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ORIGINAL ARTICLE

Inflammatory lymphangiogenesis in a rat transplant model of interstitial fibrosis and tubular atrophy

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Summary

We have previously reported *de novo* lymphangiogenesis in human renal allograft nephrectomy specimens that exhibited interstitial fibrosis and tubular atrophy (IFTA). This study examined whether a similar pathology developed in an experimental model of renal transplantation in the rat. Renal transplants were carried out in rats comprising both isografts (Lewis kidneys → Lewis rats) and allografts (Fisher kidneys → Lewis rats). Animals were immunosuppressed in the immediate postoperative period and sacrificed at 12 months. Experimental readouts included lymphatic vessel number and location, inflammatory cell infiltration, interstitial fibrosis, renal function, blood pressure and proteinuria. Rat allografts demonstrated the characteristic features of IFTA with increased macrophage and T cell infiltration and scattered B cells aggregates. Rat allografts exhibited impaired renal function and proteinuria. Although there was no difference in the number of perivascular lymphatic vessels, there was a striking 18-fold increase in the number of interstitial lymphatic vessels in renal allografts. Furthermore, the lymphatic vessel number correlated with the extent of interstitial fibrosis. This rat allograft model of IFTA demonstrates a marked increase in the number of interstitial lymphatic vessels and mirrors previous work in failing human renal allografts.

Introduction

Chronic transplant dysfunction is a common and poorly understood process that is responsible for the loss of 5% of renal allografts per annum [1]. It is characterized histologically by the development of interstitial fibrosis and tubular atrophy (IFTA) and has a multifactorial pathogenesis that includes ischaemia reperfusion injury, direct alloantigen dependent injury and calcineurin inhibitor toxicity. The clinical course is characterized by progressive deterioration in renal function with associated hypertension and proteinuria. Although modern immunosuppressive agents effectively combat T cell mediated injury and have significantly improved acute rejection rates after renal transplantation, this has not translated into an improved long-term outcome with the chronic changes of IFTA remaining a leading cause of graft loss [2]. Therefore, alternative therapeutic targets must be identified for this condition that currently has no effective treatment.

There is a growing interest in the role of the lymphatic system in the context of renal transplantation. It is now possible to distinguish between vascular and lymphatic endothelial cell populations using recently described lymphatic specific markers including the CD44 hyaluronan acid receptor (LYVE-1) [3], mucin-type glycoprotein podoplanin [4], homeobox transcription factor Prox-1 [5] and vascular endothelial cell growth factor receptor 3 (VEGFR-3) [6].

The renal lymphatic system is involved in tubulointerstitial homeostasis and plays an important role in

immune surveillance. In the normal healthy kidney, the lymphatic vessels are found in a perivascular location surrounding large intrarenal blood vessels and are absent from the tubulointerstitium [7]. However, significant changes in the intrarenal lymphatic system may occur in both acute and chronic transplant disease in humans. Kerjaschki *et al.* demonstrated marked lymphangiogenesis in the vicinity of nodular infiltrates in a subset of human allografts exhibiting acute rejection (~10% of renal biopsies) [8]. Infiltrating macrophages expressed vascular endothelial growth factor C (VEGF-C), a key lymphangiogenic growth factor that signals via VEGFR-3 [9]. Our previous work demonstrated *de novo* tubulointerstitial lymphangiogenesis in human allograft nephrectomies exhibiting IFTA with interstitial lymphatic vessels being present in all nephrectomy specimens studied [10].

Although lymphangiogenesis may accompany both acute or chronic transplant dysfunction it remains unclear whether the manipulation of *de novo* lymphangiogenesis might be of therapeutic utility and future interventional studies in experimental models are required. In this study, we examined lymphangiogenesis in a rodent model of renal allograft dysfunction that exhibited IFTA, inflammatory cell infiltration, renal dysfunction and proteinuria. We demonstrated a striking increase in the number of interstitial lymphatic vessels compared with isografts. Such chronic models will provide future opportunities to manipulate lymphangiogenesis and determine whether it will beneficially impact upon outcome.

