduction in cross-sectional area of PDPN$^+$ cortical lymphatics in both adriamycin-treated and non-adriamycin-treated kidneys compared to controls	No significant reduction in infiltrating macrophage or T-lymphocytes (IHC) No significant reduction in tubulointerstitial fibrosis (histology) No significant reduction in collagen I (IHC and qPCR)
Lyve1-Cre;R26R-DTR mice	Transgenic mice expression DTR in LYVE-1$^+$ cells and their progeny. Tested in murine UUO and IRI with seven day follow-up after a single IP dose of 1.25ng/kg bodyweight DT[54]	Significant reduction in cross-sectional area of LYVE-1$^+$ lymphatic vessels assessed at three days after DT administration	Reduction in DCs, macrophages, T- and B-lymphocytes, neutrophils and NK cells in UUO (flow cytometry) Reduction of inflammatory cytokines in UUO (qPCR) Reduction in fibrosis (qPCR, IHC and Sirius red)
LYVE-1 or VEGFR-3 soluble fusion proteins	Soluble LYVE-1 or VEGFR-3 proteins hypothesised to inhibit lymphangiogenesis through sequestering lymphangiogenic growth factors. Injected *via* tail vein before or after UUO or IRI induction in mice[54]	Significant reduction in cross-sectional area of LYVE-1$^+$ lymphatic vessels assessed at seven days after UUO surgery	Reduction in DCs and T-lymphocytes in UUO kidneys (flow cytometry) Reduction of inflammatory cytokines in UUO (qPCR) Reduction in fibrosis (qPCR, IHC and Sirius red)

DC, dendritic cells; DT diphtheria toxin; DTR, diphtheria toxin receptor; ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; IP, intraperitoneal; IRI, ischemia reperfusion injury; L-NAME, nitro-l-arginine methyl ester; NK, natural killer; qPCR, quantitative polymerase chain reaction; STZ, streptozocin; UUO, unilateral ureteral obstruction

FIGURE LEGENDS

Figure 1. Development, structure and function of kidney lymphatics.

During early nephrogenesis at embryonic day (E)12.5 the kidney is devoid of lymphatics. Thereafter, kidney lymphatic development proceeds in three distinct phases. First, the appearance of a plexus of lymphatic endothelial cells in the kidney at E14.5. Then, the remodeling of lymphatic endothelial cells into a patent vascular network by E16.5. Finally, the extension of these vessels towards the renal cortex at E18.5. All stages are presented in the context of important morphological and differentiation events during renal development. The specification of the blood vasculature[19,20], ureteric bud branching and nephron generation[21], stages of nephron differentiation[18,22], the appearance of stromal derivates such as pericytes and mesangial cells[23] and initiation of urinary function[17,24] are taken from the indicated references.

In the adult kidney, lymphatics reside in the cortical interstitium and drain to large lymphatic vessels in the hilum. The renal medulla is devoid of lymphatics. Drainage begins in the cortical interstitium. Increased pressure in this compartment causes extracellular matrix-bound anchoring filaments to force lymphatic endothelial cells (LEC) apart, thus allowing tissue fluid, immune cells (such as dendritic cells and neutrophils) and small molecules (such as soluble antigen and antibodies) enter lymphatic capillaries.

Figure 2. Lymphatic expansion in chronic renal injury and its targeting using pro-lymphangiogenic therapies.

