

## The Role of Lymphatic Vessels in Renal Injury

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### Abstract

Progressive kidney diseases are characterized by the recruitment of inflammatory cells to the tubulointerstitium, tubular atrophy and fibrosis. The number of lymphatic vessels increases in the interstitium during this process (i.e. neolymphangiogenesis). Here we will describe the current evidence for neolymphangiogenesis during renal injury, summarize the major factors involved and discuss the functional consequences. Data are emerging that central profibrotic players like TGF- $\beta$  also mediate the release of lymphangiogenic factors such as VEGF-C and VEGF-D in the kidney. Furthermore, in other organs activation of TGF- $\beta$  by VEGF-C has been described. The complex interactions of profibrotic and lymphangiogenic factors might explain why lymphangiogenesis was found to be associated with fibrosis in some models, but a reduction in fibrosis in others. It is likely that the functional consequences of lymphangiogenesis depend on the stage of the disease course and the microenvironment where it takes place. Studies are needed where lymphangiogenesis is switched on or off at defined time points. However such data are not yet available in models of renal diseases.

**Keywords:** Lymphatic vessels; Lymphatic endothelial cells; Renal inflammation; Renal allograft; Glomerulonephritis; Tubulointerstitial inflammation; Tubulointerstitial fibrosis

### Introduction

The lymphatic system drains fluid, antigens and cells from the interstitial space to the blood [1]. Formally, these vessels were thought to be a "passive" system mainly involved in fluid homeostasis. With the establishment of markers for lymphatic endothelial cells we witnessed a major interest of the scientific community in this system, reflected by a rapidly increasing number of studies on lymphatic biology. Now it is well established that lymphatic vessels and lymphatic endothelial cells play active, key roles in immune surveillance, response to infectious agents, and allograft rejection. Lymphatic endothelial cells orchestrate the exit of cells from tissues and promote acquired immune responses. Many aspects of lymphatic biology and pathophysiology have been reviewed elsewhere and are described in other parts of this special edition [1]. Here we will summarize evidence for neolymphangiogenesis in the injured kidney, describe the factors and mechanisms involved, and finally discuss the functional consequences of lymphangiogenesis for renal diseases. The role of lymphatic vessels in renal malignancies is beyond the scope of this review.

### Anatomy of the Renal Lymphatic System

The development of the renal lymphatic system has recently been described in rats [2]. At day E17 the podoplanin positive lymphatic vessels were restricted to the renal hilus [2]. These extended close to the interlobar vessels until day E20 [2]. Between days one and 20 after birth, the vessels continued to spread along the arcuate arteries with some following the interlobular vessels into the cortex [2]. No lymphatic vessels were detected in the rat medulla. VEGF-C, the

potential driving force of this development, was mainly expressed by tubular epithelial cells [2].

In the normal human kidney the lymphatic capillaries commence in proximity to the interlobular arteries (reviewed in [3]). The lymphatic precollectors are localized along arcuate vessels at the bases of the pyramids. With the interlobar blood vessels of the renal columns these finally drain into hilar collector lymphatics [3,4]. The vessel number decreases towards the outer cortex. No lymphatic vessels were described in glomerula and within the normal medulla. The renal medulla possesses a high tonicity necessary for maintaining the concentration gradient. Interestingly this does not result in lymphatic vessels in this area as described e.g. for the skin [5]. The cause of this is currently unknown. Therefore particular micro-compartments of the normal kidney are devoid of lymphatics, which is likely a reflection of their function. It could be hypothesized that in the renal medulla lymphatic vessels would interfere with the concentration gradient. In glomerula many antigens are deposited in the mesangium from the circulation. Here the absence of lymphatics might prevent unnecessary immune responses.

Recently, morphological evidence was provided that some lymphatics might drain via the renal papilla towards the pelvis in rats with unilateral ureter obstruction [6]. Furthermore, a connection between renal pelvis and lymphatics was illustrated by lymphography in a patient with chyluria [6]. Whether a subset of renal lymphatics drain into the renal pelvis needs further evaluation.

Therefore the human renal microanatomy of the lymphatic system is comparable to other parenchymal organs and is similar in the rodent kidney. The lack of lymphatic vessels in the renal medulla and in glomerula is functionally reasonable. How this is mediated during development and the functional consequences need further studies.