Materials and methods

Rodent transplant model

Kidneys from donor male Lewis rats (isografts) or Fisher rats (allografts) were transplanted heterotopically into male Lewis recipients [11]. Briefly, the left donor kidney was isolated and perfused with University of Wisconsin solution. After a left native nephrectomy was performed in recipient rats, the donor renal artery and vein were anastomosed to recipient aorta and inferior vena cava with an end-to-side anastomosis using aortic and vena caval conduits. The donor ureter was attached to recipient bladder using a bladder cuff technique. A right native nephrectomy was performed on the 10th postoperative day to make this a functional transplant model. All recipients received cyclosporine (5 mg/kg) intraperitoneally for the 10 days following transplantation to prevent episodes of acute rejection. The experimental groups included allografts (Fisher $\rightarrow$ Lewis, $n = 7$) and control isografts (Lewis $\rightarrow$ Lewis, $n = 5$). The experiment was terminated after 52 weeks or when animals exhibited features of uraemia. There was no statistical difference in the study duration between the groups.

Renal function

Serum creatinine, creatinine clearance, urine protein excretion and systolic blood pressure were determined using methods previously described [12]. In brief, rats were placed in individual metabolic cages for 24 h for the collection of urine with free access to food and water. Urine was assayed for creatinine (standard autoanalyzer techniques) and protein (Lowry method). Rats were then placed in a restraining Perspex tube and their mean systolic blood pressure was recorded by computerized tail-cuff plethysmography (IITC Life Science, Woodland Hills, CA, USA). Animals were killed under halothane anaesthesia and a blood sample taken by cardiac puncture and serum assayed for creatinine. The 24 h urine protein excretion was calculated from the urine volume and urine protein concentration and expressed as mg/24 h. The 24 h creatinine clearance was calculated by the formula '24 h creatinine clearance = (urine creatinine concentration $\times$ urine flow rate)/serum creatinine concentration' and expressed as ml/min.

Immunohistochemistry

Immunohistochemical staining was undertaken on deparaffinized 4 μm tissue sections cut from formalin-fixed paraffin embedded tissue. A standard streptavidin horseradish peroxidase method was used. Epitopes were de-masked using heat-induced antigen retrieval in a microwave oven for 15 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and tissue sections were incubated in avidin/biotin blocking kit (Vector Laboratories, Peterborough, UK) and serum free protein block (DAKO, Aachen, Germany). Sections were stained with antibodies against the lymphatic endothelial marker podoplanin (Sigma, Dorset, UK, diluted 1:100), the macrophage marker ED-1 (AbSerotec, Oxfordshire, UK, diluted 1:100), the T cell marker CD3 (Abcam, diluted 1:100) and the B cell marker B220 (AbSerotec, diluted 1:100). Tissue sections were incubated with primary antibody overnight at 4 °C and then incubated with the appropriate secondary antibodies at room temperature for 1 h followed by Vectastain RTU ABC reagent for 30 min (Vector Laboratories). The reaction was visualized using 3′3-diaminobenzidine (DAKO) and the nuclei were lightly counterstained with haematoxylin.

Lymphangiogenesis was assessed with a Leica ll microscope coupled with QCapture Pro computer aided photography system. Lymphatic vessel numbers were determined by selecting 10 random cortical fields and counting the number of fully formed podoplanin positive vessels with a visible lumen. Lymphatic vessels were classified by location as either perivascular if associated with

an arteriole or venule or interstitial if no blood vessel was evident in the immediate vicinity. $CD3^+$ T cell and ED-1^+ macrophage infiltration was quantified by counting the number of DAB positive cells in 10 random high power fields (400× magnification). All histological assessments were performed by a single blinded investigator.

To measure interstitial fibrosis, tissue sections were stained with picrosirius red to detect fibrillar collagen. Sections were deparaffinized, rehydrated and stained with 0.1% Sirius solution, rehydrated in graded alcohols and incubated in picrosirius solution for 2 min. Quantification was carried out in a blinded manner by computer assisted image analysis (PhotoShop) of 10 sequentially selected fields of renal cortex and medulla and expressed as the percentage of the total surface area positive for staining.

Extraction of RNA from formalin fixed paraffin embedded tissue and real-time polymerase chain reaction

RNA was extracted from 10 μm sections cut from formalin-fixed paraffin embedded tissue using a Qiagen RNASE Easy kit (Qiagen, W. Sussex, UK) and performed according to the manufacturer's instructions. One hundred nanograms of RNA were reverse transcribed using Superscript II Reverse Transcriptase and Random Primers (Invitrogen, CA, USA). Samples of 5 ng of cDNA were analysed for VEGF-C using a Taqman Gene Expression Assay and TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Cheshire, UK) under standard cycling conditions. Each sample was measured in duplicate on an ABI prism 7000 sequence detection system under standard cycling conditions. Reactions were normalized against 18S alone reactions on the same plate.

