The role of lymphatics in renal inflammation

Harald Seeger1,*, Marco Bonani1,2,* and Stephan Segerer1,3

1Division of Nephrology, University Hospital, Zurich, Switzerland, 2Department of Visceral and Transplantation Surgery, University Hospital, Zurich, Switzerland and 3Department of Anatomy, University of Zurich, Zurich, Switzerland

Correspondence and offprint requests to: Stephan Segerer; E-mail: Stephan.segerer@usz.ch
*Both authors contributed equally to this work.

Abstract
Progressive renal diseases are characterized by tubulointerstitial inflammatory cell recruitment, tubular atrophy and fibrosis. Various aspects of the recruitment of leukocytes have been extensively studied, but the exit routes (i.e. the lymphatic vessels and their biology) have only recently found attention. Similar to the recruitment of inflammatory cells, the exit is coordinated by an orchestrated interaction of chemotactic cytokines and adhesion molecules. During inflammatory injury, new routes are created by the de novo formation of lymphatic vessels, i.e. neolymphangiogenesis. These newly formed lymphatic vessels help to cope with the increase in interstitial fluid related to inflammation. Here, we review some aspects of lymphatic biology and the current knowledge about lymphatic vessels in renal inflammation.

Keywords: lymphatic endothelial cells; lymphatic vessels; renal allograft; renal inflammation; tubulointerstitial inflammation

Introduction

The lymphatic vessel network drains interstitial fluid and returns it to the blood (Figure 1). During the last decades, our knowledge of this system has evolved substantially. The focus has long been on immune surveillance and response to infectious agents, but new functions in lipid transport and fat metabolism as well as in the regulation of salt storage and hypertension have emerged (reviewed in [3, 4]). The interest in lymphatic vessels has also reached the kidney. The first part of this review gives a general introduction into the biology of lymphatic vessels, whereas the second part will focus on the role of lymphatic vessels in the kidney.

Anatomy and physiology of the lymphatic system

The majority of vascularized tissues (except for the central nervous system and bone marrow) contain a lymphatic capillary network, which returns an estimated 1–2 L of lymph to the venous circulation every day. The lymphatic vasculature is a unidirectional open system and displays many features which discriminates it from the blood vascular system. It is composed of blunt-ended capillaries (30–80 μm in diameter) devoid of a basement membrane or pericytes (Figure 1, [5]). They consist of single-layered ‘oak leaf’-shaped partly overlapping cells, which are interconnected via discontinuously arranged ‘button-like’ junctions and mostly lack the continuous ‘zipper-like’ interendothelial tight junctions present in the endothelial layer of blood vessel endothelial cells (BECs). The resulting interjunctional gaps are thought to represent sites of entry for interstitial fluid, macromolecules and immune cells [6, 7]. Lymphatic endothelial cells (LECs) are highly endocytic and permeable to proteins thereby allowing transecellular uptake of fluid and macromolecules. Filaments connect the lymphatic capillaries to the perivascular matrix leading to an increase of the vessel lumen and widening of the intercellular gaps in tissue swelling, facilitating fluid entry in this context [8].

Lymphatic capillaries drain into precollecting vessels, followed by larger collecting lymphatics [9]. Precollectors exhibit some perivascular smooth muscle cells (SMCs), whereas bigger collecting vessels contain continuous endothelial junctions, a basement membrane, an SMC layer and bileaflet valves. These allow for the centripetal flow of lymph augmented by rhythmic contractions [10] (Figure 1).

Characterization of LECs and formation of lymphatic vessels

LECs express a distinct set of genes which discriminates them from BECs [11, 12]. Unfortunately, these markers are not specific and also expressed in various other cell types (see Table 1). The most widely employed LEC markers are podoplanin [13, 14], the lymphatic vessel hyaluronan receptor (LYVE-1, [15]), vascular growth factor receptor 3 (VEGFR-3), which is the receptor for...
VEGF-C and VEGF-D, and the prospero-related homeo-box transcription factor 1 (Prox1, [16]).