## What is the Evidence for Lymphangiogenesis in Rodent Models of Renal Injury?

The formation of lymphatic vessels (i.e. neolymphangiogenesis) has been described in many forms of inflammation. The development of lymphatics under inflammatory conditions reflects developmental lymphangiogenesis in many ways with a prominent role of the VEGFR-3 and VEGF-C [7]. The growth factors VEGF-C and VEGF-D are triggered by proinflammatory cytokines (e.g. interleukin-1 $\alpha$ , -1 $\beta$  and Tumor Necrosis Factor (TNF)- $\alpha$ ), as shown in various cell types [8-12]. Induction of NF-kappaB e.g. by inflammatory stimuli such as TNF- $\alpha$ , IL-1 $\beta$  or bacterial lipopolysaccharides might result in enhanced sensitivity of lymphatic endothelium to VEGF-C and VEGF-D [13].

All chronic renal diseases lead to tubulointerstitial inflammation, tubular atrophy and widening of the interstitium through deposition of extracellular matrix (interstitial fibrosis, [14-16]). Many aspects of this pattern are reflected by the remnant kidney model in the rat, in which a prominent increase of lymphatics and an increased VEGF-C expression was detected mainly in interstitial mononuclear cells in fibrotic regions [17].

Unilateral Ureteral Obstruction (UUO) is a rapid model of renal interstitial fibrosis. In rats, UUO led to the expression of the profibrotic factor TGF- $\beta$ 1 and VEGF-C by tubular epithelial cells and infiltrating inflammatory cells [6]. In cultured tubular epithelial cells and macrophages, the expression of VEGF-C was upregulated by TGF- $\beta$ 1 [6]. Lymphangiogenesis and fibrosis was reduced *in vivo* by an inhibitor of TGF- $\beta$ 1 receptor [6]. Similarly, induction of lymphangiogenesis by TGF- $\beta$ 1 has been shown in peritoneal fibrosis [18]. Therefore although TGF- $\beta$  was found to reduce the proliferation of human dermal lymphatic endothelial cells and lymphangiogenesis during wound repair in the skin [19-21], TGF- $\beta$  seems to promote lymphangiogenesis in the kidney [6]. Further data comes from a study, where TGF- $\beta$ 1 increased VEGF-C expression in macrophages and tubular epithelial cells *in vitro* [22]. In this study a mouse UUO model was used, in which macrophages significantly contributed to lymphangiogenesis as clodronate depletion diminished both fibrosis and lymphangiogenesis [18]. Furthermore, transfer of macrophages deficient in VEGF-C expression resulted in decreased lymphangiogenesis in the obstructed kidney. The authors speculated that VEGF-D reversed the direct inhibitory effects of TGF- $\beta$ 1 on lymphangiogenesis resulting in a net stimulatory effect via TGF- $\beta$ 1 induced expression of VEGF-C. *In vitro*, VEGF-C protein was higher in M2-polarized macrophages as compared to M1-polarized bone marrow-derived macrophages [22]. The Blockade of VEGF-C and VEGF-D signaling decreased lymphangiogenesis as could be expected [22].

Adriamycin causes severe proteinuria in rats. In a chronic unilateral adriamycin nephrosis model, kidneys were evaluated up to 30 weeks of age [23]. Proteinuria was high after six weeks, but no signs of fibrosis or lymphatic vessel formation were described. Lymphatic vessel density increased significantly about three-fold during the next six weeks. At the same time, signs of tubular-epithelial activation (expression of osteopontin and VEGF-C) developed and the number of interstitial myofibroblasts increased. At that timepoint the number of macrophages and collagen deposition was not yet altered and most of the macrophages present stained for VEGF-C [23]. The expression of VEGF-C was stimulated in tubular epithelial cells *in vitro* by fetal calf serum (but not albumin). Hence this study suggests that

proteinuria, induces lymphangiogenesis via VEGF-C by tubular epithelial cells prior to the development of fibrosis [23].

Acute ligation of renal lymphatics resulted in increased urine volume and sodium excretion, whereas chronic obstruction caused proteinuria and reduced renal function after two weeks. Tubular damage, tubulointerstitial fibrosis and mesangial expansion were detectable by routine morphology [24].

In summary in rodent models a close association between renal fibrosis and an increased number of lymphatic vessels has been illustrated. Various forms of injury like renal ablation, ureter obstruction and proteinuria all promoted an increase in lymphatic vessels. The lymphangiogenic factors (VEGF-C and D) might be expressed by tubular epithelial cells and infiltrating mononuclear cells. Importantly there seems to be a reciprocal interaction between lymphangiogenic factors and TGF- $\beta$ .