Statistical analysis

All analysis was carried out using GraphPad Prism. Groups were compared using unpaired *t* test and data expressed as mean ± SEM. A *P*-value less than 0.05 was considered statistically significant.

Results

Allografts exhibit interstitial fibrosis and tubular atrophy

Histological examination of allografts at the end of the study revealed glomerulosclerosis and tubulointerstitial changes characterized by increased deposition of fibrillar collagen and tubular atrophy (Fig. 1a). In contrast, isograft controls exhibited minimal glomerular and tubulointerstitial disruption (Fig. 1b). Quantification of interstitial fibrosis revealed significant deposition of fibrillar collagen in both the cortex (16.6 ± 1.6 vs. 3.7 ± 1.6% surface area, allograft vs. isograft, $P = 0.0017$) and medulla (12.1 ± 1.4 vs. 5.4 ± 1.9% surface area, allograft vs isograft, $P = 0.0382$) of allografts compared with isografts (Fig. 2a–f).

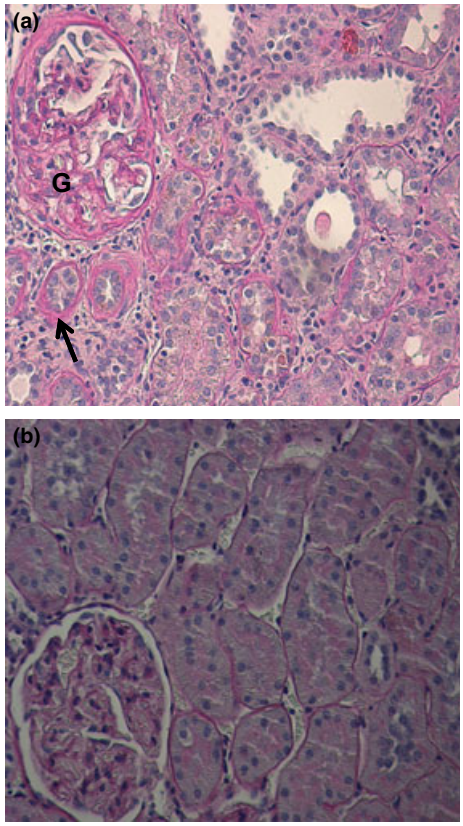

Figure 1 Rat allografts exhibit glomerular and interstitial disease. Representative Periodic Acid Schiff (PAS) staining of allografts (a) demonstrates glomerulosclerosis (G) with mesangial expansion and tuft effacement. Tubulointerstitial changes include duplication of the basement membrane and tubular atrophy (example arrowed). PAS staining of isografts (b) reveals preservation of normal tubular and glomerular architecture (all images ×200).

Rats with allogeneic transplants develop renal failure and hypertension

At the termination of the experiment rats with allografts had significant renal dysfunction (Table 1) indicated by an elevated serum creatinine (180 ± 63 vs. 64 ± 7 μmol/L, allograft vs. isograft, $P < 0.05$) and a reduced creatinine clearance (0.7 ± 0.25 vs. 1.8 ± 0.4 mL/min, allograft vs isograft, $P < 0.05$). Rats with allografts also exhibited significant proteinuria (357 ± 114 vs. 23 ± 6 mg/24 h, allograft vs. isograft, $P < 0.05$) and systolic hypertension (systolic BP 155 ± 15 vs. 120 ± 7 mmHg, allograft vs isograft, $P < 0.05$).

