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Lymphangiogenesis in renal inflammation and transplantation

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A thesis submitted for the degree of Doctor of Philosophy

The University of Edinburgh

2012
DECLARATION

I declare that the work presented in this thesis is my own and has not previously been published or presented towards another higher degree except where clearly acknowledged in the text.

The experimental design of the work presented in this thesis was that of the author and his supervisors Dr Jeremy Hughes and Miss Lorna Marson. All experimental work was performed by myself with the exception of flow cytometric cell sorting which was carried out by Mrs Fiona Rossi. Mr Badri Shrestha and Dr John Haylor performed the rodent renal transplants and assays to measure renal function. Finally the murine renal transplant model was developed by Miss Lorna Marson and performed by Dr Feng Qi.

................................................                                        ................................................

David George Vass                                               Dated
ABSTRACT

The lymphatic system plays an important role in both tissue homeostasis and inflammation. During the surgical procedure there is complete disruption of lymphatic drainage of the allograft kidney. The time course and nature of lymphatic reconnection following transplantation is poorly understood. In addition to the extra-renal lymphangiogenesis required for lymphatic reconnection, some patients may develop de novo lymphatic vessels within the renal parenchyma during acute rejection or chronic allograft damage. This work sought to examine the time course and mechanism of lymphangiogenesis and the role of macrophages in this process.

Injection of carbon black and Evan's blue into the rat kidney resulted in rapid transit to the draining hilar renal lymph node. Surgical disruption of the lymphatic drainage of the kidney prevented trafficking of carbon black to the renal lymph node at 24 hours. At day 6 there was macroscopic and microscopic evidence of carbon black localisation in the renal lymph node suggesting functional reconnection. Careful histological analysis of hilar renal tissue indicated that the large lymphatic trunks were replaced by a network of small proliferating lymphatic vessels.

Assessment of intra-renal lymphangiogenesis was undertaken in 2 distinct experimental models of renal transplantation. In a murine model of acute allograft rejection there was no evidence of increased lymphatic vessel number at day 7. In a collaboration with Sheffield University, tissue from a rat model of interstitial fibrosis and tubular atrophy was examined. The rat tissue exhibited a prominent macrophage and T-cell infiltration at 12 months but there was no difference in the number of perivascular lymphatic vessels. In contrast, there were numerous lymphatic vessels evident in the interstitium that were absent in control isograft tissue. Interestingly, the number of lymphatic vessels correlated with the extent of fibrosis. Analysis of vascular endothelial cell growth factor-C (VEGF-C) mRNA expression did not show any increase in allografts.
The model of unilateral ureteric obstruction (UOO) was employed as a model of rapidly progressive inflammatory fibrosis. UOO was associated with rapid and prominent interstitial lymphangiogenesis. This was associated with a marked increase in macrophage and T-lymphocyte infiltration and increased whole kidney mRNA expression of VEGF-C. The role of macrophages in lymphangiogenesis was explored by administration of macrophage depleting liposomal clodronate. No effect upon lymphangiogenesis was found but liposomal clodronate failed to deplete ED-1 positive macrophages in the kidney. A macrophage isolation strategy was thus employed using the myeloid CD11b marker cells and flow cytometric cell sorting and immunomagnetic bead sorting. Although gene expression studies demonstrated increased ED1 mRNA expression by CD11b enriched cells, no difference in VEGF-C mRNA expression between CD11b cells obtained from obstructed kidneys versus cells from sham controls was evident. Lastly, despite extensive efforts, immunostaining for VEGF-C was unsuccessful.

In summary, lymphangiogenesis can reconstitute the lymphatic drainage of the kidney and is prominent in both chronic allograft injury and the acutely obstructed kidney in the rat. Although VEGF-C is the likely driver of lymphangiogenesis direct evidence of macrophage VEGF-C production was not found.
Acknowledgements

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Much of the in vivo work presented within this thesis would not have been possible without the practical skills and knowledge from the irrepressible Mr Spike Clay. I am also grateful to Mr William Munnigal whose skills in animal husbandry supported much of this work. Mr Bob Morris, Mrs Susan Harvey and Miss Melanie McMillian provided an excellent service in processing histological specimens.

I would like to acknowledge Mrs Ali Zhang for her guidance in cell culture and many of the molecular biology techniques presented in this work. Finally I would like to thank all members of the PIG lab past and present who no doubt contributed in some way to this thesis.
DEDICATION

This thesis is dedicated to my parents and friends at the University of Edinburgh.
PRESENTATIONS AND PUBLICATIONS

Presentations

Oral presentations were made at the following conferences:

- UK Renal Association 2010, Manchester
- European Society of Organ Transplantation 2009, Paris
- British Transplantation Society 2009, Liverpool
- Royal College of Surgeons, School of Surgery, Edinburgh 2008
- Scottish Society of Experimental Medicine, Edinburgh 2008

Poster presentations were made at the following conferences:

- UK Renal Association 2010, Manchester
- British Transplantation Society 2010, London

Publications arising directly from the work


Related work

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Chapter 1  Introduction

This thesis will examine the mechanism and time course of lymphatic regeneration in the rodent kidney and the role of macrophages in rejection associated and inflammatory lymphangiogenesis.

1.1. Historical background

Physicians have observed components of the lymphatic system since ancient times. Hippocrates (460-377 B.C) described “white blood in nodes” and used for the first time the expression ‘chyle’ in one of history’s earliest known medical texts, On the Glands (Adams 1849). Some of the earliest insights in lymphatic vessels emanated from Herophilus (335-280 B.C.) who noted the existence of lymph nodes as “milky veins”, later writing “for nature has made, in the whole of the mesentery, peculiar veins, destined for the nourishment of the intestines….these veins terminate in certain glandular bodies, whilst all the rest are carried upwards to the portae” (Dobson 1925; Dobson 1927).

During the European renaissance, the first official differentiation between veins and lymphatic vessels was made by the Italian surgeon Gasparo Asselli (1581-1626) in 1622 when he observed the “venae albae et lactae” of a well fed dog and concluded that chyle was absorbed from the intestines and transported to the liver, which since the time of Galen of Pergamon (131-201AD), had been regarded as the blood forming organ that transformed nutrients into blood (Chikly 1997). His single manuscript was published posthumously in 1627 (Aselli 1627), one year before the historic publication of
William Harvey’s (1578-1657) “De Motu Cordis” which described the vascular circulation (Harvey 1628).

In 1652 Bartholin (1616-1680) published a description of the thoracic duct in man (which transports lymphatic fluid into the systemic circulation). Further experiments were performed in which he ligated lymphatics near the liver, which resulted in proximal filling of the vessels with a clear watery fluid, thus demonstrating that lymph flowed from and not to the liver. He also noted other vessels distinct from the lacteals which carried fluid across the intestines correctly identifying a separate system of vessels. In 1653 he published his book “Vasa lymphatica”, where he named the vessels he discovered as lymphatics (Bartholin 1653). Olfo Rudbeck (1630-1702) was probably the first anatomist to recognise the lymphatic system as part of the circulation. Using the physiological techniques of Harvey, he described that the lymphatic network is a system of closed vessels similar to arteries and vein, with lymph flowing away from tissues and that lymph eventually returned to the circulation through the thoracic duct. Shortly afterwards he published his work “De circulatione sanguinis”, writing “the glands of the body draw out from the body a serous liquid like residue …..glands situated near the crural veins, the sides of the heart, the oesophagus, and the mammary veins, carry a water liquid to the chylous ducts by virtue of certain vessels” (Rudbeck 1653). His work coincided with that of Bartholin, and when he discovered a copy of “Vasa lymphatica “ in a bookshop in Hamburg, Germany Rudbeck immediately accused Bartholin of plagiarism forming the basis of a bitter feud, which continued for the remainder of their respective careers! (Leeds 1977)
In the 18th century there was growing understanding of fundamental lymphatic vessel function and physiology that was principally initiated by William Hunter (1718-1783), with his brother John Hunter (1728-1793) and students Hewson (1739-1774) and Cruikshank (1745-1800). The brothers worked with a variety of techniques including mercury injections to trace the path of lymphatic vessels (Highley 1859; Loukas, Bellary et al. 2011). William Hunter had an aversion to publishing and his contribution to the lymphatic system was brought to public attention by the publications of his brother and former students. Hewson published in 1768 on the lymphatics of reptiles and fish and maintained that Hunter's discoveries took place “many years prior” to his own work in amphibians and fish (Owen 1846). Cruikshank published *The Anatomy of the Absorbing Vessels* in 1786 which was a collection of the illustrations of the lymphatic network performed up until that point and included a mercury injection tracing of the lymphatic drainage of the breast (Uren, Howman-Giles et al. 2003). In the year following Cruikshank’s publication, Paolo Mascagni (1755-1815), an Italian Professor of Anatomy produced a comprehensive and stunning atlas of human lymphatic vessels with 41 plates of the human body and delineated the wide extent of the lymphatic system (Mascagni 1787) leading to the physiological investigations of the 19th and early 20th centuries.

Until the mid 19th century the question of how lymph was formed remained unanswered. The German physician Ludwig (1816-1895) first began to perform physiological experiments with the lymphatic system. In 1850 he published his theory that lymph is formed by diffusion of fluids through the
vessel walls into the surrounding tissue driven by capillary blood pressure (Noll 1850). This work laid the foundation for one of the most famous names in the history of lymphatics, Ernest Starling (1866–1927). Starling demonstrated through a series of classic experiments that it was the balance between the hydrostatic and oncotic pressures between capillaries and tissues that allowed lymph to form. He was able to document predictable changes in interstitial flow based on changes in experimental conditions (Starling 1896; Bayliss and Starling 1902). Cecil Drinker (1887–1956), Professor of Physiology at Harvard Medical School was able to fully prove that significant changes in protein concentrations in either blood or tissue could lead to changes in lymph formation (Warren 1942).

In the 21st century there has been a renaissance in the study of lymphatic cell biology due to the advent of lymphatic endothelial cell specific markers which for the first time permit differentiation from blood vascular endothelial cell populations. This has shed light on their molecular characteristics leading to an exponential increase in our understanding of their role in disease processes including inflammation and tumour metastases.

1.2 Organisation and function of the lymphatic system

The lymphatic system plays an integral role in regulating tissue fluid balance and serves as a major transport route for immune cells and interstitial macromolecules. As blood travels along the branching arteries to capillaries there is efflux of plasma fluid and proteins. Much of this is reabsorbed by post-
capillary venules; however, due to osmotic forces caused by protein extravasation there is a small net fluid flux from the circulation. This fluid bathes the interstitium and is absorbed by the lymphatics in tissues around the body (Aukland and Reed 1993), it is eventually returned to the circulation via the thoracic duct. In healthy adult individuals, the lymphatic system returns approximately 1-2 litres of interstitial fluid with 20-30g of protein per litre to the venous circulation every day (Tammela and Alitalo 2010)

1.2.1 Initial lymphatic capillaries

The initial lymphatics (or lymphatic capillaries) are blind-ending structures with wide lumina and thin walls and therefore more closely resemble the sinusoidal vessels of the liver and spleen rather than the capillaries of the circulation. These capillaries possess many inter-endothelial junctions which behave like one-way valves (Alitalo, Tammela et al. 2005; Tammela and Alitalo 2010). Because of the high permeability of the initial lymphatics there is little exclusion of interstitial molecules. The protein composition of lymph is nearly identical to that of interstitial fluid and their oncotic pressures are essentially equal, but less than that of plasma. In contrast to blood vessels, lymphatic vessels have a discontinuous or fenestrated basement membrane and lack tight interendothelial junctions and therefore are permeable to interstitial fluid (Swartz 2001; Alitalo, Tammela et al. 2005). Lymphatic vessels range in size between 10-60 µm and are anchored by filaments that tether the initial lymphatic to the surrounding connective tissue (Swartz 2001). Their fibres attach the basal lamina of the extracellular matrix (ECM) and are composed of
a structure similar to that of elastin. These are highly sensitive to interstitial stresses exerting radial tension on lymphatic capillaries and deforming endothelial cells so microvalves open drawing fluid inwards in response to increase in interstitial volume (Randolph, Angeli et al. 2005). As the vessel fills overlapping cell-cell junctions close and intraluminal pressure returns to baseline. These events create a ‘tissue pump’ by which small interstitial stresses draw fluid into the lymphatic vessels but prevent leakage back into the interstitium (Ikomi and Schmid-Schonbein 1996). This cell-cell junction overlap has been referred to as the primary valve system of the lymphatic vessels (Figure 1.1).

1.2.2 Collecting lymphatic vessels

Lymph from the initial lymphatic capillary drains into the collecting lymphatic vessels. In contrast to the capillaries these vessels are not tethered to the ECM and contain smooth muscle so they have intrinsic pumping activity (Aukland and Reed 1993). They also contain valves preventing retrograde flow of lymph. Smooth muscle exhibits spontaneous contractions, but other factors also impact upon the contractile force including skeletal muscle compression (Mortimer, Simmonds et al. 1990), respiration (Negrini, Ballard et al. 1994) and increased blood flow and pressure (Parsons and McMaster 1938). The segment of collecting lymphatic area between each valve is termed a lymphangion.
1.2.3 Lymph node arrangement

All collecting lymphatic vessels pass through lymph nodes. There are hundreds of lymph nodes in the adult body and they vary in size between 1 to 10mm in diameter. Each node is invested with a fibrocollagenous capsule from which fibrous trabeculae extend into the node to form a supporting network. The collecting lymphatic vessels can be further divided into afferent (prenodal) or efferent (postnodal) vessels. The convex surface of the lymph node is penetrated by a number of afferent lymphatic vessels, while at the hilum there is an efferent vessel which transports fluid to larger collecting lymphatic structures. Afferent lymphatics drain into a major subcapsular sinus around the periphery of the node with extensions into the cortical sinuses and the medulla (Woolf 1998). Within the medulla is a network of interconnected channels called medullary sinuses which merge to form the efferent lymphatic vessel at the hilum. Naive lymphocytes migrate from the blood vasculature through specialised lymph node postcapillary venules lined by distinctive endothelial cells (high endothelial venules [HEVs]) to enter the paracortical regions of these organs.

The superficial cortex of the lymph node contains B cells aggregated into primary follicles (MacLennan 1994). Following stimulation by antigen these become secondary follicles and develop foci of active proliferation termed germinal centres. Cells in the follicles are in intimate contact with dendritic cells (DCs).
DCs are highly specialised, with the capacity to acquire and present antigen to naive T cells and they exhibit high expression of co-stimulatory molecules. They are the only class of antigen presenting cells that can activate naive T cells, stimulate clonal expansion and initiate the immune response. Immature DCs reside mainly within the epithelial and connective tissue of organs (Randolph, Angeli et al. 2005). Once they have captured antigen they traffic to the lymph nodes and present antigen to CD4 T cells (Figure 1.2). This is a highly regulated process dependent on chemokines and conformational change in DC shape.

1.3 Lymphatic vessel development in health

1.3.1 Topography of lymphangiogenesis in the embryo

In the embryo the blood vascular system develops from the mesodermal precursor cells called angioblasts, which invade the organ primordial and ensemble in-situ to form the capillary plexus (Wigle and Oliver 1999). This is termed vasculogenesis. It is followed by sprouting of the primary capillary plexus and remolds into a mature vascular network, a process termed angiogenesis. The lymphatic vessels in humans develop at 6-7 weeks of gestation (Wigle and Oliver 1999; Oliver 2004). Studies on mammalian
embryos have shown that there are eight lymph sacs; three paired and two unpaired (Sabin 1902; Sabin 1904). Lymph sacs are venous derivatives that grow by sprouting into all parts of the body, except for the central nervous system and the bone marrow, which remain free of lymphatics. At the junction of the subclavian and anterior cardinal veins, 2 jugular lymph sacs develop by endothelial cell budding from the anterior cardinal veins. Later in development the remaining lymph sacs originate from the mesonephric veins and veins in the dorsomedial edge of the Wolffian bodies. In particular the retroperitoneal lymph sac forms near the primitive inferior vena cava and the posterior lymph sacs originate near the iliac vein (Gray 1985). Further validation of this view has been obtained through studies using dynamic imaging in the embryos of zebra fish (Hogan, Bos et al. 2009).

1.3.2 Molecular pathways of lymphangiogenesis

The specific regulatory gene pathways which govern lymphatic vessel differentiation \textit{in utero} are poorly understood. The application of transgenic models has identified that the nuclear transcription factor Prospero-related homobox-1 (Prox-1) has an important role in lymphangiogenesis. Prox-1 is first detected at embryonic day (E) 10.5 in a polarised manner in a subset of mouse endothelial cells in the cardinal vein (Wigle and Oliver 1999). Prox-1$^+$ endothelial cells normally bud from veins giving rise to primitive lymph sacs, initially in the jugular and mesonephric regions. Overexpression of Prox-1 suppresses the expression of several genes specific for blood vascular endothelial cells. The committed endothelial cells migrate along a VEGF-C
gradient directed by mesenchymal cells to form primitive lymph sacs. Prox-1 knockout (−/−) mice fail to promote budding of lymphatic vessels from the anterior cardinal veins (Wigle and Oliver 1999). As a consequence the lymph sacs and rest of the lymphatic system fail to develop. This phenotype is embryonic lethal at E14.5 though the embryo exhibits normal blood vascular development suggesting that Prox-1 activity is required for the maintenance of the venous endothelial cells and differentiation towards lymphatic phenotype (Wigle, Harvey et al. 2002). Mice that are heterozygous for Prox-1 possess a discontinuous lymphatic endothelium and exhibit abnormal fluid and lipid accumulation in the interstitium and die shortly after birth (Wigle and Oliver 1999). The precise mechanism by which Prox-1 induces lymphatic vessel sprouting is yet to be established. In murine embryos scattered mesenchymal cells which co-express CD45 and LYVE-1 were detected in the regions of lymphatic vessel growth suggesting that CD45+ leukocytes may integrate into lymphatic vessels (Buttler, Kreysing et al. 2006; Buttler, Ezaki et al. 2008).