LECs do not only differ from BECs in their gene expression pattern but are also heterogeneous among themselves depending on the segment of the lymphatic tree and the tissues they reside in [11, 17]. In human skin, for example, two functionally different subpopulations of LECs were demonstrated, characterized by their expression levels of podoplanin and transcripts such as CCL21, CCL27 and the atypical chemokine receptor DARC [11].

The migration of LECs and formation of the lymphatic vasculature (termed lymphangiogenesis) are orchestrated by a large set of genes. VEGFR-3 and its main ligand VEGF-C are essential in this process and embryonic deletion of VEGF-C results in a lack of the lymphatic vasculature in mouse embryos [18]. VEGFR-3, while also expressed in BECs in early development, becomes restricted to LECs in later life [19]. Podoplanin is essential for the separation of the developing lymphatic vasculature from blood vessels via interaction with platelets. Gene targeting of podoplanin in mice resulted in dilation and malfunction of lymphatic vessels [20].

**Neolymphangiogenesis in inflammation**

Neolymphangiogenesis in the adult occurs in various conditions associated with inflammation, such as acute and chronic infections, disorders of immune regulation (e.g. rheumatoid arthritis and psoriasis), wound healing, tumour growth/metastasis and transplant rejection [4, 9]. The functional role of neolymphangiogenesis is not quite understood, but it seems to be primarily a protective response and aids in clearance of tissue oedema and inflammatory infiltrates [21–24]. Neolymphangiogenesis shares many pathways with developmental lymphangiogenesis including the pivotal role of the VEGFR-3/VEGF-C axis in stimulating lymphatic proliferation.

In tissue inflammation, pro-inflammatory cytokines, such as interleukin-1α, -1β and tumour necrosis factor (TNF), result in the release of VEGF-C and VEGF-D by
macrophages, dendritic cells (DCs), granulocytes, mast cells and fibroblasts [25–29]. The induction of nuclear factor-kappaB by inflammatory stimuli activates Prox1, and both nuclear factor-kappaB and Prox1 activate the VEGFR-3 promoter. This can enhance the sensitivity of lymphatic endothelium to VEGF-C and VEGF-D [30]. The cytokine lymphoxygen α, mainly secreted by T cells, also contributes to neolymphangiogenesis in mice [31]. Data from renal transplant inflammation in humans and inflamed cornea in mice have shown that lymphangiogenesis does not solely occur by continuous sprouting from neighbouring lymphatics but also includes incorporation of lymphatic progenitor cells into the growing lymphatic vessels [32, 33]. In salt-induced lymphangiogenesis, stimulation of tonicity-responsive enhancer-binding protein (TonEBP) leads to VEGF-C secretion by macrophages in the skin, stimulating lymphatic vessel growth [34]. A secreted splice variant of VEGFR-2 acts as a VEGF scavenger and prevents lymphangiogenesis in tissues devoid of lymphatics, such as the cornea [35].

Role of the lymphatic vasculature in inflammation

Afferent lymphatic vessels transport antigens and immune cells, mostly conventional DCs and T cells, from tissue to draining lymph nodes [36]. They also serve as drains for non-cellular structures. Peripheral tissue antigens can enter the lymph node via afferent lymphatics directly [37, 38].

Proteins expressed by LECs can directly modulate the local inflammatory milieu. The atypical chemokine receptor D6 takes up chemokines which results in digestion and local chemokine clearance [39, 40]. The deletion of this receptor in mice leads to chronic inflammation of the skin [39]. DARC is another atypical chemokine receptor expressed by lymphatic precollection vessels [41]. In contrast to D6, DARC is involved in the transcellular transport and presentation of chemokines [42]. Therefore, lymphatic vessels are important players in the regulation of both local as well as systemic inflammatory reactions.