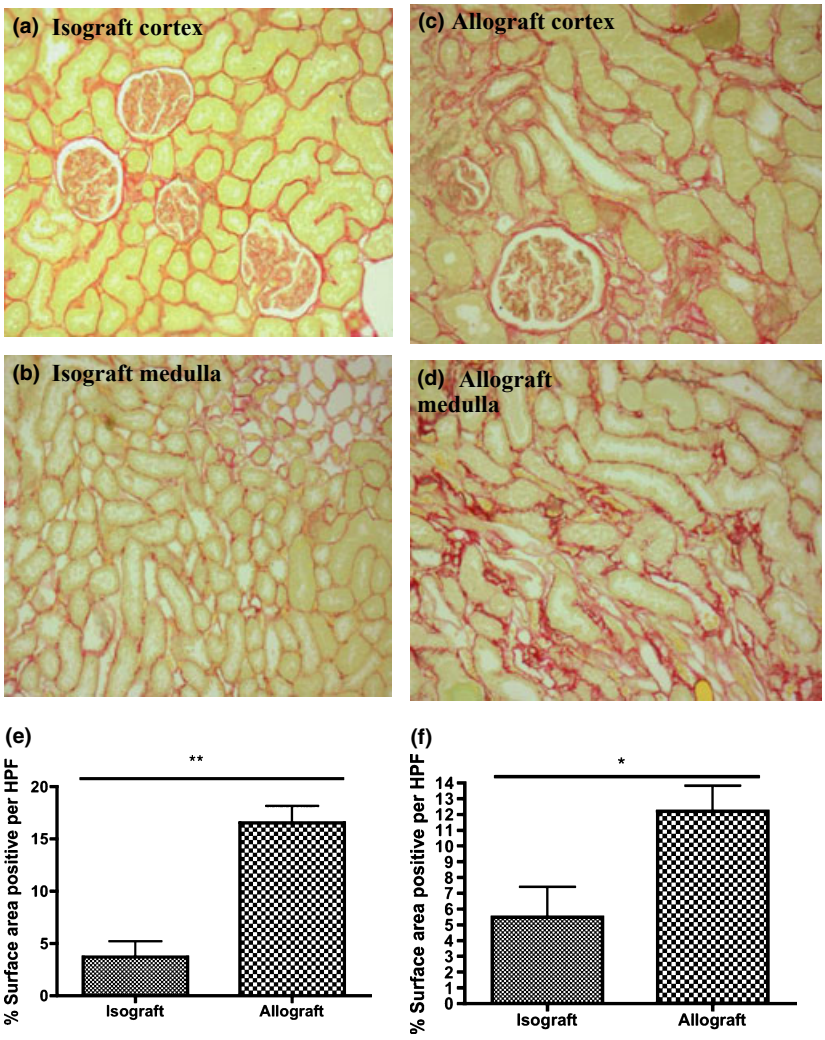

Figure 2 Rat allografts exhibit significant collagen deposition. Representative images (×200 magnification) of fibrillar collagen deposition shown by Picrosirius red staining of the renal cortex and medulla of isografts (a and b) and allografts (c and d). Quantification of fibrillar collagen deposition by computer image analysis demonstrates increased scarring in the cortex (e) and medulla (f) of allografts (**$P < 0.01$ and *$P < 0.05$).

Table 1. Allografts exhibit renal dysfunction, proteinuria and systolic hypertension.

	Isograft	Allograft
Proteinuria (mg/24 h)	23 ± 6	357 ± 114*
Serum creatinine (μmol/L)	64 ± 7	180 ± 63*
Creatinine clearance (mL/min)	1.8 ± 0.4*	0.7 ± 0.25
Systolic blood pressure (mmHg)	120 ± 7	155 ± 15*

All data are mean ± SEM, *$P < 0.05$.

Immunophenotyping of the inflammatory cellular infiltrate

The mononuclear cell infiltrate consisted predominantly of ED-1$^+$ macrophages (Fig. 3) and CD3$^+$ T-lymphocytes (Fig. 4a–c). Isograft control tissue contained a small number of mononuclear cells whilst allografts exhibited significant numbers of ED1$^+$ macrophages (10 ± 1.7 vs. 3.1 ± 0.4 cells/hpf, allograft vs. isograft, $P < 0.05$) and CD3$^+$ T-lymphocytes (49.4 ± 4.1 vs. 9.4 ± 2.9 cells/hpf, allograft vs isograft, $P < 0.05$). The B220$^+$ B-lymphocyte infiltrate of allografts was sparse and located in dense aggregates adjacent to large blood vessels within the cortex as has been described previously in chronic kidney allograft rejection (Fig. 4d).

Allografts exhibit significant interstitial lymphangiogenesis

Lymphatic vessels were very rarely encountered in the tubulointerstitium of isografts and were predominantly located adjacent to large and medium sized blood vessels (Fig. 5). The number of lymphatic vessels situated in a perivascular location was comparable between experimental groups (2.95 ± 0.15 vs. 2.57 ± 0.32 perivascular lymphatic vessels/field, allograft vs. isograft, $P > 0.05$). In