Vascular endothelial cell growth factor (VEGF-C) is a key lymphangiogenic mitogen. VEGF-C −/− embryos fail to develop lymphatic vessels and primitive lymph sacs and do not survive beyond E15.5. Prox-1+ cells in this genotype do not sprout and remain in association with the cardinal vein later disappearing, presumably by apoptosis (Karkkainen, Haiko et al. 2004; Kuchler, Gjini et al. 2006; Yaniv, Isogai et al. 2006). It is noteworthy that Vascular Endothelial Growth Factor Receptor 3 (VEGFR-3) the receptor for VEGF-C is expressed by endothelial cells in the murine cardinal vein and dorsal aorta. At embryonic day E12.5 VEGFR-3 is expressed in developing venous and lymphatic
endothelium but this is largely restricted to lymphatic vessels in adult life (Tammela and Alitalo 2010). Unfortunately transgenic mice have limited value in the study of VEGFR-3 function as VEGFR-3 gene targeted mice die at E10.5 due to abnormal remodeling of the vascular plexus leading to cardiovascular system failure (Dumont, Jussila et al. 1998; Alitalo, Tammela et al. 2005). Further work is required to delineate the pattern of lymphatic development in vivo.

1.4 Characteristics of lymphatic endothelial cells

1.4.1 Differential molecular profiles of lymphatic and vascular endothelium

Blood endothelial cells (BEC) and lymphatic endothelial cells (LEC) differ by morphology and protein expression patterns. At protein level, a growing number of discriminative molecules have been identified. BECs express the endothelial antigen Pathologische anatomie leidan endothelium (PAL-E) (Schlingemann, Dingjan et al. 1985; Kriehuber, Breiteneder-Geleff et al. 2001), neural-cadherin (N-Cadherin) (Petrova, Makinen et al. 2002; Podgrabinska, Braun et al. 2002; Yaniv, Isogai et al. 2006) and C-X-C chemokine receptor (CXCR-4) (Petrova, Makinen et al. 2002; Hirakawa, Hong et al. 2003). LECs express Prox-1 (Wigle, Harvey et al. 2002), lymphatic vessel endothelial receptor (LYVE-1) (Banerji, Ni et al. 1999), podoplanin (Breiteneder-Geleff, Soleiman et al. 1999; Kriehuber, Breiteneder-Geleff et al. 2001) and VEGFR-3 (Kaipainen, Korhonen et al. 1995). Other markers of endothelial origin include
CD31, CD34 and factor-VIII which are expressed on both BEC and LEC populations (Erhard, Rietveld et al. 1996; Sauter, Foedinger et al. 1998). The advent of lymphatic specific markers has led to a recent renaissance in the study of lymphatic cell biology both in vivo and in vitro and are further described below.

*Podoplanin*

Podoplanin is a 38 KDa membrane mucoprotein and is one of the most highly expressed genes of lymphatic endothelium (Breiteneder-Geleff, Soleiman et al. 1999). It was originally found on the surface of rat glomerular epithelial cells (Rishi, Joyce-Brady et al. 1995) but also found on a variety of normal cells including mesothelia, osteocytes, neuronal cells and type 1 alveolar cells. Studies on podoplanin -/- mice have uncovered many of the regulatory functions of this molecule. This phenotype is lethal immediately after birth on account of respiratory failure due to impaired formation of alveolar air spaces and reduction in the number of differentiated type 1 alveolar cells (Ramirez, Millien et al. 2003). Neonates also show cutaneous lymphoedema and impaired lymphatic transport (Schacht, Ramirez et al. 2003). Podoplanin is predominantly expressed on the basolateral surface, where it forms a complex with CCL21 and it is shed into the stroma, establishing a perilymphovascular CCL21 gradient promoting migration of inflammatory cells (Kerjaschki 2006).
LYVE-1
LYVE-1 is a highly glycosylated member of the LinK superfamily of Hyaluronic Acid (HA) binding receptors and is located on the surface of lymphatic endothelium. (Banerji, Ni et al. 1999) There is considerable homology in the amino acid sequence with the inflammatory homing receptor CD44, a molecule which binds HA on blood vascular endothelium (Johnson and Jackson 2008). This led to speculation that LYVE-1 may support adhesion of CD44 positive leukocytes, however, in vivo work in mice lacking the LYVE-1 gene display normal trafficking of dendritic cells from skin to lymph nodes (Gale, Prevo et al. 2007).

Prox-1
Prox-1 is a nuclear transcription factor which regulates lymphangiogenesis. (Wigle and Oliver 1999) The homeobox gene was originally cloned by homology to Drosophila melanogaster Prospero. As described earlier this is expressed by a subpopulation of endothelial cells that by budding and sprouting give rise to lymphatic cells in utero. In Prox-1 null mice, this process is arrested although vasculogenesis is unaffected (Wigle, Harvey et al. 2002).

VEGFR-3
Vascular Endothelial Cell Growth Factor–C (VEGF-C) promotes lymphangiogenesis through its interactions with the tyrosine kinase receptor VEGFR-3 or Flt3. (Machnik, Neuhofer et al. 2009) Although implicated in lymphatic cell biology it has an important role in formation of the primary cardiovascular network as VEGFR-3 knockout embryos die early in utero as a
consequence of cardiovascular failure (Dumont, Jussila et al. 1998; Alitalo, Tammela et al. 2005).

1.4.2 *In vitro generation of LECs*

Primary culture of human dermal microvascular cells identifies two distinct populations of endothelial cell (BECs and LECs). In the past several attempts have been made to obtain LECs by isolating pure populations from lymphatic rich tumors. Due to the lack of specific markers it was not possible to define the histogenic origin of the cells and the lymphatic cells from the tumour were not stable in culture, limiting the value of their study. Using the above characterised markers, Kriehuber et al used a triple fluorescence FACS strategy to obtain immunopurified populations of LECs from human dermis (Kriehuber, Breiteneder-Geleff et al. 2001). Pure LECs were defined by podoplanin$^+$ CD34$^+$ and CD45$^-$. BECs expressed CD34 but were devoid of podoplanin and CD45 expression.

In culture the expression of LYVE-1 and podoplanin persisted for 7 days which was not induced in BECs indicating a degree of phenotypic stability. During culture of ECs, podoplanin$^+$ LECs are surrounded by BECs and when placed in matrigel both cell types form homotypic tubes which are wrapped around each other, consistent with their intimate association *in vivo* (Kriehuber, Breiteneder-Geleff et al. 2001). In cultures of microvascular endothelial cells LECs proliferate alongside BECs, however isolated LEC culture requires substitution with a fibronectin matrix and VEGF to induce growth. This may be
explained by the observation that VEGF-C mRNA and protein are produced by BECs and not LECs. Stimulation with VEGF-C in vitro initiated proliferation of LECs but did not protect BECs from apoptosis.

1.4.3 Molecular pathways of angiogenesis

Blood vessels in the adult are normally quiescent and require stimulation to proliferate with angiogenic growth factor signals. At a molecular level there is equilibrium between pro-angiogenic and anti-angiogenic molecules. For the initiation of angiogenesis this balance must skew towards increased pro-angiogenic growth factors (Hanahan and Folkman 1996; Carmeliet and Jain 2000). This is largely through the vascular endothelial cell growth factor family which started with the discovery of VEGF in 1989 (Keck, Hauser et al. 1989; Leung, Cachianes et al. 1989). Since then other vascular growth factors (VEGF-A, -B, -C, -D and PGF), the effects of which are mediated by three tyrosine kinase receptors VEGFR-1/Flt-1, VEGFR-2/Flt-2 and VEGFR-3/Ft-3 have been characterised (Carmeliet 2000; Dvorak 2000; Karkkainen and Petrova 2000; Carmeliet, Moons et al. 2001; Ferrara 2001). VEGF-A interacts with VEGFR-1 and VEGFR-2 (VEGFR-2 also signals with VEGF-E) and mediates cell proliferation, migration and angiogenesis of BECs.

Recent studies have revealed that VEGF-A can also support lymphangiogenesis through interaction with VEGFR-2 (Nagy, Vasile et al. 2002; Hong, Lange-Asschenfeldt et al. 2004; Kunstfeld, Hirakawa et al. 2004). VEGF-A predominantly binds to VEGFR-1 on BECs but VEGFR-2 is also
expressed on LECs (Kriehuber, Breiteneder-Geleff et al. 2001; Hirakawa, Hong et al. 2003). VEGF-A potently induces proliferation and migration of lymphatic cells in vitro and overexpression of VEGF-A in vivo induces lymphangiogenesis in tissue repair and inflammation (Hong, Lange-Asschenfeldt et al. 2004; Kunstfeld, Hirakawa et al. 2004)

VEGFR-3 however, is involved mainly in lymphangiogenesis, often being co-expressed with VEGF-C at sites where lymphatic vessels sprout and in disease (Kukk, Lymboussaki et al. 1996; Dumont, Jussila et al. 1998). VEGF-C and VEGF-D are secreted as homodimers which undergo extensive proteolytic processing of their NH₂ and COOH carboxy terminal domains following secretion (Kirkin, Mazitschek et al. 2001). Processing of these growth factors modulates their biological effects. The secreted 31 KDa form of VEGF-C/D predominantly activates VEGFR-3 while the mature fully processed 21KDa form activates both VEGFR-2 and -3, (Dixelius, Makinen et al. 2003) forming heterodimers which may lead to unique combinatorial signals by the intracellular domains of both the receptors. Thus both lymphangiogenesis and angiogenesis can be coordinated from a single molecule. VEGF-A, platelet-derived growth factor (PDGF) and VEGF-B are formed through alternative splicing. Stimulation of VEGFR-3 with its specific ligand induces rapid tyrosine phosphorylation of Shc and activation of mitogen activated protein kinase (MAPK) pathways resulting in increased cell motility, actin reorganisation and proliferation (Al-Rawi, Mansel et al. 2005). Recent work has shown that VEGFR-3 is a strong activator of Stat3 and Stat5 proteins (Korpelainen, Karkkainen et al. 1999).
Sprouting of new lymphatics from pre-existing lymphatic vessels appears to be a critical mechanism for the formation of new lymphatic vessels in the adult (He, Rajantie et al. 2005). In adults, the expression of VEGFR-3 becomes confined to the lymphatic endothelium, as well as to activated macrophages and dendritic cells (Schoppmann, Birner et al. 2002; Chen, Hamrah et al. 2004).

There is evidence that circulating bone marrow endothelial progenitor cells (BMEPCs) participate in lymphangiogenesis. Early in vitro work described a CD133/CD34 population of endothelial cell progenitors, which had the ability to differentiate into both lymphatic and endothelial cell populations (Religa, Cao et al. 2005). The in vivo implications of this will be discussed later.

1.4.4 Biological effects of VEGF-C

VEGF-C has been shown to induce lymphatic vessel sprouting in the chorioallantoic membrane (Oh, Jeltsch et al. 1997) and when overexpressed in transgenic mice lymphatic vessel hyperplasia was observed in the skin and increased lymphatic vessel density was observed in the ear (Jeltsch, Kaipainen et al. 1997). Increased lymphatic vessel density was also seen following adenoviral overexpression in athymic mice. One of the earliest inhibition studies was performed in transgenic mice expressing soluble VEGF receptor 3. This chimeric protein consisted of the ligand binding portion of this extracellular part of VEGFR-3 and was expressed under the control of the
human keratin promoter (Makinen, Jussila et al. 2001). These transgenic mice developed a phenotype characterised by oedema of the feet and dermal fibrosis.

VEGF-C gene therapy has been reported to promote new lymph vessel formation and reduce oedema in mouse tail skin. This is of clinical interest as these may present novel therapies for lymphoedema. Lymphoedema is a pathology of tissue and fluid balance which is characterized by a chronic accumulation of protein rich fluid in the tissues which leads to swelling. Primary oedema is due to developmental abnormalities (Milroy’s syndrome) and secondary oedema is dysfunction following surgery and radiotherapy (Radhakrishnan and Rockson 2008). Genetic studies in patients with Milroy’s syndrome show some patients are heterozygous for missense mutations of the VEGFR-3 receptor (Irrthum, Karkkainen et al. 2000; Karkkainen, Ferrell et al. 2000). As a therapeutic advance, Tammela and colleagues showed that lymphatic capillaries induced by adenoviral VEGF-C or VEGF-D expression mature into collecting vessels in a mouse model of axillary lymph node dissection (Tammela, Saaristo et al. 2007).

1.5 Inflammatory lymphangiogenesis

During acute tissue injury there is a cascade of events, which begins with recruitment of inflammatory cells in response to the milieu of chemokines and cytokines. The lymphatic vascular system and the molecular pathways regulating inflammatory responses are intimately associated and LECs in
some tissues express nuclear factor-kappaB (NF-κB) (Saban, Memet et al. 2004). Activation of NF-κB in LECs upregulates VEGFR-3 and Prox-1 expression which renders the lymphatic vessels more sensitive to VEGF-C and VEGF-D produced by leukocytes.

1.5.1 Macrophage mediated lymphangiogenesis.

With inflammation monocytes very rapidly enter the tissues, differentiate into macrophages, phagocytose debris and produce VEGF-A and VEGF-C which contribute to new blood and lymph vessel formation. Macrophages have been seen to contribute to the formation of new lymph vessels through the expression of VEGF-C which has been detected at the mRNA and protein level in a number of experimental models in kidney, skin and eye (Alitalo, Tammela et al. 2005; Baluk, Tammela et al. 2005; Baluk, Yao et al. 2009). Moreover, it has recently been shown that a subfraction of circulating VEGFR3+CD14+ monocytes also strongly express VEGF-C and VEGF-D upon recruitment to peritumoral sites or with in vitro incubation of tumour necrosis factor-alpha (TNF-α) and lipopolysaccharide (Schoppmann, Birner et al. 2002). In both human studies and in vivo models VEGF-C+ macrophages colocalise with new peritumoral lymph vessels, strongly suggesting a role for these cells in lymphangiogenesis. It is known that proinflammatory cytokines upregulate VEGF-C and recent data has identified a role for 1L-1β in initiating neovascularisation in corneal inflammation. Subsequent inhibition of the downstream signaling pathway NF-κB abrogated new lymph vessel development in this model (Watari, Nakao et al. 2008).
Further evidence for a role of macrophages in lymphangiogenesis comes from systemic depletion studies with liposomal clodronate. Clodronate is a bisphosphonate commonly used for metabolic bone disease. When encapsulated with liposomes it is actively phagocytosed by macrophages inducing subsequent apoptosis. Administration of liposomal clodronate inhibited de novo lymphangiogenesis in several studies including corneal inflammation (Maruyama, Li et al. 2005; Watari, Nakao et al. 2008) and diabetic wound healing (Maruyama, Asai et al. 2007).

1.5.2 Macrophages in non-inflammatory lymphangiogenesis

In a series of elegant experiments Machnick et al demonstrated increased density and hyperplasia of the lymphatic capillary network in skin from rats fed a high salt diet to induce hypertension. The mechanisms underlying these effects on lymphatics involve activation of tonicity-responsive enhancer binding protein (TonEBP) in mononuclear phagocytes present within the interstitium of the skin (Machnik, Neuhofer et al. 2009). TonEBP binds the promoter of the gene encoding VEGF-C and causes VEGF-C secretion by macrophages. Administration of clodronate prevented the formation of de novo lymphatics in rats fed a high salt diet.
1.5.3 Bone marrow derived cells and macrophage transdifferentiation.

In addition to local production of VEGF-C by infiltrating macrophages there is evidence that bone marrow derived cells may have a more direct role in lymphangiogenesis. Religa et al demonstrated in mice with enhanced green fluorescent green protein (EGFP) labeled bone marrow that CD34^+/VEGFR-3 and CD34^+/VEGFR-2 cells were incorporated into newly formed lymph vessels in a model of corneal inflammation (Religa, Cao et al. 2005). Depletion of bone marrow cells by irradiation remarkably suppressed lymphangiogenesis. Further, transplantation of isolated EGFP^+/VEGFR-3^+ or EGFP^+/VEGFR-2^+ resulted in incorporation of EGFP^+ cells into newly formed lymphatic vessels. Maruyama reported on a subpopulation of CD11b^+ macrophages which transdifferentiated into lymphatic endothelial cells, initially forming cellular aggregates and then integrating into sprouting lymphatic vessels. (Maruyama, Li et al. 2005) This study was reinforced by the finding that activated murine peritoneal CD11b^+ macrophages formed tube-like structures and expressed lymphatic endothelial cell–specific markers in vitro (Figure 1.3).