## What is the Evidence for Lymphangiogenesis in Human Kidney Diseases?

Consistent with the data from rodent models, the number of lymphatic vessels was found to be increased in the cortex of the human kidney during chronic diseases [25-28]. This was studied in various forms of chronic kidney diseases like IgA nephropathy, diabetic nephropathy, lupus nephritis, ANCA-associated glomerulonephritis and tubulointerstitial nephritis [27,28]. In contrast to chronic renal diseases the number of podoplanin positive lymphatics was significantly lower in acute tubulointerstitial nephritis [27]. Sites of nodular interstitial infiltrates (reflecting tertiary lymphatic tissue) were surrounded by a high number of lymphatic vessels [27]. There was no difference between the underlying chronic diseases, with the exception of diabetic nephropathy which was associated with a higher number of lymphatics compared to all other diseases [28]. In one study the number of lymphatics correlated significantly with the severity of tubulointerstitial fibrosis [28]. As in the rodent models mononuclear cells (monocytes/macrophages/dendritic cells) were found to express VEGF-C in the inflamed tubulointerstitium [28]. The other main site of expression of VEGF-C was tubular epithelial cells [28].

Therefore an increased number of lymphatic vessels are present in the human kidney affected by chronic kidney diseases. As in rodent models VEGF-C was found to be expressed by infiltrating mononuclear cells and tubular epithelial cells.

## Lymphatic Vessels in Renal Allografts

Renal allograft implantation leaves the graft without a functional lymphatic drainage, which however does not seem to significantly affect renal function [29]. Early studies illustrate a rapid regeneration of the lymphatics within the first weeks (reviewed in [3]). In experimental models lymph flow continuously increased in volume in the early period after transplantation. This likely reflects the role of lymphatics to resolve interstitial edema in the graft and consequent injury [30].

In rat renal allografts impaired renal function, proteinuria, interstitial inflammation and fibrosis were found to be present at twelve months after transplantation [31]. There was an 18-fold increase in interstitial lymphatic vessel number, whereas the number of perivascular vessels remained unchanged. The interstitial lymphatic vessel number correlated with the severity of interstitial fibrosis [31]. Cyclosporine treated rat renal allografts displayed robust activation of

the VEGF-C/VEGFR3 axis with significant lymphangiogenesis. Switching animals to the mTOR inhibitor Sirolimus led to a significant reduction in lymphangiogenesis and less chronic allograft damage, implicating a function of the mTOR pathway in this process [32].

Kerjaschki et al. were the first to describe podoplanin-positive lymphatic vessels in human renal allograft biopsies [33]. Prominent neo-lymphangiogenesis was present in late allografts, but not early in acute rejection or in normal control kidney cortex [33]. Proliferation of lymphatic vessels was documented in this study, as well as prominent expression of VEGF-C by macrophages [33]. Evidence for CCR7 positive cell recruitment to lymphatics was shown by the expression of the corresponding ligand CCL21 by lymphatic endothelial cells [33]. Atypical chemokine receptors like DARC, D6 and CXCR7 are expressed by lymphatic endothelial cells and might modulate the recruitment of inflammatory cells to the lymphatics. CXCR7 was found to be expressed by lymphatic vessels in renal allografts and the number of these vessels increased during rejection episodes [34].

Podoplanin positive lymphatics were described in areas of cellular infiltration in about 2/3<sup>rd</sup>s of renal allograft biopsies with a prominent increase in lymph vessel density [35]. Renal allograft function was significantly better in patients who had a high lymph vessel density after one year [35]. Other studies found a negative association between lymphatic vessel number and renal function [36].

Currently there is evidence that the known lymphangiogenic factors (e.g. VEGF-C and D) are both expressed by infiltrating cells as well as tubular epithelial cells in renal allografts. Therefore the process could be inhibited by reducing the number of infiltrating cells or by a down regulation of pro-lymphangiogenic factors in tubulo-epithelial cells. Inhibiting the Rho kinase pathway in tubular epithelial cells in a non-life supporting acute rat transplantation model resulted in significant reduction in the number of lymphatic vessels as early as day four [37]. This approach also reduced the number of macrophages. It will be important to test such approaches in chronic renal allograft models.

Therefore in renal allografts lymphangiogenesis is an important early response to interstitial edema after ablation of lymphatics of the transplanted organ. Later lymphatics are likely involved in the response to the allograft. Neo-lymphangiogenesis has been documented in chronic renal allograft injury. The functional impact of these newly formed vessels and therapeutic inhibition needs further studies. The next paragraph summarizes results of extrarenal transplant models.