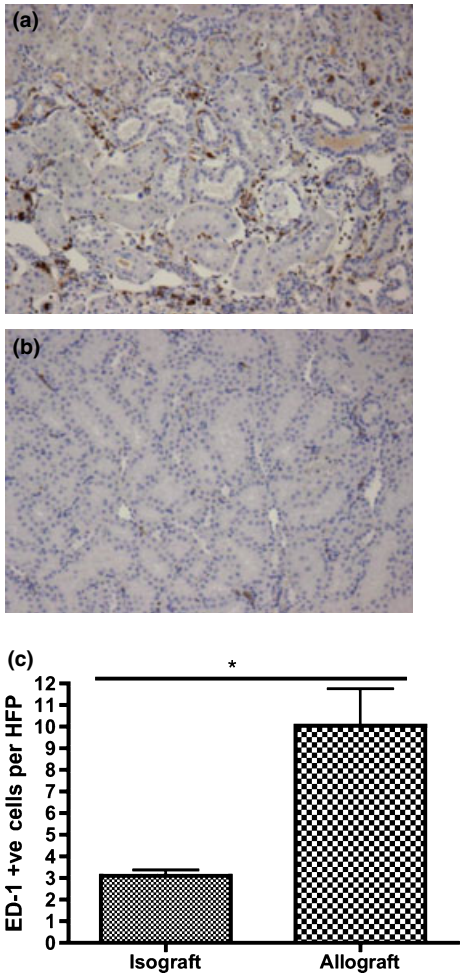

Figure 3 Rat allografts exhibit significant macrophage infiltration. Representative photomicrographs of ED-1$^+$ macrophage distribution in renal allograft (a) and isograft (b) (×200 magnification). Macrophage infiltration was quantified by counting the number of ED-1$^+$ cells per high power field with allografts exhibiting increased numbers of ED-1$^+$ macrophages compared with isograft controls (*$P \leq 0.05$) (c).

contrast, there was a striking 18-fold increase in the number of lymphatic vessels evident in the tubulointerstitium of allografts compared with isografts (9.3 ± 1.8 vs 0.53 ± 0.34 tubulointerstitial lymphatic vessels/field, allograft vs isograft, $P < 0.05$) (Fig. 6).

Although a small number of interstitial lymphatic vessels were located in the vicinity of nodular infiltrates that contained both B- and T-lymphocytes, the majority of interstitial lymphatic vessels were distinct from these cellular aggregates and located in areas of tubulointerstitial fibrosis. Indeed, there was a significant correlation between the extent of fibrosis and lymphatic vessel density (Fig. 7, $R = 0.53$, $P < 0.05$). We examined the expression of the lymphangiogenic growth factor VEGF-C by undertaking qRT-PCR from formalin fixed tissue and although this revealed a modest 1.9-fold increase in VEGF-C expression in allografts (2.1 ± 0.01 vs. 1.1 ± 0.08, allograft vs. isograft) this did not reach statistical significance. Evaluation of lymphatic endothelial cell proliferation was assessed by dual immunostaining with the proliferation marker PCNA and podoplanin, but this did not reveal any PCNA$^+$ lymphatic endothelial cells.

Discussion

The study of lymphatic endothelial biology has been greatly assisted by the advent of recently generated lymphatic specific markers and lymphangiogenesis has been reported in several conditions, such as corneal inflammation [13] and tumour metastasis [14,15]. The lymphatic system plays an integral part in immune cell trafficking, transporting antigen presenting cells into the regional lymph nodes, whereas blood vessels serve as conduits for the effector arm of the immune response.

In this study, we demonstrate *de novo* lymphangiogenesis in a rat model of renal transplantation associated with impaired renal function, proteinuria and systolic hypertension (Table 1, Fig. 6). The renal allograft exhibited features of IFTA (Figs 1 and 2) that are evident in failing human transplants as well as an inflammatory infiltrate comprising predominantly of macrophages and T cells (Figs 3 and 4). B cells were identified in scattered aggregates though the kidney suggesting that they may be forming lymphoid follicles, however, we did not immunostain for the characteristic high endothelial venules (PNAd) or plasma cells. Lymphatic vessels are normally situated in a perivascular location in normal kidneys and we identified comparable numbers of perivascular lymphatic vessels in renal allografts and isografts (Fig. 5). In contrast, many interstitial lymphatic vessels were evident in 1 year following transplantation in all of the allograft kidneys examined (Fig. 6). Double immunostaining for podoplanin and PCNA did not demonstrate active proliferation of lymphatic vessels within allografts and this finding was in keeping with the limited expression of VEGF-C at this time point. This suggests that the interstitial lymphatic vessels of renal allografts were well established and relatively quiescent. The time course of interstitial lymphangiogenesis in this model remains unclear at present as the interstitial lymphatic vessels were noted to be nonproliferative and quiescent. Further studies will need to be undertaken at earlier time points. These findings support and extend recent work by Rienstra *et al.* that involved the transplantation of kidneys from female Dark Agouti rats to male Wistar Furth rats [16]. Increased staining for the lymphatic marker LYVE-1 was noted in the renal allografts. In our study, we used male Fisher rats as donors and the combined body of

