This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e.g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

- This work is protected by copyright and other intellectual property rights, which are retained by the thesis author, unless otherwise stated.
- A copy can be downloaded for personal non-commercial research or study, without prior permission or charge.
- This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author.
- The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.
- When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.
Figure 1.1 Lymphatic vessels open in response to increase interstitial pressure.
Lymphatic vessels are highly sensitive to interstitial stresses exerting radial tension on lymphatic capillaries. The resultant deformation of the endothelial cells results in the opening of microvalves that draws fluid inwards in response to an increase in interstitial pressure (A). As the vessel fills its overlapping cell-cell junctions close and intraluminal pressure returns to baseline. (B). This cell-cell junction overlap has been referred to as the primary valve system of the lymphatic vessels.
Figure 1.2 Schemata illustrating the sequence of immune activation during inflammation and tissue injury

In response to inflammation or injury professional antigen presenting cells capture antigen and present antigen-derived peptide in association with MHC Class II molecules to helper T-lymphocytes in lymph nodes. This is the initiation of a humoral and cell mediated specific immune response.
**Figure 1.3 Role of macrophages in inflammatory lymphangiogenesis.**

Macrophages have been implicated in the development of inflammatory lymphangiogenesis. Following tissue injury macrophages are recruited to sites of inflammation in response to pro-inflammatory cytokines and chemokines. Upon arrival to the tissues macrophages may orchestrate *de novo* lymphangiogenesis through expression of VEGF-C inducing local proliferation of existing lymphatic vessels. Alternatively macrophages or bone-marrow derived cells may transdifferentiate into lymphatic endothelial cells. (МΦ- macrophage, TonEBP- tonicity enhanced binding protein)

*De novo* lymphatic vessel

Local production of VEGF-C by infiltrating МΦ
Potentially via the activation of the transcription factor TonEBP

МΦ transdifferentiation into lymphatic endothelial cell.
Figure 1.4 Monocyte and macrophage development.
Common stem and myeloid progenitors develop within the bone marrow. When released into the peripheral circulation monocytes have a Ly6C\text{high} phenotype and differentiate into a phenotypically distinct Ly6C\text{low} subset which replenish the tissue resident macrophage populations. Ly6C\text{high} cells represent “inflammatory” monocytes and are recruited to sites of inflammation. In vitro studies have shown that macrophages may adopt either a classically or alternatively activated phenotype in response to environmental stimuli.
Figure 1.5 Interstitial fibrosis and tubular atrophy (IFTA) is a progressive and accumulative process resulting in intraparenchymal scarring.

At the time of transplantation there are multiple causes of graft injury ranging from ischaemia reperfusion injury (IRI) to acute rejection. Over time there is ongoing scarring and inflammation which results from macrophage influx and myofibroblast proliferation with deposition of extracellular matrix (ECM) leading to nephron loss and eventually impaired graft function. (Mϕ-macrophages)
Figure 1.6 The parallel patterns of lymphangiogenesis following renal transplantation.
Following disruption of the lymphatic drainage of the kidney there is extra-renal lymphatic vascular regeneration and reconnection. During acute or chronic allograft injury intra-renal lymphangiogenesis has been reported. Its role as a pathological entity is yet to be established.

Allograft

Lymphatic repair

Lymphatic reconnection
- important for control of Interstitial fluid and protein balance

Intra-renal lymphangiogenesis
Documented in a subset of human patients with acute rejection and interstitial fibrosis and tubular atrophy in human biopsies.
? Beneficial or detrimental.
Table 2.1  Table outlining antibodies used in immunohistochemistry on fixed, paraffin embedded tissue. Included are the respective fixation, antigen retrieval methods and appropriate isotype controls