1.6 Lymphatic vessels in the kidney

The distribution of the normal renal lymphatic system has been investigated in various animals using the injection of various dyes, electron microscopy and microradiography. Lymph leaves the kidney by two routes – a superficial capsular network and a deeper hilar system. Connections between the two have been described (Bell, Keyl et al. 1968; Cockett, Roberts et al. 1970;
Holmes, O'Morchoe et al. 1977). The finer lymphatics which form the initial tributaries of these systems within the renal parenchyma have been the subject of much controversy during the 20th century. In 1869 Rindowksi described lymphatic vessels in association with the afferent and efferent arterioles and further work suggested there was an extensive network around Bowman’s capsule (Rindowsky 1869). This early work was refuted by Vogel (Vogel 1891) and later by Stahr (Stahr 1900) and Kustsuan (Kutsuna 1939). Many of these studies were limited by poor imaging techniques. In a further study of canine and guinea pig kidneys Peirce performed parenchymal injections with Indian Ink and Trypan blue coupled with various preservation techniques (Peirce 2nd 1944). This demonstrated that the lymphatics accompany the interlobular, arcuate and intralobular vessels, surrounding them in an irregular anastomotic network which eventually drained into the perirenal lymphatics at the hilum. Several studies have reinforced these findings. With the advent of lymphatic specific markers light microscopy studies have confirmed the perivascular location of intrarenal parenchymal lymphatic vessels (Ishikawa, Akasaka et al. 2006).

1.6.1 Adverse renal effects of lymphatic obstruction.

At present there is little understanding on the role of kidney lymphatics on tissue homeostasis. Physiological studies following acute lymphatic ligation in rats resulted in increased diuresis and sodium excretion when compared to the non-manipulated contralateral control kidney. Glomerular filtration rate and urea excretion were not affected. This study looked at the acute physiological
response to disturbance of the lymph circulation in rats with no longer term study (Wilcox, Sterzel et al. 1984). Recent work demonstrated that rats with lymph vessel ligation demonstrated chronic renal failure with elevated serum creatinine, reduced creatinine clearance and proteinuria (Zhang, Guan et al. 2008). Renal histology was characterised by tubular atrophy and interstitial fibrosis and mesangial expansion. Gene expression studies demonstrates that upregulation of TGF-β and Smad2/3 play a significant role in inducing renal fibrosis in experimental models (Sato, Muragaki et al. 2003; Okada, Kikuta et al. 2005)

1.6.2 Interstitial fibrosis and lymphangiogenesis

Renal fibrosis is considered the final common pathway for almost all forms of renal diseases that progress to end stage renal failure, including immunologically mediated glomerulonephritis, hereditary diseases and metabolic disorders. Fibrosis is often accompanied by infiltration of inflammatory cells which induces tubular damage leading to progressive fibrosis by production of cytokines and growth factors. (Ferenbach, Kluth et al. 2007). The severity of interstitial leukocyte accumulation, of monocytes/macrophages and T-lymphocytes, is associated with renal function at time of biopsy (Alexopoulos, Seron et al. 1990; Segerer, Mac et al. 1999). The relation of lymphatics to renal fibrosis has been studied in experimental models. Acute ureteric obstruction causes dilation and reflux into intrarenal lymphatics, providing an alternative drainage of an acutely obstructed kidney (Cuttino, Clark et al. 1978; Albertine and O'Morchoe 1979). Interestingly, in
chronic obstruction Tamm-Horsfall protein leaks from the tubules and can be found in lymph nodes in the renal hilum (Howie 1987). In a porcine reflux nephropathy model Hobson noted that fibrosis follows the distribution of lymphatics postulating that an irritant entered the lymph, extravasated into the interstitium and stimulated fibrosis (Hobson J 1975; Colvin 2004). In remnant rat kidney there was massive proliferation of podoplanin+ lymphatic vessels in the fibrotic tubulointerstitium. These areas contained large numbers of mononuclear cells that expressed VEGF-C (Matsui, Nagy-Bojarsky et al. 2003). In other organs lymphatic proliferation was confirmed in human idiopathic pulmonary fibrosis and bleomycin-induced pulmonary fibrosis in rats (Teles-Grilo, Leite-Almeida et al. 2005; El-Chemaly, Malide et al. 2009). Sakamoto et al investigated the expression of D2-40 positive lymphatic vessels in a wide range of renal diseases which was affected by the duration of inflammation and progression to fibrosis (Sakamoto, Ito et al. 2009). They confirmed the absence of corticomedullary lymphatics in normal control kidney but identified significant lymphangiogenesis at the sites of tubulointerstitial damage and fibrosis. In addition to mononuclear cells strong expression of VEGF-C was demonstrated in the proximal tubules. These findings were seen in biopsies with IgA nephropathy, focal glomerulosclerosis, type 2 diabetic nephropathy, tubulointerstitial nephritis and lupus nephritis. Emerging data from the same group investigated the roles of TGF-β and VEGF-C in the development of lymphangiogenesis in unilateral ureteric obstruction model. The authors demonstrate that lymphatic vessels proliferate and show correlation with TGF-β expression. Specifically, TGF-β induced VEGF-C expression both in vivo and in vitro. It is yet to be established whether
manipulation of VEGF-C levels in chronic renal injury models has any impact on prognosis (Suzuki, Ito et al. 2012).

1.6.3 Unilateral Ureteric Obstruction: A model of renal interstitial fibrosis

A model of renal fibrosis that encompasses many aspects of other models of renal disease is unilateral ureteric obstruction (UOO) (Klahr 1998; Klahr and Morrissey 1998). Many quantifiable pathophysiological events occur over the span of one week making this an attractive model to study. The advantages of using UOO as a model of renal fibrosis include the absence of exogenous toxin, the lack of a uraemic environment and the availability of the contralateral kidney as a control.

Complete UOO induces a rapid sequence of events in the kidney which leads to reduced renal blood flow and glomerular filtration rate within the first 24 hours (Vaughan, Marion et al. 2004) (Reyes, Lefkowith et al. 1992). Prolonged ureteric ligation induces marked hydronephrosis of the affected kidney and interstitial accumulation of inflammatory cells and tubular dilatation. Apoptosis of tubular cells increases rapidly and a large number of factors can initiate this process (Truong, Petrusevska et al. 1996). Several of these may be pertinent to obstructive nephropathy such as hypoxia, ischaemia, cytokines and growth factors, TNF-α, reactive oxygen species and mechanical stretch (Klahr and Morrissey 2002)(Chevalier, Forbes et al. 2009). Progression to severely hydronephrotic kidney takes place over 1-2 weeks with loss of renal parenchyma. In addition there is proliferation of interstitial fibroblasts with
myofibroblast transformation leading to excess deposition of extracellular matrix and fibrosis (Stahl and Felsen 2001).

1.7 Monocyte and Macrophage Biology

1.7.1 Differentiation and functional characteristics of monocytes and macrophages

For the past century macrophages have been recognised to be an important immune effector cell. They were the first cell to be assigned a function in host defence and their phagocytic properties were described by Elie Metchnikoff who was awarded the Nobel Prize for Medicine and Physiology in 1908. (Cavaillon 2011) In conjunction with their key role in the immune system macrophages have vital homeostatic and developmental roles, which are independent of their involvement in immune responses.

Monocytes are known to originate from long-term haematopoietic stem cells (LT-HSC) in the bone marrow under the influence of colony stimulating factor-1 (CSF-1) and the transcription factor PU.1 (Rees 2010). LT-HSC gives rise to multi-potent progenitors, which have the capacity to develop into both common myeloid and lymphoid progenitors. Common myeloid progenitors develop into granulocyte and monocyte precursors which in turn yield monocyte and dendritic cell progenitors eventually giving rise to monocytes. During monocyte development, myeloid progenitors sequentially give rise to monoblasts, pro-monoblasts, pro-monocytes and finally monocytes (Gordon
and Taylor 2005) which enter the peripheral blood and circulate for several days and enter the tissues to become “resident macrophages” (Volkman and Gowans 1965).

Mature monocytes constitute 5-10% of peripheral blood leucocytes in humans and exhibit different degrees of granularity. Haematopoietic stem cells have to continuously regenerate and release mature blood cells in order to replenish tissue specific macrophages of the bone (osteoclasts), central nervous system (microglial cells), liver (Kupffer cells), spleen and peritoneum (Gordon and Taylor 2005). This is promoted through release of CSF-1 and IL-34 which are the ligands for the receptor CSF-1R expressed by monocyte progenitors and macrophages/dendritic cells (Rees 2010). Their binding promotes transcription of PU.1, which skews stem cell development towards the monocytic lineage (Auffray, Sieweke et al. 2009). In humans $3 \times 10^{11}$ monocytes are released from the bone marrow into peripheral blood each day in order to maintain homeostasis (Rees 2010). In addition there is also a pool of pro-monocytes which can be rapidly released into the circulation under times of stress and inflammation. In order to enter the circulation from bone marrow, monocytes must transmigrate across blood vascular endothelial cells. The CCR2-CCL2 (MCP-1) axis has a key role in attracting pro-monocytes into the circulation (Serbina and Pamer 2006).
1.7.2 Monocyte heterogeneity in humans.

Monocytes have a characteristic morphological appearance with an oval or kidney shaped nucleus and a large volume of cytoplasm (Gordon and Taylor 2005). The introduction of monoclonal techniques has unearthed considerable heterogeneity in monocyte phenotype and function. Circulating bone-marrow derived monocytes can be characterised into subsets that depend on the pattern of expression of cell surface receptors (Ingersoll, Spanbroek et al. 2010). Human monocytes were initially characterised by their high levels of expression of CD14 (which is component of the receptor for LPS) (Ziegler-Heitbrock 2007). CD14+ cells may be further separated according to their differential expression of CD16 (also known as FcyRIII) (Passlick, Flieger et al. 1989). CD16− cells account for 90% of cells and have high levels of expression of CCR1 and CCR2 and are considered to represent the phenotype of a “classical” monocyte (Gordon 2003; Park, Svetkauskaite et al. 2004). Increased expression in CD16 is associated with upregulation of MHC Class II and CD32 with reduced expression of CCR2. These monocytes have enhanced capacity for transendothelial migration with this subset believed to represent the precursors of resident tissue macrophages (Sunderkotter, Nikolic et al. 2004).

1.7.3 Monocyte heterogeneity in mice.

In addition to monoclonal techniques, the use of transgenics has facilitated significant understanding and functional characterisation of mouse monocyte
subsets. In a series of classical experiments using adoptive transfer of GFP expressing monocytes Geissmann and colleagues identified phenotypically and functionally distinct subsets of circulating monocytes in the mouse (Geissmann, Jung et al. 2003). There are two broad categories of monocytes according to CX3CR1 expression with a short lived “inflammatory subset” that homes to areas of inflammation, with high levels of expression (CX3CR1<sub>low</sub>Ly6C<sub>high</sub>). This is contrast to the second monocyte subset that persists longer in tissues and serves as a precursor for resident myeloid cells (CX3CR1<sub>high</sub>Ly6C<sub>low</sub>) (Figure 1.4).

**1.8 Macrophage Activation**

Macrophages can respond to endogenous stimuli that are rapidly generated after injury or infection. In addition they may respond to signals which are produced by antigen specific immune cells which give rise to more prolonged activation states in macrophages. Macrophage activation can be systematically divided into two main classes in line with the Th1/Th2 dichotomy referred to as “classically” and “alternatively” activated macrophages (Gordon 2003). This paradigm is derived from *in vitro* studies of murine and human cells (Edwards, Zhang et al. 2006) and the phenotypic generation and interactions *in vivo* are likely to be more complex.
Figure 1.4
1.8.1 Classically Activated Macrophages.

The term classically activated has been used to designate effector macrophages that are produced during cell mediated immune responses. The classically activated phenotype or M1 macrophage described *in vitro* occurs in response to stimulation by interferon-γ (IFN-γ) and lipopolysaccharide (LPS) and is characterised by the production of IL-1β, tumour necrosis factor (TNF) and reactive oxygen species (ROS). This pro-inflammatory response promotes cytotoxic activity against phagocytosed organisms. Further *in vitro* work has generated sub-classification of M1 phenotype in response to their pattern of stimulation. M1a macrophages are classically activated by LPS and IFN-γ or another pro-inflammatory stimulus and in addition to the above cytokine profile show increased expression of MHC Class II and co-stimulatory molecules (CD40, CD80 and CD86). M1b macrophages are activated by LPS only and express inflammatory cytokines but do not produce IL-12 and therefore fail to drive Th-1 responses.

1.8.2 Alternatively Activated Macrophages.

Whilst M1 macrophages are considered to be “pro-inflammatory” a second population of functionally distinct “alternatively” activated macrophages exist initially defined *in vitro*. This phenotype is induced following exposure to either IL-4 and IL-13 (M2a), immune complexes/TLR ligands (M2b), or with IL-10 and glucocorticoids. The hallmarks of M2 macrophages are high levels of IL-10, IL-1ra production and CCL17 and CCL22 secretion. These cells are
considered to be anti-inflammatory with poor antigen presenting capability or cytotoxic potential. M2 macrophages promote scavenging of debris, angiogenesis and tissue remodeling. They are also implicated in facilitating metastatic spread when situated in the stroma of tumours (tumour associated macrophages).

**1.9 Macrophage depletion in vivo**

The most widely published method of macrophage depletion is the administration of liposomal dichloromethylene-bisphosphonate (clodronate) first described by van Rooijen (Van Rooijen 1989). Once ingested by macrophages the phospholipid bilayers of the cells are disrupted under the influence of the lysosomal phospholipases. The free clodronate reaches toxic intracellular concentrations, causing loss of mitochondrial membrane potential via inhibition of adenosine triphosphate (ATP)/adenosine disphosphate (ADP) translocase leading to apoptosis (Green 2003).

Clodronate released into the circulation from dead macrophages and leakage of lysosomes does not readily enter non-phagocytic cells and are rapidly cleared from the circulation. Such an approach has been used in rodent models of ischaemia reperfusion injury (IRI) (Ko, Boo et al. 2008), transplantation (Jose, Ikezumi et al. 2003) and unilateral ureteric obstruction (UOO) (Sung, Jo et al. 2007).
1.10 Renal transplantation

1.10.1 Historical background.

Renal transplantation is the most successful treatment for patients with end-stage renal failure, since it enhances both quality of life and survival compared with dialysis. The first successful kidney transplant was performed on the 23rd December 1954 in Peter Bent Brigham Hospital, Boston. Transplant surgeon Dr Joseph Murray performed a live donor transplant between identical twins Richard and Ronald Herrick (Mayer, Dmitrewski et al. 1997). The procedure was hailed a success with Richard making a remarkable recovery following the procedure. Unfortunately he died 7 years later after developing recurrence of his primary renal disease.

Recent developments in immunosuppressive therapy have significantly reduced the loss of allografts from acute rejection after renal transplantation (Cohen, St Martin et al. 2006). However, the long-term attrition of such grafts remains a major clinical problem and is mainly due to interstitial fibrosis and tubular atrophy (Solez, Colvin et al. 2008). This remains a considerable challenge to the transplant community accounting for the loss of 5% of grafts per annum (Pascual, Theruvath et al. 2002; Joosten, van Kooten et al. 2003; Afzali, Taylor et al. 2005). In patients who return to dialysis because of a failed graft estimated annual percentage rate of death is more than three fold higher compared to those who retain functioning grafts (Kaplan and Meier-Kriesche
2002). These findings illustrate the importance of preserving renal function and promoting allograft survival.

1.10.2 Donor Age.

Donor age is a strong predictor of poor long-term graft survival and imparts greater clinical significance with expansion in the use of marginal donors (Strandgaard and Hansen 1986; Moore, Farney et al. 2006; Stratta, Sundberg et al. 2006). Kidneys from older donors show an increased frequency of delayed graft function and elevated baseline serum creatinine. Age related loss of renal mass predominantly occurs in the cortex with relative sparing in the medulla. Histologically there is glomerulosclerosis with enlargement of the remaining glomeruli (Epstein 1996). After 1 year following transplantation donor age accounts for 30% of the variance in graft outcomes (Gjertson 1997).

1.10.3 Peri-transplant Injury and ischaemia reperfusion injury

The success rate of transplanted organs from cadaveric donors is consistently inferior to that of living donors (Haberal, Gulay et al. 1992; Najarian, Gillingham et al. 1994; Terasaki, Cecka et al. 1995; D'Alessandro, Pirsch et al. 1998). This is apparent with the success of live donor transplants even in the face of significant major histocompatibility (MHC) mismatch (Terasaki, Koyama et al. 1994). In experimental models of surgically induced intracranial hypertension, there is upregulation of MHC Class I and II antigens and
the co-stimulatory molecules B7 suggesting the potential immunogenicity (Shoskes, Churchill et al. 1990; Shoskes, Parfrey et al. 1990; Pratschke, Kofla et al. 2001). Further studies in rodents have shown that these triggers increase the subsequent infiltration of T-lymphocytes and macrophages into the kidney with increased production of the inflammatory mediators IL-1 and TNF-α (Venkateswaran, Dronavalli et al. 2009).

Ischaemia reperfusion injury (IRI) is an unavoidable consequence of solid organ transplantation. It occurs immediately after cessation of arterial inflow during organ procurement and is related to the time until the graft is reperfused with oxygenated blood (Arumugam, Okun et al. 2009). Reperfusion with oxygenated blood is important in restoring the substrates for oxidative metabolism but this can result in the production of free oxygen radicals. This causes activation of the vascular endothelium with upregulation of adhesion molecules and cytokine production (Weyrich, Ma et al. 1993; Takada, Nadeau et al. 1997). Immunologically active soluble proteins (or their transcripts) such as IL-1 and IL-6 have been demonstrated in transplanted organs (Forsythe 2007). During ischaemia IL-12 and IL-18 are upregulated in response to damage resulting in upregulation of IFN-γ, contributing to the earlier effects on MHC class expression (Lentsch, Yoshidome et al. 1999). In experimental isograft transplantation, graft histology with features of interstitial fibrosis and tubular atrophy (IFTA) can be reproduced by prolonged ischaemia highlighting the potential long term impact on graft function (Tullius, Heemann et al. 1994).
1.10.4 Calcineurin Inhibitor Nephrotoxicity.