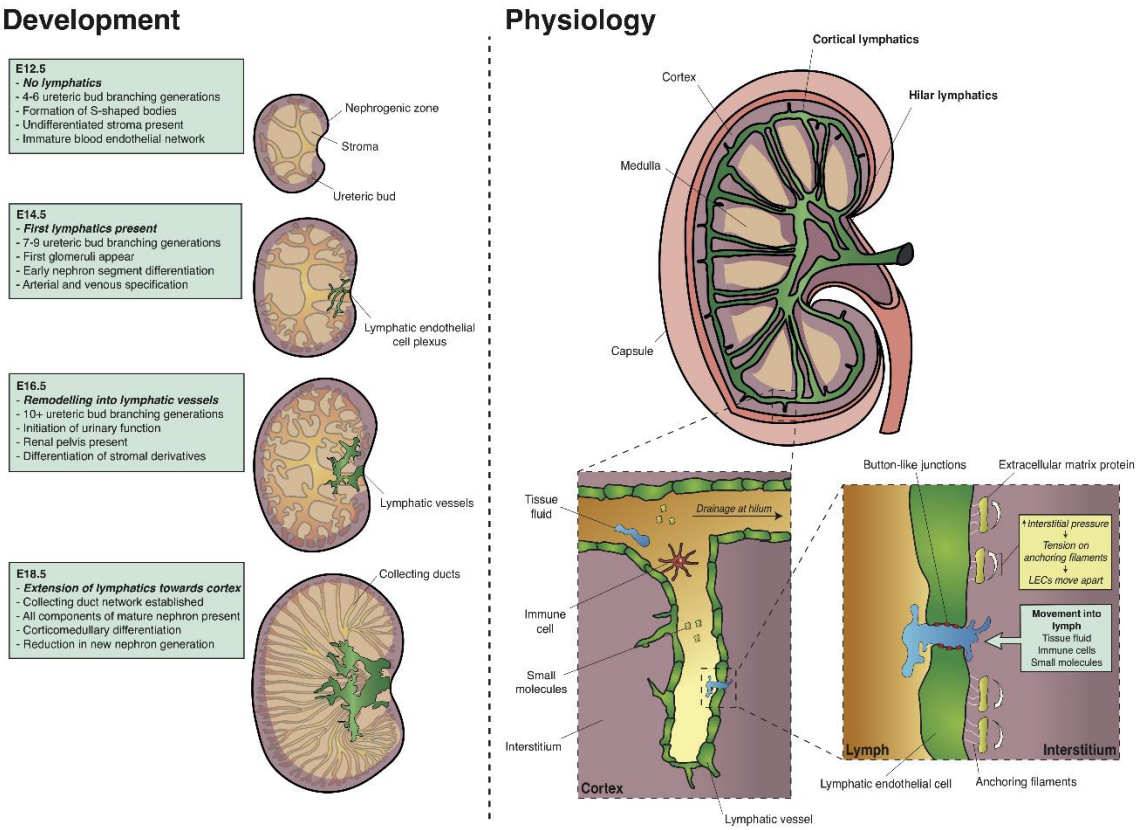
In mouse models of chronic renal injury and in human CKD, lymphangiogenesis; the expansion of lymphatics *via* proliferation and sprouting of existing lymphatic endothelium occurs and is considered the predominant mechanism of lymphatic expansion in disease. A number of cell types in the inflammatory milieu including injured tubular epithelium, activated T- and B-lymphocytes, neutrophils and dendritic cells and activated fibroblasts secrete growth factors (VEGF-C, VEGF-D, CTGF) and inflammatory mediators (LT-α, TNF-α, TGF-

β) which act directly or indirectly on lymphatic endothelium to support lymphangiogenesis. The box indicates other possible factors which have been implicated in lymphangiogenesis in other organs but have not been explored in the context of renal injury. Some studies suggest that lymphvasculogenesis, the transdifferentiation of other cell types, such as macrophages, into lymphatic endothelial cells and their integration into lymphatic vessels are an alternative mechanism of lymphatic expansion in chronic renal injury. The premise of pro-lymphangiogenic therapies, such as growth factors or genetic approaches in mice, is to augment the expansion of lymphatics to increase the clearance of the activated immune cells. A number of studies show that this alleviates renal inflammatory and reduces fibrotic remodeling in the kidney.

Figure 3. Context-specific lymphatic functions in transplant rejection and polycystic kidney disease

In chronically rejecting renal allografts from humans and rodent models, lymphatic vessels are found in close association with inflamed regions containing lymphocyte-rich infiltrates. Injured tubular epithelium and cells within infiltrates secrete guidance cues for lymphatic endothelium including CXCL11 and CXCL12 with cognate G protein-coupled receptors (CXCR4, CXCR7) expressed on lymphatic endothelium. Conversely, lymphatic endothelial cells secrete CCL21; a potent agent of chemotaxis for immune cell subtypes, and CD9+ CD63+ exosome-rich endothelial vesicles (EEV). EEVs contain immunomodulatory proteins (CSF-1) and factors involves in leukocyte migration (CX3CL1, CCL2, CCL5).

In the early stages of murine polycystic kidney disease, lymphatics are found in close association with cyst epithelium, potentially suggesting molecular crosstalk between lymphatic and cyst epithelium or the transport of cyst solute to lymph. Kidney lymphatics themselves are malformed, which may suggest that defects in the kidney lymphatic vasculature result in defective tissue fluid and immune cell clearance and contribute to cyst expansion and decline of renal function in polycystic kidney disease.