Mediators of cell recruitment and cell movement into lymphatic vessels

During DC mobilization and migration from peripheral tissue to lymph nodes, the interaction of the chemokine receptor CCR7 with its ligands CCL19/21 is thought to be a key mechanism. LECs in inflamed tissues, but also under non-inflammatory conditions, secrete the chemoattractant CCL21/SLC, thereby establishing a gradient which attracts CCR7-positive activated DCs to the afferent lymphatic vessels aiding in relocation to lymph nodes [43–45]. Podoplanin, which has been shown to bind CCL21 along the basolateral side of the LECs and the perivascular stroma, seems to be closely involved in creating the perivascular gradient. Interestingly, CCR7 expression on DCs is absolutely required for extravasation into initial lymphatics [46]. CCR7 is also crucial for the exit of CCR7-positive effector/memory T cells from peripheral tissues via afferent lymphatics, suggesting that CCL21 might be involved in CCR7-positive T-cell trafficking [47, 48].

A subtype of LECs (characterized by low expression of podoplanin and high expression of DARC) in precollection vessels of human skin secrete CCL27, thereby leading CCR10-positive T cells through precollection lymphatics [41].

In a mouse model of contact hypersensitivity, it was demonstrated that lymph node migration of CXC4 expressing dermal DCs and Langerhans cells could be substantially reduced using a CXC4 antagonist, suggesting a role of the CXC4 ligand CXCL12, a molecule expressed by dermal lymphatics [49].

Sphingosine-1 phosphate (S1P) produced by LECs causes egress of T cells expressing the corresponding
receptor via efferent lymphatics, whereas increased tissue S1P results in the arrest of T cells in inflamed tissues [50, 51].

The mechanisms of transmigration of inflammatory cells into lymphatic vessels are still incompletely understood. In vitro assays suggest a function for the adhesion molecules intracellular adhesion molecule 1 and vascular cell adhesion molecule 1, both of which are strongly up-regulated upon TNF stimulation in cultured murine and human dermal LECs. Blocking antibodies to both molecules inhibited adhesion and transmigration of lipopolysaccharide-activated macrophage-derived DCs [52]. Incubation of DCs with CCL21 led to dramatic acceleration of the otherwise slow process of lymphatic transmigration [44]. Remarkably, the adhesion molecule JAM-A appears to impede DC trafficking via afferent lymphatics [53].

Anatomy of lymphatics in the normal kidney

The anatomy of renal lymphatics has been studied in various mammals e.g. rabbit, rat, mouse, sheep, dog and cat [54–58]. No prominent differences between species were described [55]. Early studies used dye injection techniques, which can be difficult in their interpretation and resolution. Studies using the modern markers (e.g. podoplanin) confirmed many aspects of this early work [58].

The initial lymphatic capillaries run in close proximity to the interlobar arteries (Figure 1). They empty into lymphatic precollectors, which follow the arcuate vessels along the bases of the pyramids. Then, lymphatics follow the interlobar blood vessels of the renal columns and finally drain into hilar collector lymphatics [57].

In the renal cortex, lymphatics come close but do not enter the glomeruli [57, 58]. Towards the outer cortex, the number of lymphatics decreases. No lymphatic vessels are present in normal renal medulla [54, 58]. It was hypothesized that fluid from the medullary interstitium moves to lymphatics associated with arcuate or possibly interlobar blood vessels.

Human kidney

While reviewing the literature, we noted that the staining patterns of endothelial markers along the vascular tree of the kidney were not well described. The most commonly used markers for lymphatic vessels were podoplanin, LYVE-1 and Prox1 [59]. Podoplanin can be localized very reliably in human paraffin-embedded tissue after heat-based antigen retrieval using the monoclonal antibody D2-40 [59–62]. LYVE-1 has been described to be less reliable, which is consistent with our experience [63]. LYVE-1 also stains some endothelial cells of glomerular capillaries in the mouse [58]. The interpretation of the nuclear staining of Prox1 is sometimes difficult particularly when only single nuclei are present on a cross section of small lymphatic vessels.

CD31 and CD34 are commonly used markers for BECs [59]. Both are not specific for BECs, and the expression of these markers along the lymphatic network and the percentage of positive lymphatic vessels is poorly described. We think that further studies are necessary using the three markers for LECs in comparison with the markers for BECs to clearly describe the human renal vascular tree.