Calcineurin inhibitors (CNIs), since their introduction in the 1980s have been the foundation of maintenance immunosuppressive regimens in solid organ transplantation. The use of CNIs has substantially reduced the risk of acute rejection and improved short-term outcomes (Olyaei, de Mattos et al. 2001; Merville 2005). However, CNIs namely cyclosporin A and tacrolimus are implicated in nephrotoxicity and contribute to the development of diabetes mellitus, dyslipidaemia and hypertension. Acute renal injury is primarily functional with serum level dependent changes in afferent arteriolar vasoconstriction resulting in decreased renal blood flow and glomerular filtration rate (Prevot, Semama et al. 2000). This is believed to be due to imbalances in prostaglandin E2 and thromboxane A2 (Butterly, Spurney et al. 2000). Clinical improvement is often rapidly seen after dose reduction.

Chronic CNI-induced damage results from further vascular injury. The classical histological lesions include de novo or increasing arteriolar hyalinosis and striped fibrosis and may be associated with tubular vacuolation and microcalcification (Kahan 1986; Mihatsch, Ryffel et al. 1993; Remuzzi and Perico 1995). It has been shown both in vivo and in vitro that cyclosporine has been associated with increased transforming growth factor-β (TGF-β) expression (Khanna, Cairns et al. 1999). Upregulation is pivotal in causing glomerular and interstitial extracellular matrix deposition eventually leading to renal impairment. With progression severe arteriolar hyalinosis can cause
vascular narrowing and ischaemic glomerulosclerosis (Mihatsch, Thiel et al. 1988). Striped fibrosis is subjectively defined by a dense stripe of cortical fibrosis and atrophic tubules adjacent to relatively spared cortex (Dell'Antonio and Randhawa 1999).

**1.11 Chronic allograft injury and interstitial fibrosis and tubular atrophy**

*1.11.1 Prevalence and histological features.*

In protocol biopsy studies histological evidence of allograft damage is common and time dependent, with serum creatinine often underestimating the degree of injury. Damage sustained to the allograft does not represent a single entity and histological progression involves a series of pathological insults accumulating from the time of reperfusion injury, acute and subclinical rejection and calcineurin inhibitor toxicity (Figure 1.5). It is common at 10 years, being present in 50% of allografts. The characteristic histological features of chronic allograft injury are interstitial fibrosis and tubular atrophy, which has replaced the now obsolete term chronic allograft nephropathy. In addition to interstitial fibrosis and tubular atrophy, other histopathological features include arteriosclerosis and glomerulopathy. Profibrotic factors such as TGF-β and the tissue inhibitor of metalloproteinase (TIMP) are expressed by kidney tissue and increased by alloimmune mononuclear infiltration. Glomerulosclerosis and tubular atrophy ensue and scarring results from excess deposition of extra-cellular matrix secreted by
myofibroblasts. Vascular rarefaction occurs (Adair, Mitchell et al. 2007). This is characterised by a relatively slow but variable rate of decline in real function often in association with hypertension and proteinuria.

1.12 Macrophages in transplantation

The process of solid organ transplantation initiates an alloimmune response from antigen specific effector cells and the production of specific alloantibody (Chadban, Wu et al. 2010). Macrophages may participate in the adaptive immune response by presenting alloantigen to primed T-lymphocytes and by affecting cell death but also mediate tissue damage independent of the adaptive immune response (Martinez-Pomares and Gordon 2007).

Although classed as a professional antigen presenting cells there is little direct evidence that macrophages have an important role in capturing alloantigen in transplantation. Dendritic cells are considered to be more effective in presenting allopeptide in association with MHC class II molecules to naïve CD4⁺ lymphocytes.

At the time of engraftment donor macrophages remain within the kidney and increase in numbers through local proliferation in response to IRI (Ysebaert, De Greef et al. 2000; Ko, Boo et al. 2008). Their numbers begin to decline after 7 to 14 days to low numbers in absence of rejection (Penfield, Wang et al. 1999). In acute allograft rejection however the vast majority of
Macrophages are recipient in origin and constitute 38-60% of infiltrating cells in human biopsies and experimental models (Hancock, Thomson et al. 1983). In patients with histological features of acute rejection macrophage infiltration was quantitatively associated with renal dysfunction whilst T-cell infiltration was not (Girlanda, Kleiner et al. 2008). These results suggest that the direct action of monocytes / macrophages have a more profound functional impact that the cytoxic effects of T-cells.

Circulating monocytes are recruited to the rejecting kidney in response to local production of cytokines and chemokines including monocyte chemotactic protein-1 (MCP-1, CCL2) (Grandaliano, Gesualdo et al. 1997) macrophage inflammatory protein 1-α (Grau, Gemsa et al. 2000) and macrophage migration inhibitory factor (MIF) (Lan, Yang et al. 1998). Studies have shown that monocytes isolated from the perfusate of rejecting allografts show increased expression of CD80 and MHC Class II which would suggest that they are activated whilst in the intravascular compartment (Scriba, Grau et al. 1998). In order to enter the renal parenchyma monocytes adhere to the endothelium via adhesion molecules intercellular adhesion molecule -1 (ICAM-1), lymphocyte function associated molecule-1 (LFA-1) and vascular cell adhesion molecule-1 (VCAM-1) (Schenkel, Mamdouh et al. 2002).

Macrophage accumulation within the rejecting renal allograft occurs by recruitment and local proliferation. This has been confirmed by immunostaining with proliferating nuclear cell antigen (PCNA) and 5-bromo-2'-deoxyuridine (BrDU) labeling (Jose, Ikezumi et al. 2003). Macrophage colony-
stimulating factor (M-CSF) is the principal growth factor regulating the proliferation, differentiation and survival of monocytes/macrophages (Pixley and Stanley 2004). M-CSF acts via the tyrosine kinase receptor c-fms (Pixley and Stanley 2004). Expression of c-fms is restricted to cells of the monocyte and macrophage lineage, even in inflammation (Le Meur, Tesch et al. 2002). In an experimental model of murine renal transplantation blockade of the CSFR1 receptor reduced macrophage proliferation within the graft by 80% yielding an overall 50% reduction in macrophage number within the graft. This resulted in reduced tubulointerstitial rejection (Jose, Le Meur et al. 2003).

In keeping with their activated phenotype macrophages express many pro-inflammatory cytokines which include IL-1, IL-12, IL-18, TNF-α and IFN-γ (Nathan 1987; Okamura, Tsutsui et al. 1998; Jose, Le Meur et al. 2003). IL-1β activates endothelial cells and promotes expression of adhesion molecules (Dinarello 1991). IL-1β mRNA and protein is increased in patients undergoing allograft rejection (Chadban, Wu et al. 2010). The functional impact of this cytokine can be seen when recipients who express high levels of endogenous IL-1 receptor antagonist experience lower rates of rejection (Teppo, Honkanen et al. 1998).

TNF-α is known to be an important mediator of tissue damage during allograft rejection (Grenz, Schenk et al. 2000). TNF-α can bind to two receptors p55 and p75 and can induce several biological effects including cytotoxicity, which is mediated through interaction with p55 receptor (Tartaglia, Weber et al. 1991). In its role as a pro-inflammatory cytokine TNF-α promotes endothelial
cell activation with upregulation of ICAM-1 expression enhancing monocyte adhesion (Pai, Bassa et al. 1996). TNF-\(\alpha\) stimulation increases production of M-CSF (Kamanna, Pai et al. 1996), MCP-1 (Pai, Ha et al. 1996) and reactive oxygen species (Timoshanko, Sedgwick et al. 2003). In a syngeneic rat cardiac transplant model the administration of a soluble TNF receptor-Ig chimeric molecule (TNFRp55-Ig) inhibited TNF-\(\alpha\) responses and reduced the early cellular influx into the graft (Ritter, Schroder et al. 2000). Other soluble mediators expressed by macrophages are believed to be involved in the pathogenesis of allograft rejection. Interleukin-12 (IL-12) is characterised as an inductive cytokine for Th1 T-cell differentiation with subsequent expression of their cytokine profile e.g IFN-\(\gamma\) and cell mediated immunity (Scott 1993).

1.12.1 Macrophage depletion in transplantation

Direct evidence for a role in macrophages in mediating kidney injury has been provided by depletion studies. Early non-specific depletion studies used tryphan blue, silica, carrageenan and anti-macrophage serum. Although some degree of protection from rejection was identified with these strategies their effects were non-specific with substantial depletion of T-cells as well as intra-graft macrophages (Pearsall and Weiser 1968; Unanue 1968). Later studies which inhibited macrophage chemokines including monocyte chemotactic protein-1 (MCP-1) and regulated upon activation normal T cell expressed and secreted (RANTES) substantially reduced intra-graft macrophage numbers but also inhibited T-cell responses (Gao, Topham et al. 2000; Gao, Faia et al. 2001) which confounded data interpretation. Specific macrophage depletion
in vascularised transplant models was first achieved by Jose and colleagues. (Jose, Ikezumi et al. 2003). Liposomal clodronate was administered on day 1 and 5 to rats following surgery which resulted in significant macrophage depletion. In contrast to other studies there was no reduction in the number of other leukocytes seen (CD4, CD8, CD25 and NK cells). There was however histological and functional reduction in the parameters of acute rejection (e.g. tubulitis, glomerulitis and serum creatinine). In addition expression of inducible nitric oxide synthase by infiltrating cells and urinary nitrite excretion was attenuated with clodronate administration.

Further studies from our research group used a transgenic conditional ablation strategy to deplete circulating monocytes and infiltrating renal macrophages in experimental model of acute allograft rejection (Qi, Adair et al. 2008). CD11b-DTR recipients are transgenic mice, which express the human diphtheria toxin (DT) receptor under the control of the CD-11b promoter such that human administration of human diphtheria toxin results in rapid and effective macrophage ablation (Cailhier, Partolina et al. 2005; Duffield, Tipping et al. 2005). In acute rejection there was evidence of microvascular capillary rarefaction at day 7 with reduced histologic features of rejection following administration of DT at day 3 and 5. This suggests that infiltrating macrophages act as effectors of tissue damage in acute allograft rejection as there was no reduction in infiltrating T- and B-cells with reduced mRNA expression of iNOS, IL-12 and IFN-γ.
As discussed earlier the pathogenesis of IFTA is multifactorial based on a series of insults resulting in cumulative parenchymal scarring. Experimental data suggests that macrophages have a role in the progression of IFTA. The application of a macrophage inhibitor Gamma lactone in a rat model of chronic graft failure resulted in preservation of graft function, reduced intragraft accumulation of macrophage and preservation of tissue architecture. (Azuma, Nadeau et al. 1995). In a series of elegant experiments Yang and colleagues transduced renal allografts *ex vivo* with adenoviral constructs expressing molecules inhibiting endogenous TNF-α and IL-12 and constructs delivering IL-10. This reduced inflammation and improved graft histology and function and supports the pathogenic role of macrophage-derived cytokines in chronic graft injury (Yang, Reutzel-Selke et al. 2003).

Published data from our group has described peritubular capillary rarefaction and interstitial lymphangiogenesis in human transplant nephrectomy tissue with interstitial fibrosis and tubular atrophy (Adair, Mitchell et al. 2007) It may be that macrophage mediated damage to the microvasculature during acute allograft rejection may cause long term tissue hypoxia activating fibroblasts and promote progressive disease. Moreover, macrophage infiltration is associated with loss of peritubular capillaries in biopsies from patients with chronic kidney disease (Adair, Mitchell et al. 2007).
1.13 Lymphangiogenesis and transplantation

1.13.1 Lymphatic disruption and reconnection

Vascular endothelial cells within the transplanted kidney form the interface between donor and recipient circulations and represent a dynamic environment for alloimmune injury. In contrast, little is understood about the role of the lymphatic system following transplantation. The procedure of heterotopic renal transplantation is well described consisting of an arterial and venous anastomosis to the recipient circulation followed by a cystoureteric anastomosis with recipient bladder (Henry 2005). The lymphatic trunks of the donor organ within connective tissue are ligated and divided with no attempt at reconnection. Early work in dog autografts demonstrated that lymphatic regeneration commenced 3 days after transplantation (Mobley and O'Dell 1967). This was assessed by injecting 2% Evans blue dye into the renal parenchyma and noting the time of its appearance in the lymphatic vessels and lymph nodes. By the twentieth day the authors commented that lymphatics were “almost indistinguishable from normal”. They failed to describe whether there was regeneration across the original lymphatic trunks or whether there was a network of regenerating vessels.

In one of the earliest studies on the lymphatic system in transplantation Pedersen and colleagues transplanted homograft kidneys into the neck of sheep whilst preserving the renal lymphatic drainage intact. This resulted in the lymphatics of the graft forming a chronic fistula so that the lymph could be
collected throughout the survival of the graft. This had no impact on graft survival and the kidney was rejected without involvement of the host lymph nodes (Pedersen and Morris 1970). Other studies in skin allografts demonstrated delayed rejection when grafted onto oedematous tissue when compared to non-oedematous controls, which would suggest that effective lymphatic drainage has a role in presenting alloantigen and hastening rejection (Stark R. B 1960) after surgical ablation of the regional lymph nodes. There is no available data regarding either the kinetics or nature of the process of lymphatic reconnection in rodents or humans. In addition, the pattern of reconnection and whether regenerating lymphatic vessels are donor or recipient derived remains uncertain.

**1.13.2 Intra-renal lymphangiogenesis**

De novo lymphangiogenesis has been described in a subset of patients with acute renal allograft rejection. Kerjaschki et al demonstrated a >50 fold increase in lymphatic vessel density in association with nodular lymphoid infiltrates with evidence of lymphatic endothelial cell proliferation (Kerjaschki 2006). Lymphatic cells around nodular aggregates expressed SLC/CCL21 which co-stained with podoplanin and suggests that these vessels actively recruit CCR7+ DCs and lymphocytes. It is unclear whether these de novo lymphatics are derived from donor or recipient (Figure 1.6). In a series of elegant experiments with gender mismatch transplants Kerjaschki and colleagues (Kerjaschki, Huttary et al. 2006) demonstrated that the majority of
lymphatic vessels are donor derived by co-localisation of a FITC labelled Y-chromosome and nuclear transcription marker Prox-1 (Kerjaschki, Huttary et al. 2006). The authors speculated that the lymphatic endothelial progenitor cells enter the connective tissue stroma as macrophages, transdifferentiate into lymphatic cells and become incorporated into developing lymphatic vessels. Recent work within our group demonstrated the extensive presence of interstitial lymphatic vessels in human allograft nephrectomy specimens with interstitial fibrosis and tubular atrophy. (Adair, Mitchell et al. 2007)

A key question is whether post-transplantation lymphangiogenesis is beneficial (e.g. by promoting efficient inflammatory cell clearance) or detrimental (e.g. by promoting antigen presentation within draining lymph nodes and stimulating the alloimmune response) (Vass, Hughes et al. 2009). There is limited data to provide a definitive answer. In cardiac transplantation interruption of the lymphatic drainage is believed to be an important facet of cardiac allograft failure by promoting myocardial fibrosis (Kong, Wang et al. 2007). In a recent study of rodent cardiac allografts there was a dramatic and persistent decrease in lymphatic vessel density whereas the outer myocardial vessels increased in size. There was restoration of lymphatic vessel number approaching that of control isografts when the hearts were re-implanted from allogeneic to isogeneic recipients, suggesting that injured lymphatics can recover in the absence of an alloimmune response (Soong, Pathak et al. 2010).
Also studies of lymphangiogenesis in renal allograft recipients have provided discordant results. Yamamoto et al found that 32 patients with acute antibody mediated rejection, acute cellular rejection or peritubular capillaritis exhibited a 4 to 9-fold increase in lymphatic vessel density whereas a study of sequential renal biopsies from 76 patients did not demonstrate any significant difference in patients with or without rejection or the features of interstitial fibrosis and tubular atrophy (Yamamoto, Yamaguchi et al. 2006). However, the lymphatic vessel density was higher in biopsy regions with cellular infiltrates and patients with lymphatic vessels associated with these cellular infiltrates exhibited better graft function, suggesting a beneficial effect of these lymphatic vessels. 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 (Dashkevich, Bloch et al. 2009).

In lung transplantation lymphatic vessel density was increased in biopsies undergoing acute allograft rejection (Dashkevich, Heilmann et al. 2010). As with intra-renal lymphangiogenesis these were associated with nodular cellular infiltrates, however no data is recorded beyond 90 days. In line with the hypothesis that de novo lymphatic vessels clear the inflammatory infiltrate emerging data from rodent liver transplants also documented de novo lymph vessels around cellular infiltrates during acute rejection. Intriguingly the
numbers of lymphatic vessels diminished in association with the reduction and clearance of the inflammatory infiltrates (Ishii, Shimizu et al. 2010).

1.13.3 Inhibition of lymphangiogenesis

In order to determine whether lymphangiogenesis is beneficial to allograft survival it is attractive to perform an inhibition study. Advances in this direction have been made in the field of tumour lymphangiogenesis and corneal transplantation thus making anti-lymphangiogenic therapy a new and promising approach. Various anti-angiogenic therapy strategies have been used to interfere with the VEGF system in different pathological models of neovascularisation. These approaches include VEGF neutralisation antibodies, VEGF receptor antibodies, recombinant soluble VEGF proteins and receptor tyrosine kinase inhibitors (He, Kozaki et al. 2002; Krishnan, Kirkin et al. 2003; Shimizu, Kubo et al. 2004; Lin, Lalani et al. 2005; Roberts, Kloos et al. 2006).