Development
E12.5
- No lymphatics
- 4-6 ureteric bud branching generations
- Formation of S-shaped bodies
- Undifferentiated stroma present
- Immature blood endothelial network
Nephrogenic zone
Stroma
Ureteric bud
E14.5
- First lymphatics present
- 7-9 ureteric bud branching generations
- First glomeruli appear
- Early nephron segment differentiation
- Arterial and venous specification
Lymphatic endothelial cell plexus
E16.5
- Remodelling into lymphatic vessels
- 10+ ureteric bud branching generations
- Initiation of urinary function
- Renal pelvis present
- Differentiation of stromal derivatives
Lymphatic vessels
E18.5
- Extension of lymphatics towards cortex
- Collecting duct network established
- All components of mature nephron present
- Corticomedullary differentiation
- Reduction in new nephron generation
Collecting ducts
Physiology
Cortical lymphatics
Cortex
Hilar lymphatics
Medulla
Capsule
Tissue fluid
Drainage at hilum
Immune cell
Small molecules
Interstitium
Cortex
Lymphatic vessel
Button-like junctions
Extracellular matrix protein
Interstitial pressure
Tension on anchoring filaments
LECs move apart
Movement into lymph
Tissue fluid
Immune cells
Small molecules
Lymph
Interstitium
Lymphatic endothelial cell
Anchoring filaments

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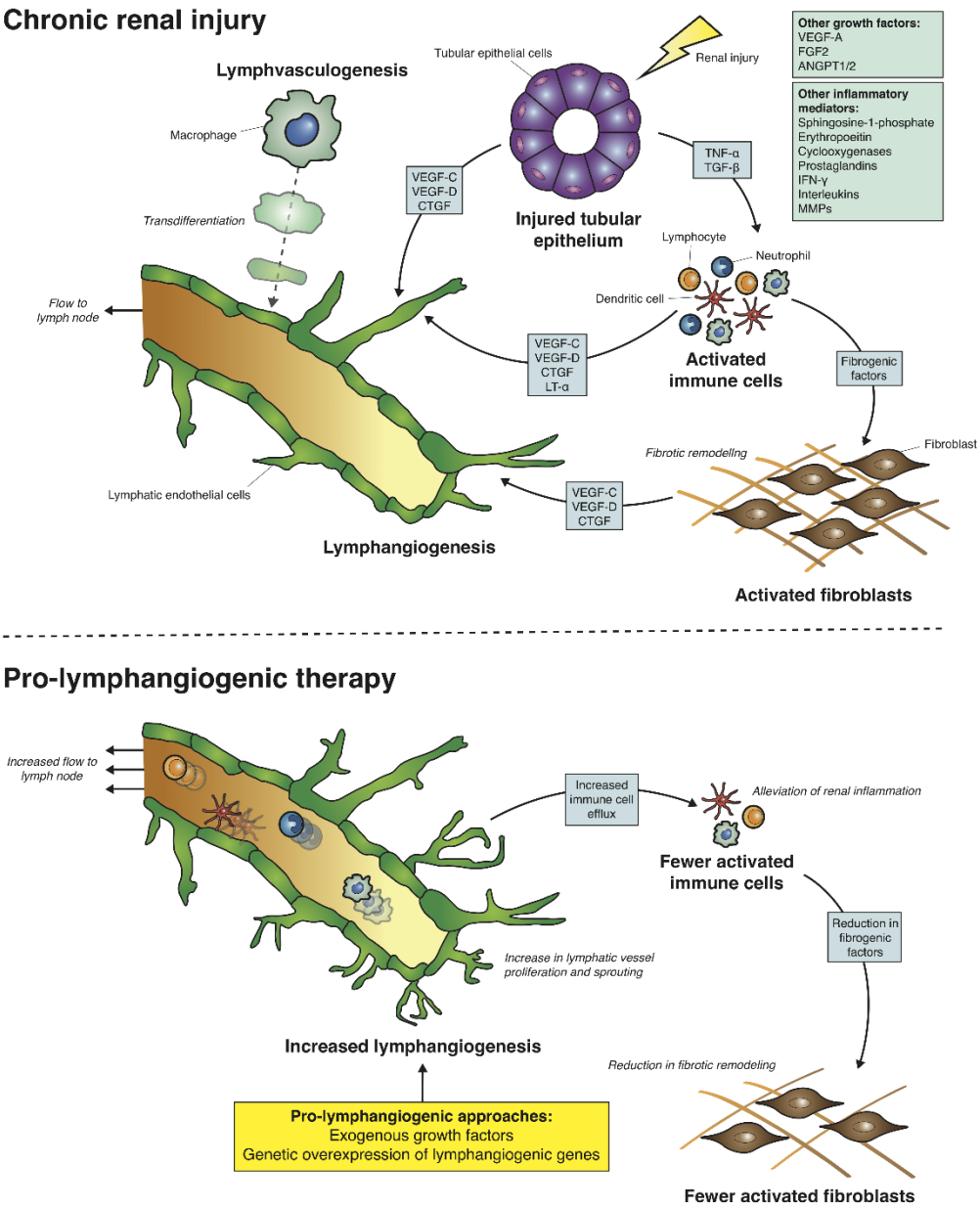
Chronic renal injury
Lymphvasculogenesis
Tubular epithelial cells
Renal injury
Other growth factors:
VEGF-A
FGF2
ANGPT1/2
Other inflammatory mediators:
Sphingosine-1-phosphate
Erythropoeitin
Cyclooxygenases
Prostaglandins
IFN-γ
Interleukins
MMPs
Macrophage
Transdifferentiation
VEGF-C
VEGF-D
CTGF
TNF-α
TGF-β
Injured tubular epithelium
Lymphocyte
Neutrophil
Dendritic cell
Flow to lymph node
VEGF-C
VEGF-D
CTGF
LT-α
Activated immune cells
Fibrogenic factors
Fibroblast
Fibrotic remodelling
Lymphatic endothelial cells
VEGF-C
VEGF-D
CTGF
Lymphangiogenesis
Activated fibroblasts
Pro-lymphangiogenic therapy
Increased flow to lymph node
Increased immune cell efflux
Alleviation of renal inflammation
Fewer activated immune cells
Reduction in fibrogenic factors
Increase in lymphatic vessel proliferation and sprouting
Increased lymphangiogenesis
Reduction in fibrotic remodeling
Pro-lymphangiogenic approaches:
Exogenous growth factors
Genetic overexpression of lymphangiogenic genes
Fewer activated fibroblasts