<table>
<thead>
<tr>
<th>Host Species</th>
<th>Antigen</th>
<th>Ig Class/Isotype</th>
<th>Target Cell</th>
<th>Fixation</th>
<th>Antigen Retrieval</th>
<th>Working Concentration</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>anti-rat</td>
<td>ED-1</td>
<td>Rat Macrophage</td>
<td>Formalin</td>
<td>Citrate</td>
<td>1:100</td>
<td>AbSerotec</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>anti-rat</td>
<td>ED-2</td>
<td>Rat Macrophage</td>
<td>Methcayrn</td>
<td>Citrate</td>
<td>1:100</td>
<td>Abserotec</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>anti-rat</td>
<td>Podoplanin</td>
<td>Rat Lymphatic endothelium</td>
<td>Formalin</td>
<td>Proteinase K</td>
<td>1:300</td>
<td>Sigma</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>anti-rat</td>
<td>B220</td>
<td>Rat B Cell</td>
<td>Formalin</td>
<td>Tris EDTA/ Borgs Decloaker</td>
<td>1:100</td>
<td>Abserote</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>anti-rat</td>
<td>CD 3</td>
<td>Rat T Cell</td>
<td>Formalin</td>
<td>Citrate</td>
<td>1:100</td>
<td>AbCam</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamster</td>
<td>ant-mouse</td>
<td>Podoplanin</td>
<td>Mouse Lymphatic endothelium</td>
<td>Formalin</td>
<td>Citrate</td>
<td>1:750</td>
<td>AbCam</td>
</tr>
<tr>
<td>Hamster IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>anti-mouse</td>
<td>LYVE-1</td>
<td>Mouse Lymphatic endothelium</td>
<td>Formalin</td>
<td>Proteinase K</td>
<td>1:200</td>
<td>AbCam</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>anti-rat</td>
<td>Prox-1</td>
<td>Rat Lymphatic endothelium</td>
<td>Formalin</td>
<td>Tris EDTA</td>
<td>1:100</td>
<td>Novus</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>anti-rat</td>
<td>PCNA</td>
<td>Rat Proliferating Nuclei</td>
<td>Formalin</td>
<td>Citrate</td>
<td>1:100</td>
<td>DAKO</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.1 Flow cytometric cell sorting of CD11b$^+$ cells.
Obstructed and sham kidneys were digested mechanically and enzymatically into a single cell suspension and immunostained with anti-CD11b-PE. Following incubation with primary antibody cells underwent flow cytometric cell sorting into CD11b$^+$ and CD11b$^-$ populations. Positive populations have a purity of 97%.
Figure 2.2 Magnetic bead immunopurification of CD11b+ cells.

Following digestion of whole kidney and immunostaining with α-CD11b-PE antibody cells were incubated anti-PE microbeads. The resulting cell suspension was passed through a MACS separator columns with CD11b+ bound magnetically to the columns.
Figure 3.1 Subcapsular injection of carbon black in the native rodent kidney
Rats underwent a midline laparotomy and injection of 5µl of carbon black into the subcapsular space. Immediate appearances of the kidney following subcapsular injection (A). Macroscopic localisation of carbon black in the isplateral renal lymph node (arrowed) at 24 hours (B).
Figure 3.1 cont.
Transverse section of the kidney 24 hours following subcapsular injection of carbon black (C). This demonstrates retention of carbon (arrow) within the subcapsular space without sequestration into the renal parenchyma.
Figure 3.2  Carbon black in parathymic nodes 24 hours following subcapsular injection.
Following laparotomy and subcapsular injection of carbon black animals were recovered and sacrificed at 24 hours. Carbon black localised to the ipsilateral renal lymph node and parathymic nodes in the superior mediastinum (arrowed).
Figure 3.3 Histology of left renal hilar lymph node 24 hours following subcapsular injection of carbon black. H&E stained section demonstrates localisation of carbon particles to mononuclear cells suggestive of phagocytosis (A). Carbon colocalisation with ED1⁺ cells (white arrow) with ‘free carbon’ black evident (yellow arrow) (B).
Figure 3.4 Localisation of carbon black to renal lymph node within 4 hours

Macroscopic evidence of carbon localisation in the ipsilateral renal lymph node 4 hours following subcapsular injection of carbon black into the kidney (A). The renal lymph is lying lateral to the descending colon (DC). H&E stained renal lymph node at 4 hours identifies carbon black (arrowed) localising to cortex of the lymph node (B).
**Figure 3.5 Demonstration of the lymphatic drainage of the native kidney**