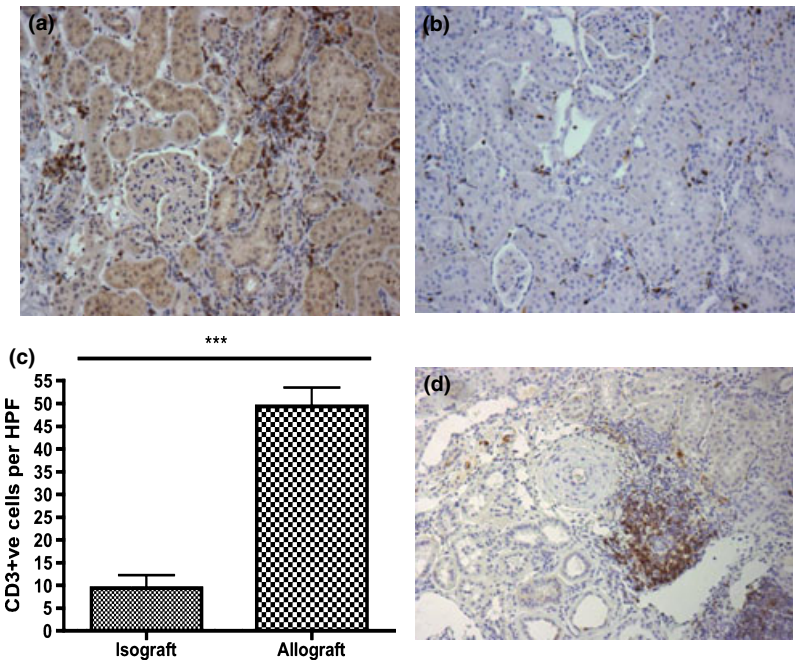

Figure 4 Rat allografts exhibit significant T cell infiltration and occasional B cell aggregates. Representative photomicrographs of CD3 immunostaining in allografts (a) and isografts (b). An infiltrate of $CD3^+$ T lymphocytes is evident in allografts. T cell infiltration was quantified by counting the number of $CD3^+$ cells per high power field with allografts exhibiting increased numbers of $CD3^+$ T cells compared with isograft controls (***$P < 0.0005$) (c). Representative image of B-lymphocyte infiltrates in allografts. Immunostaining against the B220 antigen demonstrated that $B220^+$ cells are concentrated in interstitial aggregates often associated with large blood vessels (all ×200 magnification) (d).

data indicates that lymphangiogenesis occurs in different rat strains and both genders. There are some important differences between the studies. Rienstra *et al.* [16] did not undertake formal counting of lymphatic vessels, but quantified the percentage of tissue area exhibiting LYVE-1 staining using computer image analysis. In contrast, we specifically counted podoplanin positive lymphatic vessels with a discernible lumen and classified them as either perivascular, the position of lymphatic vessels in health, or interstitial lymphatic vessels. This approach revealed the novel finding that the number of perivascular lymphatic vessels was completely unchanged in allografts and that the dramatic lymphangiogenic process evident in allografts was primarily an interstitial phenomenon. It is possible that interstitial lymphatic vessels may initially arise from perivascular lymphatic vessels, but the nature of the connections between the interstitial and perivascular lymphatic vessel networks will require further study.

There are two key aspects to the lymphangiogenesis associated with renal transplants. First, the transplantation surgery disrupts all lymphatic connections of the donor kidney and the transplanted organ requires to be 'reconnected' to the lymphatic system of the recipient. Animal studies using canine autografts demonstrated evidence of early reconnection at day 3 following transplantation reaching functional maturity at day 14 [17]. There is no available data regarding either the kinetics or nature of the process of lymphatic reconnection in rodents or humans. Second, lymphangiogenesis has been noted to accompany acute cellular rejection and chronic transplant