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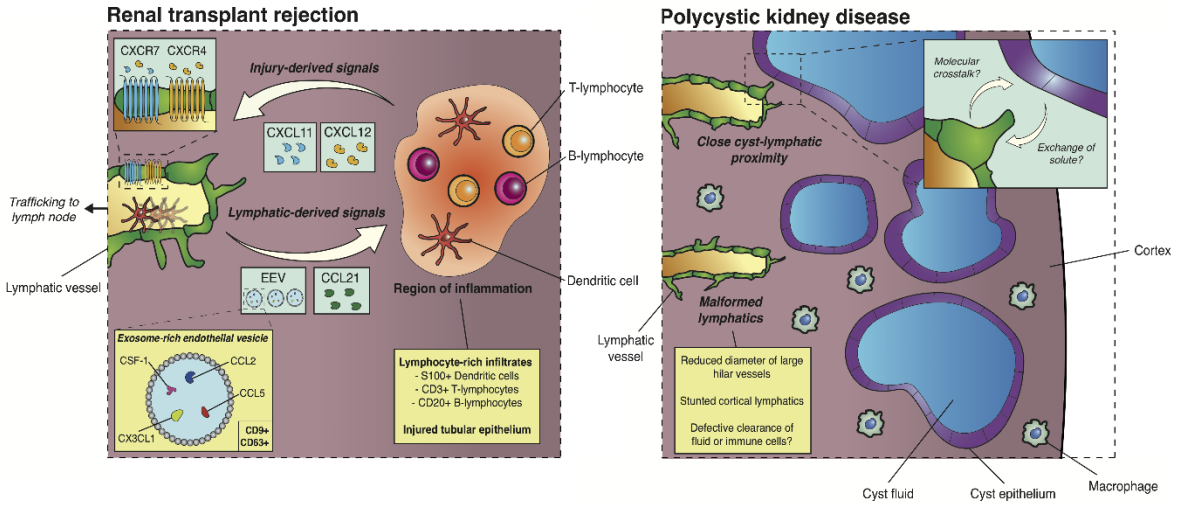
Renal transplant rejection
CXCR7
CXCR4
Injury-derived signals
CXCL11
CXCL12
T-lymphocyte
B-lymphocyte
Trafficking to lymph node
Lymphatic-derived signals
Lymphatic vessel
EEV
CCL21
Region of inflammation
Dendritic cell
Exosome-rich endothelial vesicle
CSF-1
CCL2
CCL5
CX3CL1
CD9+
CD63+
Lymphocyte-rich infiltrates
- S100+ Dendritic cells
- CD3+ T-lymphocytes
- CD20+ B-lymphocytes
Injured tubular epithelium
Polycystic kidney disease
Molecular crosstalk?
Exchange of solute?
Close cyst-lymphatic proximity
Cortex
Malformed lymphatics
Lymphatic vessel
Reduced diameter of large hilar vessels
Stunted cortical lymphatics
Defective clearance of fluid or immune cells?
Cyst fluid
Cyst epithelium
Macrophage

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