Rats underwent a midline laparotomy and the kidney was dissected free from its lateral attachments to the perinephric fat. Following medial reflection of the kidney 10µl of 2% Evan’s Blue is injected intraparenchymally. This delineates the lymphatic trunks (LT) running posterior to the renal artery into the renal lymph node (LN),(A). High power image of the a lymphatic trunk entering the renal lymph node overlying the renal artery (RA) and renal vein (RV),(B).
Figure 3.6 Intra-operative photograph of lymphatic disruption procedure.
Rats underwent a midline laparotomy. The small bowel was retracted and the descending colon mobilised in order to expose the aorta, inferior vena cava and left kidney. The renal pedicle is dissected free from the surrounding connective tissue and stripped off the renal artery and vein (A). The renal artery and vein are separated creating a plane between the two structures which facilitates “skeletalisation” of the renal artery (B).
Figure 3.7 Lymphatic reconnection occurs by day 6.
Rats underwent a midline laparotomy and disruption of lymphatic drainage of the *in situ* kidney. This was followed by injection of carbon black into the subcapsular space (day 0) (A). Macroscopic evidence of carbon localisation in the ipsilateral renal lymph node is evident at day 6 following lymphatic disruption. The kidney is outlined by dashed white line.
Figure 3.8 After surgical disruption of the lymphatic drainage of the kidney there is no macroscopic evidence of carbon localisation in the ipsilateral renal lymph node.

Rats underwent a midline laparotomy and disruption of lymphatic drainage of the in situ kidney. This was followed by injection of carbon black into the subcapsular space (day 0) (A). At 24 hours the animal was sacrificed. Examination of the ipsilateral renal lymph node revealed absence of carbon localisation (arrow), confirming disruption of lymphatic drainage (B).
Figure 3.8 (cont).

The left renal lymph node was examined 6 days following lymphatic disruption surgery. Sections were stained with H&E which reveals carbon localisation within phagocytic cells (arrowed) located in the lymph node cortex (A). Magnified image of phagocytic cells with carbon localisation (B).
Figure 3.9 Photomicrographs of the normal renal hilum sectioned in a transverse orientation.

The renal hilum from a normal kidney was removed en bloc and serial sections were performed in a transverse orientation. Haematoxylin & Eosin stain demonstrates the renal artery (RA) and vein (RV) with the lymphatic trunks (LT) lying posterior to the renal artery which enter the lymph node (LN) (A) Podoplanin+ endothelium is evident within the lymphatic trunk (arrowed), (B).
Figure 3.10 Transverse section of the renal hilum 7 days following lymphatic disruption.

Podoplanin immunostaining of the renal hilum revealed an absence of large podoplanin\(^+\) lymphatic trunk and emergence of a network of small podoplanin\(^+\) (arrow) lymphatic vessels of variable size seen at low magnification.
Figure 3.10 (cont)

High power photomicrograph of de novo podoplanin+ lymphatic vessels. In the renal hilum following restoration of lymphatic drainage from the rodent kidney.
Figure 3.11  Dual expression of lymphatic markers confirm lymphatic phenotype.

The network of podoplanin+ vessels also express the nuclear transcription factor Prox-1 which confirms the lymphatic phenotype of these vessels.
Figure 3.12  Dual Immunostaining confirms that the *de novo* lymphatic vessels are proliferating.

Immuohistochemistry was performed with podoplanin and the proliferation marker PCNA. This confirmed the diffuse network of podoplanin+ lymphatic vessels (arrowed) (A) and at high power a number of these vessels co-localised with PCNA (arrowed) (B).
Figure 4.1 Schemata outlining the experimental protocol of acute murine rejection model.

Renal transplants were carried out between Balb-c donors and FVB recipients (allografts) and FVB mice (isografts). At day 7 animals were culled and tissue harvested for histological analysis.
Figure 4.2 Murine allografts undergoing acute rejection do not exhibit de novo lymphangiogenesis.