### Blockade of Lymphatic Formation in Non-Renal Allograft Models

In murine islet, heart and cornea allograft models lymphangiogenesis was inhibited by VEGFR3 blockade [38]. Treatment of mice after islet transplantation with either Sunitinib (a kinase inhibitor which blocks VEGFR3) or treatment with a monoclonal VEGFR3 antibody led to a reduction in inflammatory cell influx and prolonged graft survival [38]. In a rat heart allograft model the transfer of VEGFR3-Ig reduced CCL21 expression and inflammatory cell recruitment, but did not affect lymphangiogenesis. A monoclonal antibody against VEGFR3 reduced lymphatic vessel number, arteriosclerosis and infiltrating cell recruitment in chronic mouse heart rejection [39]. Overexpression of soluble VEGFR3 prolonged the survival of corneal allografts [40].

These data suggest that in models of organ transplantation the inhibition of lymphangiogenesis or lymphatic endothelial activation might be beneficial with respect to allograft rejection. However it could be envisioned that sustained blockade of lymphangiogenesis within the renal allograft, a highly vascularized and strongly perfused organ, might cause serious complications such as interstitial edema leading to increased interstitial pressure with consecutive reduction of glomerular filtration pressure. Therefore experiments in kidney transplantation models are needed to confirm the above results in renal allografts.

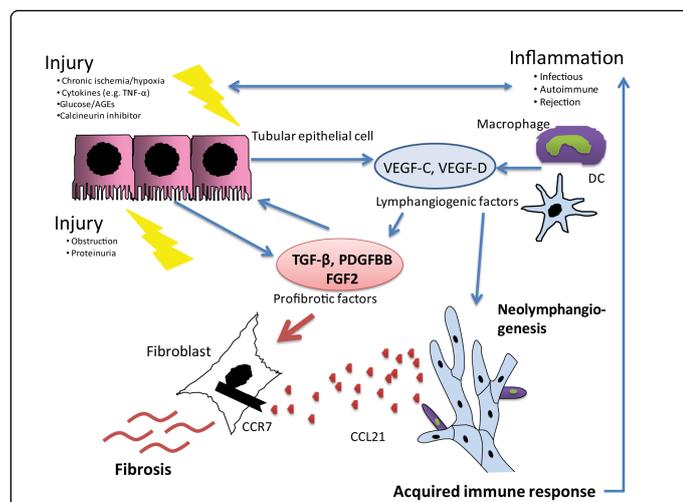
### Cells Involved in Renal Neolymphangiogenesis

The tubulointerstitium of endogenous kidneys and renal allografts are infiltrated by lymphocytes, dendritic cells and macrophages during progressive diseases. CD68 was the marker most commonly used in the kidney and stains a mixed population of DCs and macrophages potentially involved in lymphangiogenesis [41]. In renal allografts from female donors transplanted into male recipients a total of 47 out of 1005 nuclei (4.5%) of the Prox1 positive lymphatic endothelial nuclei demonstrated a Y chromosome [42]. Lymphatic endothelial cells in normal skin and gastrointestinal biopsies, taken from female bone marrow recipients who received a male donor graft, did not contain a Y chromosome [42]. Furthermore, lymphatic vessels contained CD68 positive cells in allograft nephrectomies [43]. Therefore in human renal allografts lymphangiogenesis under inflammatory conditions does not solely occur by continuous sprouting, but also includes incorporation of lymphatic progenitors into the growing lymphatic vessels and CD68 positive cells [42]. Besides the direct incorporation, the CD68 positive cells are a rich source for lymphangiogenic growth factors.

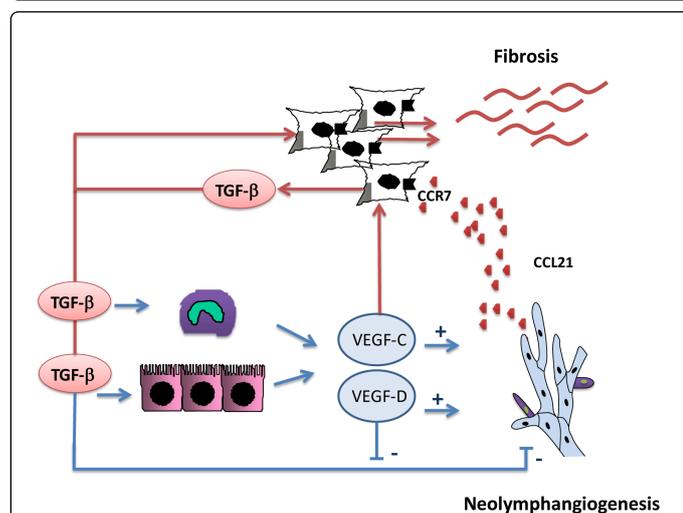
### Summary

Until now neolymphangiogenesis has been demonstrated in various renal diseases and in injured renal allografts. Diabetic nephropathy was found to be associated with the highest number of lymphatics (which awaits conformation). Both macrophages/DCs and tubular epithelial cells express lymphatic growth factors (Figure 1A). The functional role of neolymphangiogenesis in the kidney has not yet been addressed systematically. Lymphatic vessels can be seen as a double edged sword [3]. In the acute situation they likely are necessary for the removal of inflammatory cells, clearance of pathogens, or interstitial edema. This acute function has been demonstrated in models of skin inflammation, where transgenic overexpression of VEGF C did reduce acute skin inflammation [44]. In later stages or chronic autoimmune diseases the lymphatics might promote on-going inflammation via acquired immune responses. Therefore the question of whether newly formed lymphatic vessels are good or bad is likely dependent on the time course of the disease.