The anatomy of the lymphatic tree in the human kidney seems to be very similar to what was described in the above-mentioned animal studies [54, 64]. Immunohistochemistry for podoplanin in well-preserved human kidneys demonstrated lymphatic vessels in the midcortex and along interlobar and arcuate arteries [65, 66]. No podoplanin-positive vessels were present in the superficial cortex, in glomeruli and in the interstitial tissue between tubuli [65]. Towards the corticomedullary junction, podoplanin-positive vessels became more common. No lymphatics were present in the inner or outer medulla. The largest lymphatic vessels were described within the renal sinus [65]. These were no longer associated with the arteries [65]. Numerous lymphatics were also described to be present within the media of the muscular sinuses veins [65].

The role of lymphatic vessels in animal models

The role of lymphatics in normal kidneys was studied through lymphatic ligation experiments in rats. This resulted in proteinuria and a reduced creatinine clearance in the second week after ligation. These changes were associated with tubular damage, tubulointerstitial fibrosis and mesangial expansion [67].

All chronic renal diseases result in the histopathological pattern of tubulointerstitial inflammation, tubular atrophy and widening of the interstitium through the deposition of extracellular matrix (interstitial fibrosis) [68, 69]. The remnant kidney model in the rat reflects some of these aspects [70]. In contrast to the normal rat kidney, a prominent accumulation of lymphatic vessels (illustrated by immunohistochemistry for podoplanin and LYVE-1) was present in remnant kidneys in association with fibrotic regions and moderate infiltration of mononuclear inflammatory cells in the tubulointerstitium of the cortex [70]. By immunohistochemistry and in situ hybridization, an increased VEGF-C expression (potentially mediating neo-lymphangiogenesis) was detected mainly in interstitial mononuclear cells, presumably macrophages [70].

Unilateral ureteral obstruction is a model of rapid interstitial fibrosis. In the normal non-obstructed kidneys, podoplanin- and LYVE-1-positive vessels were present adjacent to large- and intermediate-sized vessels, but not within the tubulointerstitium of the cortex [71]. In the obstructed kidney, the number of podoplanin-positive lymphatics increased in the cortex but also in medulla and in the renal pelvis [71]. Induction of VEGF-C paralleled TGF-β1 expression. In vitro TGF-β1 induced significant up-regulation of VEGF-C in proximal tubular epithelial cells (human) as well as in mouse collecting duct cells and macrophages [71]. Administration of a TGF-β Type-I receptor inhibitor (LY364947) to rats with unilateral ureteral obstruction resulted in significant reduction of VEGF-C
and LYVE-1 messenger RNA expression and reduced the number of lymphatic vessels. This study illustrates an important new link between lymphangiogenesis and interstitial fibrosis via TGF-β-mediated induction of VEGF-C.

Furthermore, these studies illustrate the importance of lymphatic vessels under normal and inflammatory conditions. The formation of new vessels might well be a response to the increased interstitial fluid and inflammatory cell accumulation in the disease process.

The role of lymphatic vessels in inflammatory diseases of the human kidney

Consistent with the data in the rat remnant kidney model, lymphatics were described to be scattered throughout the interstitium in the cortex of human end-stage kidneys ($n=3$), with an increased number compared to normal renal cortex [66].

We localized podoplanin-positive lymphatic vessels in renal biopsies from patients with acute interstitial nephritis, chronic interstitial nephritis and patients with IgA nephropathy [60]. Sites of interstitial inflammation were associated with a high number of lymphatic vessels. The mean number of podoplanin-positive vessels was significantly higher in renal biopsies from patients with chronic interstitial nephritis or chronic IgA nephropathy as compared to biopsies with acute tubulointerstitial nephropathy [60]. This illustrates that the inflammatory process has to persist for some time before neolymphangiogenesis becomes apparent. Tertiary lymphatic organs, which are structured accumulations of B cells, T cells and DCs, becomes apparent. Tertiary lymphatic organs, which are structured accumulations of B cells, T cells and DCs, were surrounded by lymphatic vessels [60]. The functional role of these structures is still obscure.