It is recognised that lymphangiogenic growth factors promote cancer cell spread to regional lymph nodes. He et al demonstrated inhibition of lymphatic metastases by inhibition of VEGFR-3 signaling by systemic delivery of a soluble VEGFR-3 immunoglobulin fusion (Ig) fusion protein by adenoviral delivery (He, Kozaki et al. 2002). Monoclonal antibodies to VEGFR-3 have been shown to be effective in inhibiting regeneration of mouse lymphatic vessels and reducing lymphatic metastases in in vivo models of orthotopic tumours (Shimizu, Kubo et al. 2004). Corneal transplantation is the most
commonly performed soft tissue transplant and, although a site of immunological privilege, neovascularisation is associated with increased graft rejection and associated with lymphangiogenesis in humans. 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 (Bachmann, Luetjen-Drecoll et al. 2009). No such studies have been conducted in solid organ transplantation. Rapamycin is mTOR inhibitor which is used in the treatment of acute allograft rejection (Huber, Bruns et al. 2007). Emerging data has demonstrated \textit{in vivo} inhibition of VEGF-C yet this has not translated into improved results in long term graft survival (Huber, Bruns et al. 2007).
1.14 Hypothesis

This thesis will examine the hypothesis that the macrophage is a key effector of inflammatory and rejection associated lymphangiogenesis in the kidney. Furthermore, manipulation of macrophage number will permit modulation of the development of de novo lymphangiogenesis.

1.15 Aims

1) To develop a rodent model of lymphatic disruption of native kidneys to permit studies of the time course and mechanism of lymphatic reconnection.

2) An acute murine rejection model and rodent model of IFTA will be used to assess the presence of de novo lymphangiogenesis within the rejecting allograft.

3) Characterisation of the rodent model of UUO will be performed in respect to impact on the lymphatic vessels within the kidney

4) To permit elucidation of the role of macrophages in inflammatory lymphangiogenesis depletion studies will be performed and the effects characterised.
2 MATERIALS AND METHODS

2.1 Materials and Reagents

Tissue culture reagents were purchased from Life Sciences Technology (Paisley, UK). Tissue culture plastics were purchased from Costar (Loughbourgh, UK) and Falcon (Runcorn, UK). Conjugated and unconjugated antibodies used are summarised in Table 2.1. All other reagents were purchased from Sigma Aldrich Co. Ltd (Poole UK) unless otherwise stated.

2.2 Rodent model of renal lymphatic disruption

Adult male Sprague-Dawley rats (250-350g) were used. Anaesthesia was induced by administration of intra-peritoneal Ketamine (75mg/ml) and Medetomidine (0.5mg/Kg). In order to preserve core body temperature all procedures were performed on a heat pad, which maintained core temperature at 36°C. The abdomen was shaved and cleaned with Betadine solution. Surgery was performed using aseptic technique. The abdomen was opened with a long midline excision. In order to gain access to the left kidney the small bowel was delivered into the wound and this was wrapped in a moist drape. Using an operating microscope (Zeiss), the descending colon was
mobilised by dissecting its peritoneal attachments and reflected medially. To increase exposure of the kidney the spleen and stomach were retracted in a cephalid direction. Using blunt dissection the renal pedicle was mobilised from its surrounding fatty tissue. Care was taken to avoid damaging the adrenal artery and vein. The dissection was extended to the level of the aorta and inferior vena cava. A plane was created between the renal artery and renal vein and these vessels were dissected free from each other. All of the surrounding connective tissue was stripped from the renal pedicle and the kidney was mobilised by dividing the peri-renal fat using electrocautery. Once the kidney was mobilised fully, it was reflected medially exposing the renal lymphatic vessels, which lie posterior to the renal artery. At 25x magnification these vessels were carefully retracted from the surface of the vessel with fine dissecting forceps and divided.

To perform a subcapsular injection the kidney was draped with a wet swab containing a small window in order to prevent contamination of the peritoneal cavity. Using a 4G needle 5µl of carbon black was injected into the subcapsular space with pressure applied to the puncture site immediately after injection to prevent the retrograde leak of dye.

When culling the kidney was removed en-bloc with the renal hilum and a cuff of aorta and inferior vena cava. This was immediately fixed in 10% formalin.
2.3 Murine model of allograft rejection

The murine model of acute renal allograft rejection was established by Miss Marson and performed by Dr Qi. Renal transplants were performed between Balb/c donors (H2-d haplotype) and FVB/nj recipients (H2-q haplotype) (allograft group), and between FVB/nj mice (isograft group). The donor kidney was harvested and perfused with cool normal saline. A left unilateral nephrectomy was performed in recipient mice and donor renal artery and vein were anastomosed to recipient abdominal aorta and inferior vena cava respectively. Donor ureter was anastomosed to recipient bladder. A contralateral nephrectomy was not performed therefore this was not a functional model. Animals were culled at day 7.

2.4 Rodent model of heterotopic renal transplantation

The rat model of Interstitial fibrosis and tubular atrophy (IFTA) in rats at the University of Sheffield was established by Dr John Haylor and performed by Mr Badri Shresha. Kidneys from donor male Lewis rats (isografts) or Fisher rats (allografts) were transplanted heterotopically into male Lewis recipients. 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 respectively 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 post-operative day in order to make this a functional transplant model. All recipients received cyclosporine (5mg/kg) intraperitoneally for the 10 days following transplantation to prevent episodes of acute rejection.

2.5 Rodent Model of unilateral ureteric obstruction

Adult male Sprague-Dawley rats (250-350g) were used. Anaesthesia was induced by administration of intraperitoneal Ketamine and Medetomidine as previously described. Surgery was performed using aseptic technique. A midline abdominal incision was made and the left ureter ligated with a 5/0 silk suture at two points and divided between the ligatures. The abdomen was closed with continuous 3/0 vicryl and the skin with a subcuticular 3/0 vicryl suture. Reversal of anaesthesia was achieved by subcutaneous administration of Atipamezole (0.5mg/Kg). Following the procedure animals received 5mls of normal saline and 5µl of Buprenorphine subcutaneously. Sham procedures were performed in a similar manner with the exception of ureteric ligation. Kidney, liver and spleen from the sacrificed rat were fixed in either 10% buffered formalin or methyl carnoy’s solution. Pieces of kidney for mRNA extraction were snap frozen in liquid nitrogen.

2.6 Macrophage depletion with liposomal clodronate

Clodronate liposomes and vehicle controls (PBS liposomes) were obtained from Professor Van Rooijen, University of Vrije (www.liposomal clodronate.com). They were prepared as previously described (Van Rooijen...
1989). The liposomes were warmed and resuspended and a dose of $5\mu l/g$ body weight (Ko, Boo et al. 2008) was administered intravenously to induce macrophage depletion.
2.7 Immunohistochemistry

2.7.1 Standard ABC immunohistochemistry protocol

Whole kidneys, spleen and liver were cut longitudinally and fixed in 10% formalin or methyl-Carnoy’s solution (60% methanol, 30% chloroform and 10% glacial acetic acid) for 24 hours with the fixative exchanged for an equal volume of PBS and methanol respectively prior to embedding in paraffin. Immunohistochemical staining was carried out on 4µm formalin fixed paraffin embedded tissue. Sections were dewaxed in xylene for 15 minutes and then rehydrated through a series of graded alcohols. Tissue sections were washed in distilled water for 15 minutes and epitopes were demasked using heat-mediated or enzymatic antigen retrieval (Table 2.1). Sections were microwaved for 15 minutes in either Citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) or Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0) after which they were immersed in cool water and washed for a further 15mins to bring to room temperature. For enzymatic antigen retrieval, slides were incubated in Proteinase K (15 µg/100ml of PBS, Sigma) at 37°C for 15 minutes.

Endogenous peroxidase activity was blocked by incubating tissue sections in 3% hydrogen peroxide (Sigma) for 15 minutes. Slides were washed in PBS and then mounted into individual sequenza cassettes. An avidin/biotin blocking kit (VECTOR) was used to prevent non-specific binding. Sections were incubated with Avidin D solution for 10 minutes. After rinsing briefly with
PBS, biotin solution was incubated for a further 10 minutes. Sections were washed with PBS. In order to prevent non-immunological binding of the specific immune sera, tissue sections were incubated in a serum-free protein block (DAKO) for 10 minutes. The primary antibody was incubated overnight at 4°C.

Once the slides were brought to room temperature, they were washed 3 times in PBS. The sections were then incubated with the appropriate secondary antibodies for 30 minutes (1:300). After rinsing with buffer, Vectastain RTU ABC (Vector laboratories) reagent was added to the sections for 30 mins. Sections were washed in buffer. The reaction was visualised using 3’3 diaminobenzidine (DAB), (DAKO) with a 5 minute incubation period. Sections were thoroughly washed in buffer to remove any excess DAB. Nuclei were lightly counterstained by immersing the slides briefly in haematoxylin and washed in distilled water. Sections were dehydrated through graded alcohols and incubated in xylene for 15 minutes. Slides were mounted with Perspex cover slips.

2.7.2 Immunohistochemistry with Envision Kit

Standard Avidin-Biotin Complex method (ABC) protocols failed to produce adequate immunostaining against the PROX-1 and B220 (CD45R) antigens. Therefore we used an EnVision kit (DAKO) which is based on dextran polymer technology. This permits the binding of a large number of enzyme molecules (horseradish peroxidase) to a secondary antibody via the dextran backbone.
This has the advantage of increasing sensitivity and minimising the non-specific background staining. Sections were dewaxed in xylene and rehydrated in graded alcohols as previously described. Following antigen retrieval sections were incubated with a combined peroxidase and protein block for 10 minutes. Slides were briefly washed in PBS and the primary antibody was incubated at 4°C overnight. Sections were washed in PBS and brought to room temperature following which the HRP polymer was incubated for 30 minutes. In order to visualise the reaction, sections were washed in PBS three times and treated with DAB for a maximum of 5 minutes.

2.7.3 Dual Immunofluorescence Staining

After deparaffinisation, antigen was retrieved in citrate buffer for 15 mins. Sections were pre-incubated with serum free protein block (DAKO) for 10 minutes and incubated with both primary antibodies overnight at 4°C. After washing in PBS the sections were incubated with the appropriate combination of secondary antibodies for 30 minutes. Sections were mounted with slides using VECTASHIELD mounting media with DAPI (VECTOR). Image acquisition was carried out using Zeiss Axioscop 2 Fluorescent microscope.
2.8 Gene expression studies

2.8.1 RNA extraction from whole tissue and cells

RNA was extracted using NucleoSpin RNA II columns (Machery-Nagel) according to the manufacturer’s instructions. Tissue samples were homogenised by bead-milling in the presence of lysis buffer RA1 and β-Mercaptoethanol. This was carried out in a Quiagen Tissue Lyser for 2 minutes at 20Hz.

When RNA was being extracted from cells, the suspension was centrifuged at 12,000g for 3 minutes. The supernatant was discarded and the pellet resuspended in β-mercaptoethanol.

The samples were loaded in a NucleoSpin Filter and centrifuged for 1 minute at 11,000g in order to reduce the viscosity and clear the lysate. The filter was discarded and 70% ethanol added to the homogenised lysate in order to adjust the RNA binding conditions. The lysate was then added to a Nucleospin RNA II column and centrifuged for 30s at 11,000g. Membrane desalting buffer was added to the silica membrane and centrifuged at 11,000g for 1 minute to dry the membrane. Contaminating DNA, which is also bound to the silica membrane was removed by incubating with rDNAse solution for 15 minutes at room temperature. The columns were washed with Buffer RA2 for 30s at 11,000g. In order to complete the washing steps to remove salts, metabolites and macromolecular cellular components, the columns were washed with
Buffer RA3 for 11,000g for 30s then repeated for a further 2 minutes. To elute the RNA, RNase-free water was added to the column and centrifuged at 11,000g for 1 minute.

2.8.2 RNA extraction from Formalin Fixed Paraffin Embedded Tissue (FFPET)

RNA was extracted from FFPET using an RNeasy FFP kit (Quiagen). Tissue sections of 10µm thickness were cut and placed in a sterile eppendorf. To de-paraffinise the sections 320µl of xylene was added to the tissue and vortexed for 10s. This was then incubated at 56°C for 3 minutes and allowed to cool to room temperature. 150µl of Buffer PKD lysis buffer was added, mixed by vortexing then centrifuged for 1 minute at 11,000g. In order to release RNA from the tissue 10µl of proteinase K was added and incubated for 15mins at 56°C followed by a further 15 mins at 80°C. After this, the lower clear phase was transferred into a new 2ml microcentrifuge tube and incubated on ice for 3 minutes and centrifuged for 15 minutes at 20,000g for 15mins. The supernatant was transferred to a new microcentrifuge tube, not disturbing the pellet which contained insoluble debris including crosslinked DNA. In order to remove genomic DNA, DNase stock solution was added and mixed by inverting the tube and incubated for 15mins. Following this reaction 320µl of buffer RBC was added to adjust binding conditions followed by a further incubation of 720µl of ethanol and this was mixed well by pipetting. The sample was then transferred to a RNeasy MiniElute spin column and centrifuged for 15s at >8000g. The flow through was discarded. Immediately afterwards 500µl of Buffer RPE was added to the column and the above
centrifuge repeated, with the further flow through discarded. A further 500µl of buffer RPE was added and centrifuged for 2 minutes to flush the membrane. The RNeasy MiniElute spin column was transferred to a new collecting tube. In order to dry the spin column of residual ethanol (which may interfere with downstream reactions) the lid of the spin column was opened and centrifuged full speed for 5 minutes. Finally, the RNA was eluted by adding 20µl of RNase-free water and centrifuged for 1 minute.

2.9 Reverse Transcription of mRNA

RNA was quantified by assessing absorbance at 340nm using a Nanodrop 1000 spectrophotometer (ThermoScientific). 1µg of mRNA was reverse transcribed using a RT kit (Applied Biosystems, Warrington, UK). The 1µg mRNA was adjusted to a volume of 10µl using RNAse free water. In order to anneal samples mRNA was mixed with 2µl of random primers and placed in a PTC Thermal Controller (MJ Research) at 70°C for 10 minutes. The samples were then mixed with 9µl reaction mix (Table 2.2) and placed on thermal controller for 1 hour at 42°C. cDNA was stored at -20°C.

2.10 Quantitative Real Time PCR

Taq Man® inventoried probes were used for mRNA detection and quantification. Briefly, TaqMan® probes are oligonucleotides that have a
Table 2.2  Illustration of reaction mix reagents and concentrations used in reverse transcription of mRNA.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume / Reaction (µl)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x RT Buffer</td>
<td>2</td>
<td>1x</td>
</tr>
<tr>
<td>25 x dNTP mix (100mM)</td>
<td>0.8</td>
<td>4 mmol</td>
</tr>
<tr>
<td>10x RT Random primers</td>
<td>2</td>
<td>1 x</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>RNAase Inhibitor</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>
fluorescent reporter dye attached to the 5' end and a quencher moiety coupled to the 3' end. These probes are designed to hybridize to an internal region of a PCR product. In the unhybridized state, the quencher molecule inhibits the fluorophore activity whilst remaining in close proximity. The polymerase activity of DNA polymerase initiates synthesis of strand in 5'→3' direction and cleaves the fluorophore molecule attached to the 5' end of the primer which generates a fluorescent signal proportional to the amount of probe cleavage.

To perform the assay 2µl of cDNA was mixed with 1µl of x20 TaqMan® probe and a further 10µl of mastermix and 7µl of RNase-free dH₂O in 96 well fastplates. The plate was run for polymerase chain reaction set for 40 cycles on an Applied Biosystems Fast Real time 7500 PCR machine using an automated program as outlined (Table 2.3).

The PCR study was carried out using the SDS Software 1.3.1. Quantification was performed using the comparative Ct method. In this analysis the computer generates a threshold cycle (Ct) value for each sample where fluorescence passes a threshold and involves comparing the Ct values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The Ct values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene.

The comparative Ct method is also known as the ΔΔCt I and involves subtracting the Ct of the internal control e.g. 18s from the sample of interest.
Table 2.3 Outline of the thermal cycles for RT-PCR.

<table>
<thead>
<tr>
<th>Step</th>
<th>Duration</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Uracil-N-glycosylase (UNG) activation</td>
<td>2 minutes</td>
</tr>
<tr>
<td>02</td>
<td>DNA polymerase activation</td>
<td>10 minutes</td>
</tr>
<tr>
<td>03</td>
<td>Melting</td>
<td>15 seconds</td>
</tr>
<tr>
<td>04</td>
<td>Annealing&amp; extension</td>
<td>1 minute</td>
</tr>
</tbody>
</table>
2.11 Tissue Digestion and Cell Isolation

Kidneys were minced into small pieces with a scalpel blade and digested in 8ml solution containing RPMI medium with Collagenase B (128µl 100mg/ml, Roche Applied Sciences) and DNAse 1 (80l 10mg/ml, Roche Applied Sciences) for 45 minutes at 37°C. The samples were centrifuged at 4°C for 5 minutes at 300g. The solution underwent further digestion with Collagenase B for 15 minutes at room temperature. The samples were centrifuged for 15 minutes and resuspended in 4 mls of RPMI. The resulting kidney homogenate was passed through a 45µm filter forming a single cell suspension. Cells were incubated in red cell lysis buffer for 10 minutes and washed with PBS -/-.

20 x10^6 cells from each animal were blocked in 10% mouse serum for 20 minutes and stained with anti-CD11b RPE (1:100 ABDserotec) for 30 minutes. Samples were washed in PBS (-/-) and centrifuged for 5 minutes at 12,000g and resuspended in Hanks solution with 1% FCS. Cells were stained with Propidium Iodide (PI) immediately prior to selection. Positive selection was made on PI negative cells. Samples were collected in Hanks solution with 10% FCS (Figure 2.1).