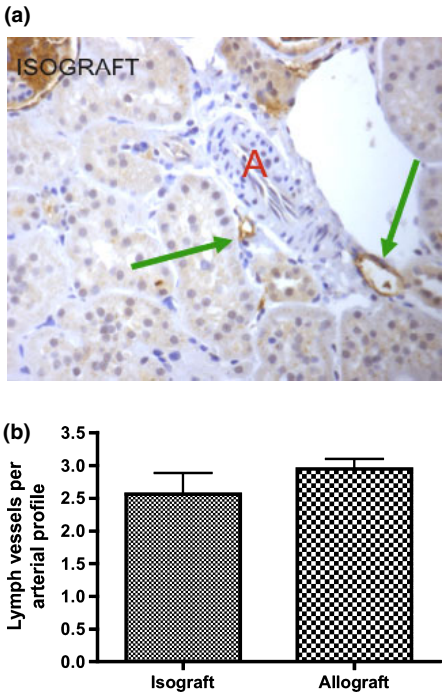

Figure 5 Rat allografts exhibit comparable numbers of perivascular lymphatic vessels. Podoplanin immunostaining reveals the normal perivascular location of lymphatic vessels in isografts (a – examples arrowed). Podoplanin is also expressed by glomerular podocytes (×200 magnification). Quantification of the number of lymphatic vessels per vascular profile (b) demonstrated no difference between allograft and isograft controls ($P > 0.05$).

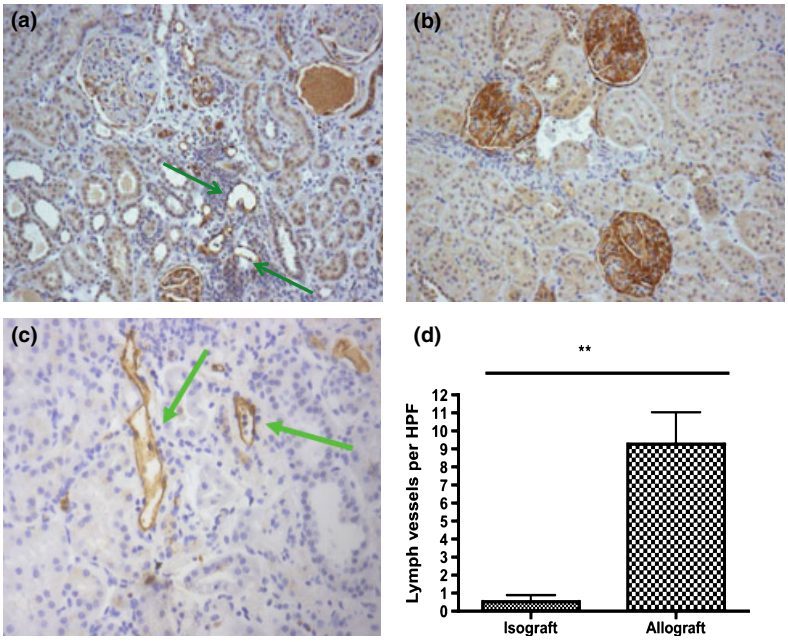

Figure 6 Rat allografts exhibit increased numbers of interstitial lymphatic vessels. Podoplanin immunostaining demonstrated the presence of interstitial lymphatic vessels in all allograft biopsies examined (a – examples arrowed) but these were rarely present in control isografts (b) (×200 magnification). High power image of interstitial lymphatic vessels (c, ×400 magnification). Quantification was undertaken by counting the number of fully formed podoplanin$^+$ lymphatic vessels per high power field (**$P < 0.005$) (d).

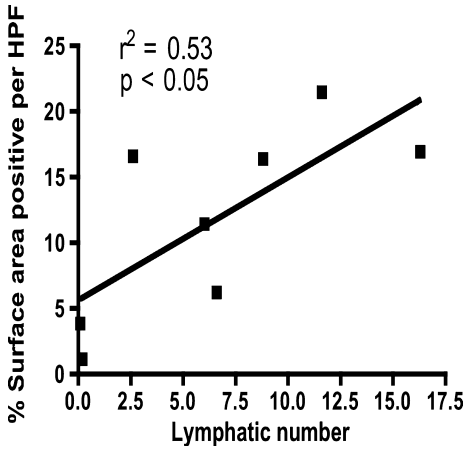

Figure 7 The numbers of interstitial lymphatic vessels correlates with renal fibrosis. The number of interstitial vessels in allografts exhibits a positive correlation with the extent of renal fibrosis quantified by picrosirius red staining.

dysfunction. Currently, there is no consensus as to whether *de novo* lymphangiogenesis in allografts is beneficial or detrimental. Previous work has inhibited the biological action of VEGF-C to block *de novo* lymphangiogenesis in experimental models [18]. This approach could be utilized in this rat model either directly after transplant surgery to inhibit the reconnection of the allograft to the systemic lymphatic system or at later time points to block *de novo* lymphangiogenesis within the kidney. The effect upon allograft structure and function could then be determined and such experiments will indicate whether the early extra-renal lymphangiogenesis or late intra-renal lymphangiogenesis is beneficial or detrimental.