In a study on patients with renal involvement in multiple myeloma, Zimmer et al. [72] used a similar approach as ours, but combined it with morphometry. The investigators compared renal biopsies from patients with multiple myeloma ($n=37$) to biopsies from patients with acute kidney injury ($n=12$) and controls (biopsies from allograft donors taken before implantation; $n=15$). Patients with multiple myeloma had a significantly higher lymph vessel length density than the two other groups. Lymph vessel length density was not associated with the degree of fibrosis but significantly associated with the degree of interstitial inflammation [72]. Active proliferation of lymphatic vessels in patients with multiple myeloma was demonstrated by staining for the proliferation marker Ki-67. Acute kidney injury biopsies demonstrated lymphatic vessels similar to normal controls.

Sakamoto et al. [59] localized podoplanin (by D2-40 staining) in 124 human kidney biopsy specimens. Podoplanin-positive lymphatic vessels in control kidney biopsies, taken 1 h after renal transplantation ($n=9$), were limited to the peritubular space of the interlobular arteries and infrequently found in tubulointerstitial areas of the normal cortex [59]. Podoplanin-positive lymphatics were present in cortical areas of tubulointerstitial inflammation and fibrosis in biopsy specimens of IgA nephropathy [60], diabetic nephropathy, lupus nephritis, anti-neutrophil cytoplasmic antibody-related glomerulonephritis and tubulointerstitial nephritis [59]. Lymphatic vessels were described to be frequently filled with inflammatory cells, suggesting that they are functioning. In diabetic nephropathy, the number of lymphatics was significantly higher in areas of tubulointerstitial fibrosis as compared with non-diabetic renal disease with a similar severity of tubulointerstitial fibrosis [59]. The authors confirmed a low number of lymphatics in acute tubulointerstitial nephritis [59]. The number of lymphatics correlated significantly with the severity of tubulointerstitial fibrosis [59]. Mononuclear cells (monocytes/macrophages/DCs) were found to express VEGF-C in the inflamed tubulointerstitium [59]. The majority of these cells were CD68 positive. Additionally, a strong expression of VEGF-C was detected in tubular epithelial cells, predominantly proximal tubular epithelial cells, which may contribute to lymphangiogenesis in the outer cortex [59]. Therefore, in various endogenous kidney diseases, neolymphangiogenesis has now been well described. The cause of injury does not seem to matter as neolymphangiogenesis was found in very different renal diseases. It is associated with the presence of inflammatory cells which release factors known to promote lymphangiogenesis (e.g. VEGF-C). Besides CD68-positive macrophages/DCs, activated tubular epithelial cells seem to be able to trigger lymphangiogenesis.

Lymphatic vessels in human renal allografts

During the explantation of a kidney, the lymphatics are dissected; thus, early after transplantation, the renal allograft has no lymphatic drainage. The lymphatic regeneration is fast, starts within the first week and a competent lymphatic off-stream is present within 2–3 weeks [73–75]. The lymph flow from a normal sheep kidney was shown to be ~1–3 mL/h [56]. After renal transplantation, the lymph production continuously increased in volume, which illustrates the importance of a functional lymphatic system to prevent interstitial oedema in the graft and consequent injury [76].

Kerjaschki et al. [77] were the first to describe 35 renal allograft biopsies, containing podoplanin-positive lymphatic vessels and nodular infiltrates, of a preliminary screen of 350 archival biopsies. Nodular mononuclear infiltrates in biopsies were associated with extensive (>50-fold) neolymphangiogenesis as compared with normal control kidney cortex ($n=6$) or biopsies taken in the acute phase of rejection with diffuse mononuclear infiltrates (six biopsies with acute interstitial rejection and four with vascular rejection). The presence of lymphatics was confirmed by additional immunohistochemical staining for LYVE-1 and Prox1. The nuclear proliferation marker Ki-67 was expressed in numerous LECs in the peritubular space and by mononuclear cells within the nodular infiltrates [77]. VEGF-C was found to be expressed by macrophages within the nodular infiltrates [77]. The LECs also expressed the lymphoid chemokine SLC/CCL21 [77]. Within the nodular infiltrates, numerous CCR7-positive cells were detected.
Lymphangiogenesis and macrophages

A common feature in both endogenous kidney diseases and renal allografts was the close association between inflammation and neoangiogenesis. The human renal tubulointerstitium contains a mixed population of CD68-positive cells, i.e. DCs and macrophages potentially involved in lymphangiogenesis [79].