2.12 Enrichment of CD11b positive Cells using indirect magnetic labelling separation.

Harvested kidneys were digested and stained with CD11b-PE as above. After incubation with primary antibody the cells were washed with buffer (0.1% BSA
in PBS-/−) for 10 minutes. Cells were then incubated with MACS anti-PE microbeads (Miltenyi) for 15 minutes and washed twice with buffer for 10 minutes. (Figure 2.2) The cell suspension was added to a MACS column and then placed in a MACS separator for 5 minutes and washed 3 times with buffer. Unlabelled cells passed through the columns and were collected whilst labelled cells were released after removal of the column from the magnet. A sample from both labelled and unlabelled fractions underwent flow cytometric analysis to determine the purity of CD11b positive cells.

2.13 Statistical Analysis

All analysis was carried out using GraphPad Prism. Groups were compared using unpaired t test and expressed as mean ± SEM. Where multiple conditions were compared one-way ANOVA for repeated measurements. P less than 0.05 was considered significant.
3. Time course and Mechanism of Lymphatic Reconnection in the Rodent Kidney

3.1 Introduction

At the time of kidney transplantation the donor renal artery and vein are anastomosed to recipient vessels. In contrast, the lymphatic vessels located within the hilar connective tissue are ligated \textit{en bloc} and divided, resulting in complete disruption of the lymphatic drainage from the graft. The renal lymphatic system plays a key role in mediating the afferent limb of the immune response and in maintaining interstitial tissue homeostasis. There is no available data regarding either the kinetics or nature of the process of lymphatic reconnection following transplant in rodents or human. Early work carried out in dog autograft kidneys suggested that there was functional restoration of lymphatic drainage between one and two weeks following transplantation. Regeneration of lymphatic vessels was assessed by intraparenchymal generation of 2\% Evans blue (Mobley and O'Dell 1967). We therefore established an \textit{in vivo} model of lymphatic disruption in order to study the time course and mechanism of lymphatic regeneration and re-connection within the native kidney.
3.2 Assessment of the lymphatic drainage of the normal rodent kidney.

The first objective was to identify the anatomy of lymphatic drainage of a normal kidney. To achieve this rats were anaesthetised, a midline laparotomy was performed and 5µl of carbon black was injected into the subcapsular space of the kidney as previously described (chapter 2). Carbon black was purchased as commercially available Indian ink, prepared by ultrafiltration through a 45nm PES membrane (Millipore) and diluted in sterile PBS (1:1 ratio) prior to injection. The animal was recovered and sacrificed 24 hours following injection having displayed no adverse effects from the surgical procedure. At post-mortem carbon black was retained within the subcapsular space (Figure 3.1) of the kidney. In addition there was macroscopic evidence of localisation of carbon black particles within the renal lymph node and the parathymic nodes in the chest (Figure 3.2). The renal lymph node is located superior to the origin of the renal artery from the abdominal aorta, and this was examined microscopically. Sections stained with Haematoxylin and Eosin displayed carbon deposits that appeared to lie within phagocytic cells and in the parasinusoidal spaces. Immunostaining with the macrophage marker ED-1 demonstrated that carbon black was evident within ED-1 positive cells suggesting that carbon particles are ingested in the lymph node or that macrophages ingest carbon particles in the renal subcapsular space and migrate to the hilar lymph node (Figure 3.3).
Figure 3.1
Figure 3.2
Further subcapsular injection was performed with assessment carried out at earlier time points. Carbon staining was discovered as early as 4 hours following subcapsular injection (n=3) (Figure 3.4). Earlier time points were not examined.

3.3 Intra-parenchymal injection of Evans Blue identifies the lymphatic trunks

Injection of carbon black successfully identified the draining lymph nodes but failed to delineate the course of individual lymphatic vessels draining the kidney. Evan’s blue is a diazo compound which binds to albumin and has a well established role in delineating lymphatic vessel anatomy. Under terminal anaesthesia intra-parenchymal injection of Evans blue failed to identify the presence of lymphatic vessels on the anterior aspect of the renal pedicle. The posterior aspect of the renal pedicle was then examined by mobilising the kidney and reflecting it medially, thereby exposing the posterior surface of the renal artery. Intra-parenchymal injection of Evans blue revealed 3-4 discrete lymphatic trunks that arose from the renal hilum and ran into the renal lymph node along the posterior aspect of the renal pedicle (Figure 3.5). This reached the renal lymph node within 30 seconds, which is in striking contrast to the kinetics seen with carbon black injection. Although early time points were not examined, by conclusion of the surgery there was no evidence of carbon staining in the renal lymph node. The difference in timing may result from the movement of particulate material (carbon black) versus solute (Evans blue).
3.4 Lymphatic disruption prevents trafficking of carbon black from the kidney

In order to study the process of lymphatic reconnection of the transplanted kidneys we first developed a novel method of disrupting the lymphatic drainage of a native kidney in situ. We surgically disrupted the lymphatic drainage by removing all tissue between the kidney and the hilar lymph node with the exception of the renal artery and vein (formal surgical 'skeletalisation') (Figure 3.6). We confirmed that this procedure completely disrupted trafficking of carbon black from the kidney to the lymph node by injecting carbon black and examining the kidney at 24 hours. There was no microscopic or macroscopic evidence of carbon localisation in hilar lymph nodes confirming the efficacy of the surgery and effective interruption of the lymphatic drainage (Figure 3.7).

3.5 Time course studies demonstrate that lymphatic reconnection is re-established by 6 days

Time course studies were undertaken to identify the point at which functional reconnection of the lymphatic system was re-established. Following the surgical procedure there was a dense inflammatory mass around the renal hilum at day 4, which made identification of the renal lymph node problematic. There was however, microscopic and macroscopic evidence of carbon staining in the renal lymph node at day 6 (n=5) following disruption (Figure
3.8). Intra-parenchymal injection of Evans blue failed to identify the presence of lymphatic trunks observed in the non-disrupted kidney.

### 3.6 Histological assessment of the renal hilum following lymphatic disruption

We then removed the tissue between the kidney and lymph node *en bloc* and undertook a careful histological assessment of every 10th serial tissue section. Staining with the lymphatic marker podoplanin yielded striking findings. In normal tissue, we noted 2 to 3 very large lymphatic trunks with a strongly podoplanin positive endothelium (Figure 3.9) reflecting the hilar lymphatic trunks delineated by intra-parenchymal injection of Evans blue. In contrast, day 6 tissue from functionally reconnected kidneys demonstrated a network of small lymphatic vessels with no large lymphatic trunks evident (Figure 3.10).

The lymphatic nature of these small vessels was confirmed by further immunostaining with prox-1 (a second lymphatic marker) as evident by positive nuclear staining. (Figure 3.11). In order to assess the proliferation status of the lymphatic vessels dual immunostaining with podoplanin and the proliferation marker PCNA was performed. This demonstrated striking numbers of PCNA⁺ nuclei located within the renal hilum. A number of the podoplanin⁺ lymphatic vessels had PCNA⁺ nuclei which indicates engagement of cell proliferation machinery (Figure 3.12). VEGF-C immunostaining was
technically problematic and this prevented identification of the pattern of this growth factor expression.

3.7 Summary

• Carbon black readily migrates to the renal lymph node as early as 4 hours following subcapsular.

• The lymphatic drainage of the kidney consists of 3-4 trunks which run into the renal lymph node, lying at the origin of the renal artery.

• We describe an in vivo model of lymphatic disruption in the native rodent kidney

• Following lymphatic disruption there is evidence of functional reconnection at day 6

• When examined histologically, the regenerating lymphatic system consists of a network of small proliferating lymphatic vessels, which is in contrast to the defined lymphatic trunks in the normal kidney.
4. Lymphangiogenesis in experimental models of renal transplantation

4.1 Introduction

Previous work in our laboratory demonstrated significant intra-renal lymphangiogenesis in human failed transplant nephrectomy specimens (Adair, Mitchell et al. 2007). This was seen in association with prominent macrophage infiltration and loss of peritubular capillaries. In the seminal study by Kerjaschki and colleagues, de novo lymphangiogenesis was detected in the acutely rejecting renal allograft as early as 4 days following transplantation (Kerjaschki 2006). These areas of lymphatic vessel proliferation were associated with nodular infiltrates in a subset of patients with allograft rejection, which contained a variable number of alloreactive B and T lymphocytes. The significance of lymphangiogenesis as a pathological entity remains unclear. Two further retrospective studies have attempted to correlate lymphangiogenesis with its impact on prognosis. These studies unearthed contrasting results. Yamamoto and colleagues observed that increased lymphatic vessel number was significantly associated with rejection episodes (Yamamoto, Yamaguchi et al. 2006) whilst a later study by Stuht and colleagues found that patients with de novo lymphatic vessel formation were found to have improved graft function and survival at one year (Stuht, Gwinner et al. 2007). Both studies were limited by patient numbers and absence of long term follow up.
The work outlined in this chapter explored whether lymphangiogenesis was evident in two distinct experimental models of renal transplantation. First we examined the process in the context of acute cellular rejection using a murine model. Secondly using a heterotopic rodent model of rat interstitial fibrosis and tubular atrophy, we assessed whether lymphangiogenesis occurs in chronic allograft damage, mirroring our previous work on human material. These studies lay the foundation for future interventional studies in order to establish whether modulation of lymphangiogenesis affects the rejection process and the long term functional and structural outcome of renal transplantation.

4.2 Characterisation of a murine renal transplant model

Renal transplants were performed between Balb/c donors and FVB/nj recipients (allografts, n=8), and between FVB/nj donor and recipients (isografts n=8), (Figure 4.1). In this model there is discordance at the H2 haplotype with acute cellular rejection evident at day 7 (Qi, Adair et al. 2008). Histological analysis was chosen on day 7 as pilot data generated by Dr Qi and Dr Adair indicated significant macrophage, T-cell and B-cell accumulation and tissue injury at this time point. Pilot studies also suggested that lymphangiogenesis was evident on day 7.
4.3 There was no evidence of lymphangiogenesis in the acutely rejecting renal allograft at day 7.

At day 7 there was no evidence of interstitial lymphatic vessels in isografts or allografts. As previously described (chapter 1), lymphatic vessels normally accompany arterial structures up to the level of the interlobular vessels. When the number of fully formed podoplanin$^+$ vessels per identifiable arterial profiles was quantified there was no difference between isografts and allografts (3.1±0.5 vs 2.7±0.2; isograft vs allograft; $p=0.34$, ns) (Figure 4.2). In order to confirm these findings further immunostaining and quantification with a second LEC marker LYVE-1 was performed. These results mirrored the earlier findings with no increase in lymphatic vessel number at day 7 (2.6±0.6 vs 2.9±0.2; isograft vs allograft; $p=0.63$,ns), thus confirming that lymphangiogenesis does not occur in this model at this time point (Figure 4.3). Further experiments with this model were not pursued. Although prominent rejection was evident at day 7 an important caveat is that *de novo* lymphangiogenesis may occur at a later time point.

4.3.1 Peri-arterial lymphatic vessels contain a variable number of inflammatory cells

A consistent and interesting finding was that many of the peri-arterial lymphatic vessels were dilated and contained a large number of inflammatory cells, which may suggest that they have a functional role (Figure 4.4). Further studies are required in this area, but data presented earlier in this thesis suggest that by day 6 there is functional reconnection of the lymphatic system.
This would suggest rapid and marked leukocyte trafficking from the allograft if the lymphatic vessels are reconnected to the systemic lymphatic system. Alternatively it may represent an accumulation of leukocytes attempting to egress from the rejecting allograft as a consequence of on-going disruption of renal lymphatic drainage.

4.4 Characterisation of a rodent model of Interstitial fibrosis and tubular atrophy

Renal transplants were performed between Fischer donors and Lewis recipients (allograft group, n=7), and between Lewis donors and recipients (isograft control group n=5). A contralateral nephrectomy was performed on day 10 to establish this as a functional model (Figure 4.5). The experiment was terminated at 52 weeks or when animals exhibited features of uraemia. There was no statistical difference in the study duration between the groups. Serum and urine biochemistry and blood pressure were performed and obtained by personal communication from Mr B Shrestha and Dr J Haylor (University of Sheffield).

4.4.1 Allografts exhibit interstitial fibrosis and tubular atrophy

Histological examination of allografts at the end of the study revealed glomerulosclerosis and tubulointerstitial changes characterised by increased deposition of fibrillar collagen and tubular atrophy (Figure 4.6). In contrast, isograft controls exhibited minimal glomerular and tubulointerstitial disruption.
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 to isografts (Figure 4.7 A-F).

4.4.2 Rats with allogeneic transplants develop renal failure and hypertension
At the termination of the experiment rats with allografts had significant renal dysfunction (Table 4.1) 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/24h; allograft vs isograft; p<0.05) and systolic hypertension (systolic BP 155±15 vs 120±7 mmHg; allograft vs isograft; p<0.05). Personal communication, Mr B Shrestha and Dr J Haylor.

4.4.3 Immunophenotyping of the inflammatory cellular infiltrate
The mononuclear cell infiltrate consisted predominantly of ED-1+ macrophages (Figure 4.8) and CD3+ T-lymphocytes (Figure 4.9). 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
Table 4.1 - Allografts exhibit renal dysfunction, proteinuria and systolic hypertension

<table>
<thead>
<tr>
<th></th>
<th>Isograft</th>
<th>Allograft</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria (mg/24hr)</td>
<td>23±6</td>
<td>357±114*</td>
</tr>
<tr>
<td>Serum Creatinine (µmol/l)</td>
<td>64±7</td>
<td>180±63*</td>
</tr>
<tr>
<td>Creatinine Clearance (ml/min)</td>
<td>1.8±0.4*</td>
<td>0.7±0.25</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>120±7</td>
<td>155±15*</td>
</tr>
</tbody>
</table>

All data is mean ± SEM, *p<0.05
large blood vessels within the cortex as has been described previously in chronic kidney allograft rejection (Figure 4.10).

4.5 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 (Figure 4.11). The number of lymphatic vessels situated in a perivascular location was comparable between experimental groups (2.9±0.2 vs 2.6±0.3 perivascular lymphatic vessels/field; allograft vs isograft; p>0.05). In contrast, there was a striking 18-fold increase in the number of lymphatic vessels evident in the tubulointerstitium of allografts compared to isografts (9.3±1.8 vs 0.5±0.3 tubulointerstitial lymphatic vessels/field; allograft vs isograft; p<0.05) (Figure 4.12).

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 as indicated by picrosirius red staining and lymphatic vessel density (Figure 4.13, R = 0.53, P<0.05). Unfortunately mRNA and frozen tissue was not available for further study. 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±0.1; allograft vs isograft; p=ns) 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.
4.6 Summary

• In a murine model of acute allograft rejection there is no evidence of *de novo* lymphangiogenesis at day 7 after immunostaining for podoplanin and LYVE-1.

• Collaborative studies using a rodent model of chronic allograft failure, revealed allografts with features of interstitial fibrosis and tubular atrophy with impaired renal function.

• Allografts have significant intra-renal infiltrate of macrophages and T-cells

• There was no difference in the number of perivascular lymphatic vessels between isografts and allografts.

• There was a striking 18-fold increase in the number of interstitial lymphatic vessels in allografts, being a feature in every biopsy examined. This is correlates with interstitial fibrosis raising the possibility that lymphatic vessel number reflects tissue damage and scarring rather than an immunological mediated process.

• These results mirror published data in human transplant nephrectomies with chronic allograft damage. (Adair, Mitchell et al. 2007).

• These findings may permit interventional studies guiding potential future therapies.
5 Inflammatory lymphangiogenesis in unilateral ureteric obstruction

5.1 Introduction

Previous work presented in this thesis described increased lymphatic vessel number in a rodent model of IFTA. These were interstitial in location and associated with accumulation of macrophages and T-cells and interstitial fibrosis. Unfortunately there was no frozen tissue or mRNA available from our Sheffield collaborators to perform gene expression studies. Lymphangiogenesis is associated with inflammation, macrophages and scarring. We therefore studied the *in vivo* model of unilateral ureteric obstruction (UUO) in the rat; an established model of inflammation leading to parenchymal scarring (Nagle, Bulger et al. 1973; Sharma, Mauer et al. 1993; Klahr and Morrissey 2002; Chevalier, Forbes et al. 2009). This allowed assessment of lymphangiogenesis in a non-immunological dependent model and facilitated the study of macrophages and VEGF-C expression.

5.2 Characterisation of the model of Unilateral Ureteric Obstruction (UUO) in the rat.

A surgical model of UUO was established in Sprague-Dawley rats. At the time of operation the left ureter was ligated with two 5/0 sutures and divided between these points. An initial time course study was conducted with the
removal of the obstructed kidney at one, two and three weeks following surgery (Figure 5.1).

Ureteric ligation results in progressive hydroureter and hydronephrosis of the obstructed kidney. Macroscopic features include dilation of the renal calyces, pale discolouration and oedema of the parenchyma, the severity of which depends on the duration of obstruction (Figure 5.2). Histological examination revealed a dense inflammatory infiltrate of mononuclear cells, which was accompanied by tubular dilation and tubulointerstitial fibrosis (Figure 5.3).