Although VEGF-C promotes lymphangiogenesis, the initial stimulus that initiates VEGF-C upregulation and the formation of new lymphatic vessels in a transplanted organ currently remains unclear. Lymphatic vessels play an important role in the maintenance of tissue fluid homeostasis and it is pertinent that ligation of the draining renal lymphatic trunks of rats induced progressive renal dysfunction with associated tubulointerstitial damage [19]. Tissue oedema frequently accompanies allograft rejection and intrarenal lymphangiogenesis may reflect a physiological response to increased interstitial fluid as seen in terminal heart failure associated with chronic myocardial oedema and an increased density of lymphatic vessels [20]. Furthermore, lymphangiogenesis in the skin may be induced by an increase in interstitial tonicity with elevated expression of macrophage tonicity-responsive enhancer binding protein (TonEBP) driving VEGF-C expression [21]. It remains unknown whether a similar process may be involved in the lymphangiogenesis associated with transplantation.

Lymphangiogenesis has been described as early as 72 h after transplantation in acute allograft rejection with a >50-fold increase in the number of lymphatic vessel numbers in the context of human acutely rejecting renal allografts compared with nonrejecting controls [8] – a finding confirmed by Yamamoto *et al.* [22]. The podoplanin$^+$ lymphatic vessels extended deep into the tubulointerstitial space and some expressed the proliferation marker Ki-67, indicating active lymphangiogenesis. A

number of these lymphatic vessels were associated with nodular infiltrates of alloreactive T and B-lymphocytes and it has been suggested that they may represent the development of organized cellular infiltrates resulting in the formation of ectopic 'germinal centres' within the rejecting kidney [23].

A key question is whether the new interstitial lymphatic vessels are derived from cells of the donor or recipient. In this study, we were unable to distinguish between Lewis and Fisher cells. However, the origin of lymphatic endothelial cells was studied in human male recipients who developed lymphangiogenesis and rejection in renal transplants derived from a female donor. In situ hybridization for the Y chromosome identified a recipient origin and suggested the involvement of recipient-derived lymphatic progenitor cells in the lymphangiogenic process [24].

Lymphangiogenesis may also occur in nonrenal transplants as increased expression of the PROX-1 lymphatic endothelial marker has been demonstrated in lung biopsies undergoing acute allograft rejection [25]. Corneal transplantation is the most commonly performed soft tissue transplant and, although a site of immunological privilege, neovascularization is associated with increased graft rejection and associated with lymphangiogenesis in humans [26]. Corneal transplantation has been used experimentally to study lymphangiogenesis and VEGF-TRAP mediated blockade of lymphangiogenesis and haemangiogenesis improves corneal graft survival suggesting an inhibition of detrimental immune responses [27]. No such studies have been conducted in solid organ transplantation.

It is important to note that intrarenal lymphangiogenesis may occur in experimental and human chronic kidney disease including the rat remnant kidney model [28] and human diabetic nephropathy [29] and IgA nephropathy [30] suggesting that it is also a response to chronic injury and scarring. Thus, the development of interstitial lymphangiogenesis is not restricted to immunological disease and may represent a biological response to diverse injurious stimuli. It is thus of interest that we noted a significant correlation between the number of lymphatic vessels and the severity of tubulointerstitial scarring in our study (Fig. 7) and this is in accord with the work of Rienstra *et al* [16].

In this present study, we have described a rodent model of renal allograft IFTA that exhibits significant interstitial lymphangiogenesis and mirrors our previous findings in humans. It is currently unclear whether the formation of new lymphatic vessels is inherently detrimental to the renal allograft and future studies involving the blockade of VEGF-C are required to determine if graft function and survival may be improved by inhibition of interstitial lymphangiogenesis.

Authorship

DGV: performed research/study, wrote manuscript. BS: performed research/study. JH: designed and performed research/study. JH and LM: designed research/study, wrote manuscript.

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