The situation now became more complex with the finding that TGF- $\beta$  (a key profibrotic factor) induces VEGF-C and VEGF-D (Figure 1). This might explain the close association between fibrosis and neolymphangiogenesis. Furthermore, in the rat heart it was shown that VEGF-C induced fibrosis by stimulating the TGF- $\beta$ 1 pathway [45]. A vicious circle can now be envisioned where VEGF-C triggers TGF- $\beta$ , which then promotes further release of polylymphangiogenic factors (Figure 1B).



**Figure 1A:** Schematic illustration of the close interactions between pro-fibrotic and pro-lymphangiogenic factors. Tissue injury promotes inflammation, release of cytokines and release of VEGF-C and VEGF-D (upper part of the panel). This results in neolymphangiogenesis. Lymphatics can clear interstitial edema, but also promote ongoing inflammation via acquired immune responses and directly activate fibroblasts (via CCL21 release, lower part of the figure).



**Figure 1B:** Schematic illustration of the close interactions between pro-fibrotic and pro-lymphangiogenic factors. TGF- $\beta$  is a central profibrotic factor, which can directly inhibit lymphangiogenesis when lymphatic endothelial cells are exposed (lower part of the figure). The direct effect on lymphatic endothelial cells can be blocked by VEGF-D (-). On the other hand TGF- $\beta$  can trigger the release of VEGF-C by tubular epithelial cells and macrophages. VEGF-C triggers neolymphangiogenesis (+) but can also trigger the release of TGF- $\beta$  (vicious circle in the upper part of the figure).

TNF: Tumor Necrosis Factor; VEGF: Vascular Endothelial Growth Factor; TGF: Transforming Growth Factor; FGF: Fibroblast Growth Factor; DC: Dendritic Cell.

Newly formed lymphatics might also directly promote fibrosis through the release of chemokines and activation of fibroblasts [46]. Although the knowledge about lymphatics in the kidney has substantially increased in recent years, the functional consequences of neolymphangiogenesis are still far from being understood.

## Future Perspectives

The interaction between pro-lymphangiogenic and pro-fibrotic pathways as described above are a very exciting field of future studies. However, certain areas await further investigation. The functional consequences of the blockade of neo-lymphangiogenesis or promotion of lymphangiogenesis (e.g. early in renal transplantation) need to be evaluated. Furthermore, only the most prominent lymphangiogenic factors have been studied in the kidney so far. We performed a search concerning the role of other known lymphangiogenic factors such as CCBE1, semaphorins, neuropilins, ephrin B2, alk, BMP9, angiopoietins 1-3, TIE, DLL4, Notch1, FGF2, sphingosine 1-phosphate in the renal literature. Whereas some have been investigated in the context of renal development or fibrosis (e.g. semaphorin) none of them has been scrutinized in the context of neolymphangiogenesis of renal injury. Finally, the lymphatic vessel tree has different segments, which can be defined by markers of lymphatic endothelial cells. The phenotype of de novo formed vessels in the kidney needs further description (Table 1). We are looking forward to exciting studies on these important vessels in the kidney.

Renal lymphatics within the normal kidney run in proximity to larger vessels. Their number decreases in the outer cortex. Glomerula and normal medulla do not contain lymphatics
Pro-lymphangiogenic factors are expressed by infiltrating mononuclear cells and tubuloeplithelial cells
Models of renal fibrosis and data from human renal diseases illustrate neolymphangiogenesis in progressive renal diseases
Lymphangiogenesis in renal allografts might be detrimental, whereas in other chronic kidney diseases the functional role remains to be defined
A booming area of studies is the interaction between pro-lymphangiogenic factors and fibrogenic growth factors
Studies are needed concerning the therapeutic impact of the blockade of lymphangiogenesis in models of renal disease and renal allograft rejection

**Table 1:** Take home points.

## Conflict of Interest

Stephan Segerer received benefits from ROCHE as a consultant, and currently from Baxter Healthcare Corporation. Otherwise the authors do not declare conflicts of interest.

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