5.2.1 ED-1 positive macrophages are a feature of the obstructed kidney

Immunostaining for the macrophage ED-1 antigen demonstrated significant expression in the inflammatory infiltrate of the tubulointerstitium of obstructed kidneys. In the normal kidney there is a sparse population of resident tissue macrophages, which exist in the quiescent state, but following ureteric ligation there is a significant accumulation of macrophages in the obstructed kidney (Figure 5.4). When quantified the number of ED-1 cells increased reaching a maximum at 2 weeks following obstruction however this number decreased at week 3. (2. ±0.9 vs 32.± 0.5 ED1+ cells/hpf; control vs week 2 UUO; p<0.05)

5.2.2  T and B-Lymphocytes traffic to the obstructed kidney

Although the mononuclear cell infiltrate consisted predominantly of ED-1 positive macrophages, CD3 positive cells were also frequently encountered in
the interstitium of the obstructed kidney. When assessed by quantifying the number of cells per high power field (x400 magnification) the number of T-lymphocytes were maximal at week 3 when compared to normal control kidney (2.6 ±0.7 vs 85.7± 6.4 CD3+ cells/hpf; control vs week 3 UUO; p<0.0001) (Figure 5.5).

The B-lymphocyte infiltrate in the obstructed kidney was much sparser than the T cells and was located in dense periarterial infiltrates of the cortex (Figure 5.6). These were not evident in the normal control kidney. This is consistent with observations in a number of experimental models and studies in human disease, including renal allograft rejection (Thaunat, Field et al. 2005; Kerjaschki 2006; Thaunat, Patey et al. 2006).

5.3 De novo lymphangiogenesis develops in kidneys which have undergone Unilateral Ureteric Obstruction.

5.3.1 Kinetics of lymphangiogenesis in the obstructed kidney

After one week of unilateral ureteric obstruction there was increased numbers of podoplanin positive lymphatic vessels in the kidney (2.1±0.8 vs 0.5±0.1; podoplanin+ vessels/hpf; week 1 UUO vs control; p=0.0806). These de novo lymphatic vessels were distributed throughout the tubulointerstitium of the cortex in the absence of adjacent vascular structures that typically accompany normal lymphatic vessels (Figure 5.7). Quantification of podoplanin+ lymphatic vessels within the cortex revealed that the numbers increased with the duration of obstruction reaching a maximum at 3 weeks (12.9±1.9 vs 0.5± 0.1
podoplanin \^{}vessels/hpf; week 1 UUO vs control; p=0.0002) (Figure 5.7). No lymphatic vessels were identified in the medulla.

5.3.2 Interstitial lymphatic vessels contain a variable number of inflammatory cells

A number of the interstitial lymphatic vessels contained inflammatory cells within their lumen, indicating that they have a role in the trafficking of the inflammatory infiltrate to draining lymph nodes. In this model there has been no manipulation on the lymphatic drainage of the native kidney. Dual immunostaining with the macrophage marker ED-1 and podoplanin confirmed the presence of a dense peri-lymphatic infiltrate with evidence of an intraluminal macrophage (Figure 5.8).

5.3.3 Serial sections with different lymphatic markers confirm lymphatic phenotype

In order to rigorously confirm the presence of \textit{de novo} interstitial lymphatic vessels additional immunostaining of serial sections for podoplanin and prox-1 was undertaken. The positive nuclear stain from PROX-1 localised to the area of membrane staining observed with podoplanin (Figure 5.9), which confirmed the lymphatic phenotype of these vessels.

5.3.4 \textit{De novo} lymphatic vessels were actively proliferating in the interstitium

We next performed dual immunostaining with podoplanin and the proliferation marker PCNA to determine if the interstitial lymphatic vessels were actively proliferating. This revealed several interstitial lymphatic vessels that
exhibited PCNA$^+$ nuclei indicating engagement of the cell cycle machinery (Figure 5.10). Interestingly a number of lymphatic vessels contained inflammatory cells, which were PCNA positive, indicating recent proliferation of these cells.

5.4 VEGF-C expression is increased in the obstructed kidney

The mRNA expression of VEGF-C, the major lymphangiogenic mitogen was assessed in whole kidney tissue. qRT-PCR revealed an increase in expression at 1, 2 and 3 weeks following obstruction compared to normal control tissue. This reached a maximum at 2 weeks (20.8± 4.10, fold increase in relative expression to control tissue, p=0.015) and started to decline in week 3 (11.3± 1.7, fold increase in relative expression to control tissue p= 0.007). (Figure 5.11).

5.4.2 Attempts at VEGF-C immunostaining were unsuccessful

Significant efforts were made trying to achieve VEGF-C immunostaining. We employed 3 different anti-VEGF-C antibodies that had been successfully used by other investigators (Kerjaschki, Huttary et al. 2006; Machnik, Neuhofer et al. 2009). Three different tissue fixatives were employed including formalin, methyl-carnoys solution, paraformaldehyde and frozen tissue. In addition, multiple antigen retrieval techniques were employed including heat mediated (citrate buffer, TrisEDTA) and enzymatic (proteinase K and Trypsin) treatment. Although we did not use a specific positive control the rat UUO tissue
exhibited significant interstitial lymphatic endothelial proliferation suggesting that VEGF-C protein should be present at a significant level.

5.5 TonEBP expression is increased in the obstructed kidney

Recent work has demonstrated a role for macrophage Tonicity Enhanced Binding Protein (TonEBP), which responds to changes in extracellular tonicity in regulating the transcription of VEGF-C. We therefore assessed mRNA expression of TonEBP at the same time points. There was a sharp increase in the expression of TonEBP at week 1 (18.52± 4.28, fold increase in relative expression to control tissue p=0.0022) which continued into the second week (23.26 ± 4.1, fold increase in relative expression to control tissue, p=0.0006), (Figure 5.12). However, there was a rapid decline in the expression at 3 weeks (3.03± 0.81, fold increase in relative expression to control tissue) Interestingly this mirrored the reduction in the expression of VEGF-C. One could speculate that at week 3 the increase in lymphatic vessels would act to promote clearance of interstitial oedema and cellular infiltrates, which in turn reduces the expression of TonEBP and VEGF-C.
5.6 Summary

- UUO in the rat is associated with tubular dilation, prominent macrophage and T cell infiltration and occasional aggregation of B-cells.
- A dramatic increase in the number of podoplanin\(^+\) interstitial lymphatic vessels is evident following UUO.
- Lymphatic vessels are also positive for Prox-1 and were noted to contain mononuclear cells suggesting they contributed to cell trafficking.
- Lymphatic vessels are PCNA positive and high levels of VEGF-C mRNA expression is evident.
- Expression of TonEBP mRNA is also increased during UUO.

6.1 Introduction

A body of evidence indicates that macrophages may play a role in lymphangiogenesis. Various roles have been described including the action of the transcription factor TonEBP in a rat model of hypertension (Machnik, Neuhofer et al. 2009). In addition transdifferentiation of macrophages and bone marrow derived cells into lymphatic endothelial cells had been described in diabetic wound healing (Maruyama, Asai et al. 2007) and corneal injury (Maruyama, li et al. 2005). Work outlined in the previous chapter indicated the occurrence of marked lymphangiogenesis in the inflamed interstitium that contained a prominent macrophage infiltrate. The experiments described in this chapter describe our attempt to dissect the involvement of macrophages in UUO-associated lymphangiogenesis using a macrophage depletion and macrophage isolation strategy. We hypothesised that macrophage depletion would attenuate intra-renal lymphangiogenesis as a result of loss of VEGF-C expressing macrophages.

The most widely published method of specific macrophage depletion is administration of liposomaly encapsulated clodronate. This has been used successfully in a number of experimental models. In a rat model of ischaemia reperfusion injury Ko and colleagues administered liposomal clodronate at
5µl/ g body weight every fifth day and obtained significant depletion in ED1+ macrophages in the kidney (Ko, Boo et al. 2008). We therefore decided to employ a similar depletion strategy to Ko and colleagues.

6.2 Characterisation of the effect of Liposomal Clodronate administration

6.2.1 Administration of liposomal clodronate failed to deplete ED1 cells in the obstructed kidney

In order to characterise the effects of depleting the mononuclear phagocyte system in vivo experiments were undertaken with administration of i.v. clodronate at a dose of 5µl per g/body weight (Figure 6.1). A pilot study was performed to assess the effect on animal welfare, as there was concern in our animal unit regarding safety of in vivo liposomal clodronate following animal surgery. Rats tolerated UUO and showed no adverse effects following intravenous administration of clodronate.

In contrast to published data (Jose, Ikezumi et al. 2003; Ko, Boo et al. 2008) liposomal clodronate failed to eliminate ED-1+ macrophages within the kidney. Quantification of the ED-1+ (Figure 6.2) infiltrate revealed no statistical difference between the number of positive pixels between PBS and clodronate treated animals in both cortex (1.4 ±0.32 vs 2.4± 0.4 % area+; PBS vs Clodronate treated; p=0.085 ns), and medulla (1.6±0.4 vs 2.4± 0.2 %area+):
PBS vs Clodronate treated; p=0.096 ns) despite the final dose being administered 24 hours prior to culling.

6.2.2 Clodronate effectively depleted ED1 macrophage populations in the spleen and liver.

Despite failing to deplete ED-1$^+$ cells in the kidney, significant macrophage depletion was evident in both liver and spleen. In the liver we observed considerable ablation of the Kupffer cell populations. As the Kupffer cell can be readily identified they were quantified by counting the number of cells per high power field. This indicated dramatic ablation (26.8±3.3 vs 1.7±0.5 ED1$^+$ cells; PBS vs Clodronate treated; ***p<0.0001) (Figure 6.3). A similar finding was evident in the spleen following clodronate administration. The splenic macrophage was quantified by computerised image analysis in view of the close proximity of macrophages. This revealed a marked decrease in ED1$^+$ (7.6±0.56 vs 0.735±0.17% area$^*$; PBS vs Clodronate treated; ***p<0.0001), (Figure 6.4). This effect on ED-1 populations in both liver and spleen are consistent with published data in the literature.

6.2.3. ED2$^+$ populations in the kidney, liver and spleen were depleted.

The ED-1 (CD68) antigen is expressed on monocytes or inflammatory macrophages whilst ED-2 (CD 163) is expressed on the membrane of resident macrophages. We observed effective depletion of the ED-2 subpopulation of macrophages in kidney, liver and spleen. In the kidney the number of ED-2
positive pixels was markedly reduced in both cortex (0.3±0.1% vs 0.01% area^+; PBS vs Clodronate treated; *p=0.032) and medulla (0.3±0.1 vs 0.02±0.01% area^+; PBS vs Clodronate treated; *p=0.03) in the group which received clodronate (Figure 6.5). Similar effects were seen in both spleen (5.3±1.0% vs 0.002% area^+; PBS vs Clodronate treated; ***p=0.003) (Figure 6.6) and liver (43±2.4 vs 0 ED1^+ cells; PBS vs Clodronate treated; **p=0.0011, n=3 (Figure 6.7).

6.3 Administration of liposomal clodronate and ED2^+ macrophage depletion in the kidney does not modulate lymphangiogenesis

6.3.1 Quantification of Lymphatic vessel density revealed no difference in animals treated with liposomal clodronate and controls.

Assessment was made on the effect of clodronate administration on interstitial lymphangiogenesis. Five doses of clodronate were administered between day 6 and day 20, with animals being culled on day 21. By quantifying the number of fully formed podoplanin positive vessels per x200 magnification field we observed no difference between the groups (9.5±1.6 vs 11.6±1; PBS vs Clodronate treated; p=0.28,ns) (Figure 6.8)

6.3.2 Clodronate administration resulted in no statistical significance in VEGF-C and Prox-1 expression between both groups.

The mRNA expression of VEGF-C demonstrated a reduction in the clodronate treated group (15.1± 2.4 vs 8.2± 2.7; fold increase in relative expression to control tissue; PBS vs Clodronate treated; p=0.099) however, this failed to
reach statistical significance (Figure 6.9). Further gene expression studies include Prox-1, interestingly this demonstrated an increase in expression in clodronate groups compared to PBS treated animals (3.1±0.5 vs 6.5±2.1; fold increase in relative expression to control tissue; PBS vs Clodronate treated) (Figure 6.10) which failed to reach statistical significance.

6.4 Gene expression studies on cell sorted populations

6.4.1 Constituent cells which may be responsible for VEGF-C expression

In our experimental model of UUO intravenous administration of liposomal clodronate effectively depleted ED2+ populations in the kidney but not ED1+ cells. This was despite effective depletion of ED1+ splenic macrophages and Kupffer cells. In order to investigate whether ED1+ cells make a significant contribution to VEGF-C expression we attempted to perform gene expression studies on macrophage isolated populations using flow cytometry and magnetic bead immunopurification.

6.5 Immunostaining for ED-1 positive cells did not permit subsequent gene expression studies following cell sorting.

Animals underwent either UUO or a sham procedure as described in chapter 2 and culled at day 14. The day 14 time point was chosen by earlier work as this was the time point demonstrating maximal VEGF-C expression. Provisional
experiments using our established protocol of digesting whole kidney (chapter 2) were performed. Following mechanical and enzymatic disruption into single cell suspension, cells were stained with anti-CD68PE (ED-1) and sorted by flow cytometric analysis into positive and negative populations.

It became apparent immediately that cells in both populations had undergone necrosis as quick-diff staining of cytospin samples revealed cell debris and “cell ghosts”. ED-1 is an intra-cellular antigen expressed on the endoplasmic reticulum within the cell. As part of the immunostaining protocol permabilistion with TRITON is necessary in order to optimise the quality of intracellular staining. The detergent destroyed RNA from both positive and negative yields.

6.6 Cell sorting of CD11b-PE stained cells resulted in a population with a purity of 97%

Given that further experiments with CD68 (ED-1) were not possible, alternative markers were sought. CD11b is a cell surface protein expressed by cells of myeloid lineage, predominately monocytes and macrophages, however, it is also expressed by neutrophils and to a lesser extent dendritic cells. As previously described animals underwent a sham procedure or UUO and culled after 14 days. Kidneys were digested into cell suspension and stained with anti-CD11b-PE. Prior to cell sorting they were stained with Propidium iodide (PI). A gate was placed on populations which were CD11b⁺ and PI⁻. Following sorting an effluent containing CD11b positive and negative
populations was obtained. A sample from CD11b\(^+\) populations were analysed and this confirmed purity of 97% (Figure 6.11)

### 6.7 CD68 and E-cadherin expression were upregulated in CD11B positive populations

In order to assess the integrity of our RNA we performed qRT-PCR on genes expected to be upregulated in either CD11b negative or positive populations. Expression of CD68 was assessed between CD11b negative and positive populations from the kidneys harvested from animals which have undergone UUO, rather than include sham controls. This was due to the presence of greater cell numbers harvested from obstructed tissue with higher numbers of CD11b positive cells than those obtained in sham kidneys. CD68 expression was significantly increased in the CD11b positive cells when compared to negative population (9.4±1.9 ;relative expression to control tissue; *p= 0.013), (Figure 6.12). This would suggest that gene expression is detectable following the isolation procedure.

E-Cadherin is a cytokeratin expressed in tubular epithelial cells. The level of expression of E-cadherin was also assessed between the two populations. This confirmed an increase in expression in the CD11b negative populations (3.1+0.4; relative expression to control tissue;** p=0.0076 Figure 6.13) This result is consistent with the CD11b positive cells being predominantly of myeloid lineage whilst CD11b negative cells are a heterogenous population compromised of tubular, endothelial, myofibroblasts and T-cells. In addition,
there was also increased expression of alpha SMA expression (Figure 6.13) in CD11b positive population however, this failed to reach statistical significance (6.1±2.9; relative expression to control tissue ns p=0.13)

6.8 RT-PCR studies demonstrated no difference in expression of VEGF-C between the CD11b negative and positive populations.

Following cell sorting, RNA was extracted and mRNA reverse transcribed into cDNA. qRT-PCR was then performed firstly with VEGF-C, the key lymphangiogenic mitogen. Expression was compared of CD11b positive cells sorted from sham and UUO animals. This revealed no difference in VEGF-C expression between sham and UUO kidneys (1.2±0.2; relative expression to control tissue; p=0.91) (Figure 6.14) As a positive control VEGF-C expression was carried out on whole tissue which had not undergone the digestion, immunostaining and cell sorting process. This identified upregulation of VEGF-C in this sample confirming the integrity of the assay.

In the absence of significant VEGF-C expression in CD11b+ populations we considered whether CD11b- cells were responsible for driving the upregulation seen in whole kidney tissue. This heterogenous population consisted of myofibroblasts, epithelial cells and lymphocytes. Using RT-PCR VEGF-C expression was assessed in CD11b- populations isolated from obstructed and control kidney. There was decreased VEGF-C expression in the CD11b negative cells from sham controls when compared to the negative populations from obstructed kidneys (1.45±0.6 vs 0.35±0.1 p=0.0537 Figure 6.14), which
failed to research statistical significance. Assessment of TonEBP expression was not performed as the isolation procedure resulted in cells being suspended *ex vivo* in solutions of varying tonicity which may impact on the gene expression profile.

6.9 Magnetic bead immunopurification resulted in a CD11b enriched population with a purity of 82%.

In view of the inability to identify which cellular population was responsible for the increase in VEGF-C identified in whole tissue we were concerned that there had been degradation in RNA integrity due to the prolonged time each cell suspension was in buffer and room temperature during the cell sorting process. In order to reduce the time required for purification we used magnetic bead sorting of CD-11b-PE positive cells with MACS columns. Animals underwent either sham or UUO procedure and culled at day 14. Following mechanical and enzymatic dissociation the cell suspension was stained with anti-CD11b-PE antibody. As described in chapter 2 the stained cells were incubated with anti-PE antibody conjugated magnetic beads and passed through MACS column selecting the CD11b-PE stained populations as CD11b⁺ cells were retained in the columns of the magnet.