In graft nephrectomies from 29 patients with chronic allograft injury, 17 of 29 graft samples showed interstitial lymphatic vessels, positive for podoplanin, LYVE-1 and VEGFR-3 [80]. In the interstitium of grafts, CD68 and VEGF-C double-positive cells were detected. In contrast, neither B cells nor T cells expressed VEGF-C [80]. In control tissue, there was no VEGF-C expression [80]. Therefore, CD68-positive DCs/macrophages might be an important trigger of neolymphangiogenesis.

To further define the source of LECs, six female donor kidneys were studied which had been transplanted into male recipients [32]. The tissue specimens showed a high rate of lymphatic endothelial proliferation as well as massive chronic inflammation. The nuclei of progenitor LECs were detected by co-localization of Prox1 by immunohistochemistry and the Y chromosome in situ hybridization [32]. A total of 47 of 1005 nuclei (4.5%) of the Prox1-positive lymphatic endothelial nuclei demonstrated a single Y chromosome and were therefore derived from circulating progenitors of the recipient’s genotype [32]. Potential candidates for lymphatic progenitors were described to be tissue macrophages [32]. In contrast, LECs in normal skin and gastrointestinal biopsies, taken from female bone marrow recipients who received a male donor graft, did not contain a Y chromosome [32]. Supportive evidence came from a study which demonstrated that podoplanin-positive interstitial lymphatic vessels contained CD68-positive cells in allograft nephrectomies [80].

Therefore, as already shown for other organs, CD68-positive cells can be involved in lymphangiogenesis in the kidney in two different ways. They can either promote lymphangiogenesis via the release of VEGFs or they can transdifferentiate and incorporate into a lymphatic endothelium [81]. It is currently unknown whether there is a difference in CD68-positive DCs or macrophages in the propagation of lymphangiogenesis.

Summary and outlook

Research on lymphatic biology is currently booming as summarized in the first part of the manuscript. In sharp contrast, our knowledge even of the physiological expression of the currently used markers of lymphatic vessels in the human kidney is still relatively limited. The current studies are consistent in the view that inflammation of the kidney (either in endogenous kidneys or renal allografts) results in the formation of new lymphatic vessels. Early studies demonstrated an enormous amount of lymph and cells to leave renal allografts via lymphatics. As the normal cortex is limited in the lymphatic drainage, new lymphatic vessels might be a necessary response to cope with this increased workload. In situations of interstitial oedema formation (e.g. allograft rejection, interstitial nephritis), these lymphatics would be important to decrease interstitial pressure. Therefore, in the early phase of inflammation, newly formed lymphatic vessels are most likely a positive response to injury. Neolymphangiogenesis seems to be promoted by lymphatic growth...
factors released by inflammatory cells, most likely CD68-positive cells (in the human kidney resembling macrophages and DCs). Additionally, bone marrow-derived cells are incorporated into lymphatic vessels of renal allografts in relatively low numbers.

The important question of whether neolymphangiogenesis is good or bad in the long run remains a matter of debate. It is possible that the higher number of lymphatic vessels might result in acquired immune responses promoted by the efflux of antigen and antigen-presenting cells. Blocking lymphangiogenesis has, for example, been shown to be beneficial in pancreatic islet transplantation [82]. It could further be hypothesized that the lymphatic drainage of sites, which normally have no direct access to the lymphatic tree, might change the normal interior milieu resulting in tissue injury. At the moment, there is little direct evidence for a negative role of lymphatics in chronic inflammation of the kidney. Neolymphangiogenesis can likely be seen as a double-edged sword promoting the increased efflux of lymph and inflammatory cells, thereby exerting a protective influence. On the other side of the sword, the increased number of lymphatic vessels might promote acquired immune responses against the endogenous kidney or renal allografts, which might be detrimental in the long run.

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