To assess the purity of retained CD11b-PE selected populations and non-retained flow through cells, a sample from each animal was harvested, fixed and assessed by flow cytometry to quantify the percentage of CD11b positive cells present. In UUO kidneys the average percentage purity populations was 68±3% whilst in the negative population there was contamination with
CD11b cells which reached 12±1% (Figure 6.15). In sham controls the purity in enriched and negative populations was (29±1% & 13±1%) respectively. Quick diff staining of each fraction confirmed cell viability in both fractions. (Figure 6.16)

6.10 VEGF-C expression was not increased in CD11b positive or negative populations.

The same gene expression studies, which were earlier performed on flow cytometric sorted populations were repeated using CD11b enriched and negative populations. Similarly, there was no difference in the level of VEGF-C expression between CD11b enriched cells from sham and UUO animals (Figure 6.17). Intriguingly, when we assessed the expression of VEGF-C in CD11b negative populations this was decreased in the kidneys from UUO tissue when compared to sham controls (Figure 6.17). These results mirror the pattern of VEGF-C expression identified in populations which were immunopurified by flow cytometry.

RT-PCR studies were also carried out using CD68, E-cadherin and alpha-SMA. The pattern of gene expression was assessed between CD11b negative populations from obstructed kidneys. CD68 was significantly upregulated in CD11b enriched populations when compared to CD11b negative populations (7.73±1.9, relative expression to control tissue **p=0.0092) (Figure 6.18). There was no difference in expression of E-Cadherin , p=0.17, ns and alpha-SMA between the two groups, p=0.53,ns (Figure 6.19).
The differential expression between the flow cytometric sorted populations and magnetic purification may represent a higher percentage of contaminating cells seen following sorting with MACS columns.

**6.11 Comparison with different housekeeping genes demonstrated no difference in the expression of VEGF-C between CD11b negative and CD11b positive populations.**

The above gene expression studies were used with 18S as the internal control. 18S (18S ribosomal RNA) is generally recommended as it shows less variance when exposed to treatment conditions, however we repeated the RT-PCR using Beta-2 macroglobulin as our housekeeping gene. When VEGF-C expression was assessed there was an increase in expression in the CD11b positive cells from UUO kidney compared to sham however, this failed to reach statistical significance (1.01±0.09 vs 2.35±0.88, p=0.3, ns) (Figure 6.20) In the CD11b negative populations there was no increase in expression (1.06±0.25 vs 0.67±0.25, p=0.33, ns).
6.12 Summary

- Liposomal clodronate depletes ED2 populations in the kidney, liver and spleen but no significant effect on ED1 cells in the kidney despite effective depletion in the liver and spleen.
- Despite obtaining effective depletion of ED2 populations their relative contribution to the inflammatory infiltrate was small, and did not impact on lymphatic vessel proliferation between the groups.
- Clodronate did not affect lymphatic vessels number or VEGF-C expression.
- Macrophage isolation and enrichment using flow cytometry and MACS columns failed to show increase in VEGF-C in the macrophage enriched populations despite increase in CD68 expression.
- Data suggests that non-macrophage cells were a significant source of VEGF-C.
7 Discussions

7.1 Characterisation of the lymphatic reconnection model

These data demonstrate the establishment of an in vivo model of lymphatic reconnection of the native kidney. Initial studies examined the normal lymphatic drainage of the rat kidney using a combination of injection techniques with carbon black and Evan’s blue. Intra-parenchymal injection of Evan’s blue demonstrated a bundle of 3 to 4 large podoplanin positive lymphatic trunks lying posterior to the renal artery draining to the renal lymph node consistent with the published literature (Zhang, Guan et al. 2008). Alternative methodology included injection of carbon black into the subcapsular space. This resulted in localisation of carbon in the renal lymph node within 4 hours of injection.

Surgical disruption of the lymphatic drainage of the rodent kidney prevented carbon trafficking to the renal lymph node at 24 hours. There was evidence of carbon localisation in the draining renal lymph node at day 6 following surgical disruption. These results suggest that a sufficient functional reconnection has taken place by day 6 to permit the trafficking of carbon particles or renal phagocytes that have ingested carbon. Earlier time points were examined but it was not possible to identify the renal lymph node due to the presence of dense inflammatory adhesions. One important consideration with this model is the kidney remains in-situ and, although considerable efforts are made during the surgical procedure to cut all the lymphatic vessels, it is possible that small
lymphatic vessels may remain so that there is incomplete disruption of renal lymphatic drainage. In order to eliminate this potential confounding factor future work could employ an isograft transplant model where complete disruption is certain.

There are a number of important differences with our model in respect to human renal transplantation. During recovery of the kidney the renal hilum is ligated with suture material to prevent lymph leak and subsequent lymphocele formation. This may alter the interstitial pressure within the kidney and the pattern of growth factor expression. Published work demonstrates that ligation of the draining renal lymphatic trunks induced progressive renal dysfunction with associated tubulointerstitial damage. This was associated with upregulation of TGF-β and smad-2 and smad-3 proteins. This is in contrast to our rodent disruption model in which lymphatic trunks are simply divided without ligation. This will likely result in extravasation of lymph into the peritoneal cavity and may alter the lymphangiogenic response. It would be interesting to compare simple division and ligation in the kinetics of lymphatic reconnection.

A further important difference between human transplantation and rodent models is the anatomical location of implantation of renal allografts. In humans allografts are anastomosed to the iliac system and lie in an extraperitoneal position. This is in contrast to rodent models which use an “aortic cuff” technique with direct anastomosis to the abdominal aorta. Accordingly different lymphatic chains are involved and this may impact on the
time course of reconnection. Given the caveats inherent in any murine transplantation model, caution must be taken when translating this to human disease.

It remains unanswered whether lymphatic regeneration is initiated and progresses in a ‘renal lymph node → kidney’ direction, a ‘kidney → renal lymph node’ direction or whether it is bidirectional. Early in vivo work by Swartz and colleagues suggests that lymphatic regeneration is mediated in the direction of lymphatic flow (Goldman, Conley et al. 2007). In the studies undertaken in our lab, serial sections taken from the tissue between the renal hilum and the renal lymph node 7 days following surgical disruption revealed a network of proliferating podoplanin positive lymphatic vessels in association with ED1⁺ macrophages. We attempted to detect VEGF-C using immunohistochemistry in order to identify the cellular source of this key lymphangiogenic growth factor – predicting VEGF-C expression by inflammatory ED1⁺ macrophages. However, these attempts at VEGF-C immunohistochemistry were unsuccessful. Having established the methodology of extracting RNA from paraffin embedded tissue of rat kidneys from our collaborators in Sheffield, it would be informative to perform future studies examining the gene expression of VEGF-C in the serial section tissue. These experiments might show a ‘gradient’ of VEGF-C expression that would suggest to the directionality of lymphatic reconnection.
7.2 Assessment of lymphangiogenesis in experimental models of transplantation.

Seminal work by Kerjascksi and colleagues demonstrated de novo lymphangiogenesis within a subset of patients undergoing acute allograft rejection (Kerjaschki 2006). This was detected as early as day 4 following transplantation. We assessed lymphangiogenesis in a murine model of acute allograft rejection. At day 7 there was no difference in the number of perivascular lymphatic vessels between isografts and allografts. Interstitial lymphatic vessels were not encountered in this model, at the time point which was selected based on data indicating prominent acute rejection at day 7. A number of perivascular lymphatic vessels contained large numbers of leukocytes with a number of ingested apoptotic cells evident within emigrating phagocytes. It is not possible to determine whether these lymphatic vessels are functional and participate in the egress of the cellular infiltrate from the allograft. However, our data suggests that restoration of lymphatic drainage is restored by day 6 albeit in a native kidney model of lymphatic disruption.

Although lymphatic vessels were not encountered at day 7 these data do not exclude the possibility that de novo lymphatic vessels may develop at later time points. Future work would incorporate longer time points after transplantation. In this model transplants are carried out between Balb/c and FVB/nj mice. Different strain combinations may elicit a different pattern of injury within the allograft and the development of lymphangiogenesis may be
dependent on this injury. It would be interesting to assess this with different strain combinations.

As lymphatic vessels were not a feature of our acute rejection model we wished to explore lymphangiogenesis using a model of chronic allograft damage and we therefore established a collaboration between our colleagues in Sheffield (Dr J Haylor and Mr B Shrestha). In this experiment, we demonstrate de novo lymphangiogenesis in a rat model of renal transplantation associated with impaired renal function, proteinuria and systolic hypertension. The renal allograft exhibited features of IFTA that are evident in failing human transplants as well as an inflammatory infiltrate comprising predominantly of macrophages and T cells. B cells were identified in scattered aggregates though the kidney although we did not immunostain for high endothelial venules (PNAd) or plasma cells which are characteristic of lymphoid follicles. 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. In contrast, we noted a striking 18-fold increase in the number of patent interstitial lymphatic vessels in allografts at one-year post transplantation. 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. Further work could use computer software to generate a 3D reconstruction of the lymphatic network from a series of serial sections stained for podoplanin as this might indicate the nature of the connection between interstitial and perivascular
lymphatic vessels.

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 (Rienstra, Katta et al. 2010). 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 data indicates that lymphangiogenesis occurs in different rat strains and both genders.

In our studies, double immunostaining for podoplanin and the proliferation marker 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 late 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 is unclear at present as the interstitial lymphatic vessels were noted to be non-proliferative and quiescent. Further studies will need to be undertaken at earlier time points.

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 (Nykanen, Sandelin et al. 2010). 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.

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 (Dashkevich, Bloch et al. 2009). 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 (Machnik, Neuhofer et al. 2009). It is unknown whether a similar process may be involved in the lymphangiogenesis associated with transplantation.

Lymphangiogenesis has been described as early as 72 hours 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 non-rejecting controls (Kerjaschki, Regele et al. 2004) – a finding confirmed by Yamamoto et al (Yamamoto, Yamaguchi et al. 2006). 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 organised cellular infiltrates resulting in the formation of ectopic ‘germinal centres’ within the rejecting kidney (Thaunat, Field et al. 2005).

A key question is whether the new interstitial lymphatic vessels are derived from cells of the donor or recipient. Lymphatic regeneration may be in response to local proliferation of existing endothelial cells or the incorporation of recipient derived bone-marrow cells or macrophages into lymphatic vessels. In this study we were unable to distinguish between Lewis and Fischer 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 (Kerjaschki, Huttary et al. 2006). In order to elucidate the origin of de novo lymphatic vessels we were very keen to undertake analysis and perform double labeling for both podoplanin (lymphatic marker) and MHC molecules specifically expressed by Lewis rats (the recipient animals) in order to ascertain whether the interstitial lymphatic vessels that arise during the evolution of IFTA are derived from recipient cells. Specific immunostaining against Lewis MHC antigens has been described by Ratcliffe and colleagues in a study examining a chimeric model of autoimmune myocarditis (Ratcliffe, Wegmann et al. 2000). We made vigorous attempts to obtain an antibody that would distinguish between the MHC molecules of Lewis and Fischer rats but following discussions with numerous manufacturers it is now recognised that
the antibodies used in previous studies (RT1A and RT1B) are not able to accurately distinguish between Lewis and Fischer MHC molecules using more modern flow cytometric assays. In addition, both donor and recipient rats were male and this precluded the use of in situ hybridization against the Y-chromosome.

It is important to note that intrarenal lymphangiogenesis may occur in experimental and human chronic kidney disease including the rat remnant kidney model (Matsui, Nagy-Bojarsky et al. 2003) and human diabetic nephropathy (Sakamoto, Ito et al. 2009) and IgA nephropathy (Heller, Lindenmeyer et al. 2007) 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.

7.3 De novo lymphangiogenesis in a model of unilateral ureteric obstruction

UUO is a progressive model of renal fibrosis representative of the histological features seen in end stage renal failure. Following obstruction there is significant accumulation of macrophages and T-cells within the kidney. Immunostaining with the lymphatic marker podoplanin revealed the presence of de novo lymphatic vessels within the interstitium which were absent in
normal control tissue. These lymphatic endothelial cells were undergoing proliferation as shown by the dual immunofluorescence with podoplanin and PCNA. A number of these vessels contained inflammatory cells which suggest that they have a role in cellular egress from the inflamed interstitium.

The finding of de novo lymphangiogenesis was accompanied by increased expression of VEGF-C in whole kidney tissue. It was not possible to identify from immunohistochemistry which cell type was expressing VEGF-C due to technical issues discussed earlier in this thesis. However, in order to support the observations of macrophage mediated lymphangiogenesis by other investigators we sought to inhibit monocyte and macrophage populations using a clodronate depletion strategy. After administering systemic liposomal clodronate at doses previously published to be effective we were surprised to observe no depletion of ED-1 cells in the obstructed kidney despite obtaining effective knock-down in macrophage populations within the liver and spleen. These data were unexpected given the published work in this field. As anticipated ED-2 cells were effectively depleted in kidney, liver and spleen following clodronate administration. Similar data was obtained from a provisional experiment using clodronate from a different stock solution. We were unable to explain the lack of ED-1 depletion in the kidney as clodronate has previously been used in rodent UUO and induced rapid and effective macrophage ablation.

Formal quantification of lymphatic vessels revealed no difference in the number of lymphatic vessels between clodronate and vehicle control treated
animals. In addition, whole kidney VEGF-C expression was comparable between the two groups. With these data we could conclude that ED-2 cells or “tissue resident” macrophages are unlikely to play an important role in driving lymphangiogenesis.

Having failed to deplete ED-1 cells which had accumulated in the inflamed interstitium, alternative strategies to identify the comparative level of VEGF-C expression in macrophages and intrinsic renal cells were sought. Using established methodology in our laboratory we pursued an immunopurification strategy using enzymatically digested kidneys. Initial experiments employed a PE-conjugated antibody against CD68 (ED-1). This antigen is located in the endoplasmic reticulum of the cell. An integral part of the immunostaining procedure involves permeabilisation of the cells which facilitates intracellular binding of the CD68 antigen. Although this produced excellent flow cytometric staining this step combined with the cell sorting process resulted in necrosis of the cell and cleavage of DNA. These experiments were abandoned and an alternative antibody identified.

CD11b is a cell surface protein expressed by cells of myeloid lineage, such as monocytes and macrophages although it is also expressed by neutrophils and to a lesser extent dendritic cells. CD11b⁺ cell populations from both sham control kidneys and UUO kidneys underwent gene expression studies. This revealed no difference in VEGF-C expression between the two populations. This suggested that the source of VEGF-C was derived from other cell types including tubular epithelial cells. RT-PCR was performed on CD11b⁻
populations, this revealed no difference in VEGF-C expression between sham controls and obstructed kidneys. These findings were somewhat surprising as neither CD11b positive or CD11 negative cell populations exhibited increased VEGF-C expression despite the marked increase in VEGF-C expression seen in whole kidney tissue.

RNA gene expression studies were performed on other constituent genes in order to ensure the integrity of mRNA harvested. Upregulation in CD68 was increased in CD11b positive groups. In order to explain these results we suggested that the inability to identify VEGF-C upregulation represented a failure in maintaining stable conditions during the enzymatic digestion and cell sorting in combination with the prolonged time at room temperature.

In order to reduce the time required to obtain RNA and the limit degradation a different method of extraction was sought. Immunomagnetic bead purification is a rapid method of isolating immunolabelled cells. However, it is associated with a higher proportion of contaminating cell populations. Following extraction of mRNA VEGF-C expression was compared between CD11b positive and negative groups. The expression pattern was representative of the expression pattern seen by flow cytometric analysis with an absence in VEGF-C mRNA upregulation evident in either group.

Taken together the results of our depletion experiments and gene expression studies have failed to provide mechanistic data that implicates macrophages in the role of inflammatory lymphangiogenesis. In order to dissect the
mechanisms further, future experiments using this model could employ alternative depletion strategies. Macrophage colony-stimulating factor (M-CSF) is the principal growth factor regulating the proliferation, differentiation and survival of monocytes/macrophages (Pixley and Stanley 2004). M-CSF acts via the tyrosine kinase receptor c-fms and effective inhibition of macrophage accumulation has been described in the obstructed rat kidney following administration of a blocking antibody. Future experiments could assess the impact on lymphatic vessel proliferation following inhibition of c-fms in vivo.

As alluded to earlier in this chapter de novo lymphangiogenesis is associated with tubulointerstitial scarring and it remains unclear if these are two separate pathological entities or indeed if lymphatic vessels occur in response to fibrosis. Macrophage depletion in rodent models of UUO results in decreased levels of interstitial fibrosis so potentially any effects of lymphatic vessel number could be attributed to attenuation of fibrosis rather than a depletion of macrophages.

In order to establish the specific gene expression activity of ED-1 positive cells within a non-manipulated system it would be advantageous to capitalise on laser capture microdissection technology. This method isolates specific cells of interest from microscopic tissue. This has developed to include immunostained cells from paraffin embedded tissue with laser microdissection isolating the labelled cell to permit extraction of RNA. Gene expression studies could then be performed on selected cell populations. This method
would avoid the potential confounding effects of the cell isolation methods using flow cytometry or magnetic beads.

Having confirmed the pattern of VEGF-C expression through gene expression studies future work could attempt to inhibit this growth factor in vivo. This could be achieved by administering blocking antibodies or adenoviral delivery of recombinant VEGFR-3-Ig fusion protein, which blocks the extracellular domain of the VEGFR3 receptor. The likely effect would be inhibition of lymphangiogenesis and the effect upon kidney inflammation could be discerned. It would, however, fail to identify the cell responsible for VEGF-C production.
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