Immunostaining with the lymphatic marker podoplanin indicated no increase in lymphatic vessel number in acutely rejecting murine allografts. Photomicrograph of day 7 allografts, delineating the fully formed podoplanin positive lymphatic lumen (arrowed) lying adjacent to blood vessels (A). Quantification of the number of lymphatic vessels per arterial profile revealed no difference in number of lymphatic vessels between allografts and isografts (B) ($p=0.39$).
Figure 4.3. Assessment with a second lymphatic marker LYVE-1 indicates no increase in the number of lymphatic vessels in the rejecting murine renal allograft.

Representative immunostaining with LYVE-1 confirms the Periarterial (ART) location of lymphatic vessels, lying adjacent to Glomeruli (A). Quantification revealed no difference in the number of peri-arterial lymphatic vessels between isografts and allografts (B) p=0.69.
Figure 4.4 High power photomicrograph of inflammatory cells contained within a lymphatic vessel.

Immunostaining with podoplanin reveals lymphatic vessel lumen (green arrows) containing a variety of cells including mononuclear cells that may represent lymphocytes (red arrow). In addition, there are large cells that contain multiple ingested cells (yellow arrow) which are likely to be macrophages.
Figure 4.5 Schemata outlining the experimental protocol of rat transplant model of interstitial fibrosis and tubular atrophy (IFTA).

Transplants were carried out between Fischer and Lewis rats (allograft group) and Lewis rats (isograft group). Rats were immunosuppressed with cyclosporin for the first 10 postoperative days. A contralateral nephrectomy was performed on day 10 establishing this as a functional model. Rats were culled at 52 weeks.
Figure 4.6 - Rat allografts exhibit glomerular and interstitial disease

Representative Periodic Acid Schiff (PAS) staining of allografts (A) demonstrates glomerulosclerosis (G). Tubulointerstitial changes include duplication of the basement membrane and tubular atrophy (example arrowed). PAS staining of isografts (B) reveals preservation of normal tubular and glomerular architecture (all images x 200)
Figure 4.7 - Rat allografts exhibit significant collagen deposition

Representative images (x200 magnification) of fibrillar collagen deposition shown by Picrosirius red staining of the renal cortex and medulla of isografts (A & B) and allografts (C & D).
Figure 4.7 cont.

Quantification of fibrillar collagen deposition by computer image analysis demonstrates increased scarring in the cortex (E) and medulla (F) of allografts (**P < 0.01 and * P < 0.05).
Figure 4.8 Rat allografts exhibit significant macrophage infiltration

Representative photomicrographs of ED-1+ macrophage distribution in renal allograft (A) and isograft (B) (x200 magnification). Macrophage infiltration was quantified by counting the number of ED-1+ cells per high power field (x400 magnification) with allografts exhibiting increased numbers of ED-1+ macrophages compared to isograft controls (*P <=0.05).
Figure 4.10 B-cells predominately form perivascular nodular infiltrates.

Representative image of B-lymphocyte infiltrates in allografts. Immunostaining against the B220 antigen demonstrated that B220$^+$ cells are concentrated in interstitial aggregates often associated with large blood vessels (A) These are absent in isograft controls (B).
Figure 4 9- Rat allografts exhibit significant T cell infiltration

Representative photomicrographs of CD3 immunostaining in allografts (A) and isografts (B). An infiltrate of CD3+ T lymphocytes is evident in allografts. T cell infiltration was quantified by counting the number of CD3+ cells per high power field with allografts exhibiting increased numbers of CD3+ T cells compared to isograft controls (C) (*P <=0.05)
**Figure 4.11 - Rat allografts exhibit comparable numbers of perivascular lymphatic vessels.**

Podoplanin immunostaining reveals the normal perivascular location of lymphatic vessels in isografts (A – examples arrowed). Podoplanin is also expressed by glomerular podocytes (x 200 magnification). Quantification of the number of lymphatic vessels per vascular profile (B) demonstrated no difference between allograft and isograft controls (P>0.05)
Figure 4.12 - Rat allografts exhibit increased numbers of interstitial lymphatic vessels.

Podoplanin immunostaining demonstrated the presence of interstitial lymphatic vessels in all allograft biopsies examined (A) examples arrowed) but these were rarely present in control isografts (B) (x200 magnification)
Figure 4.12. cont.

High power image of interstitial lymphatic vessels (C). Quantification was undertaken by counting the number of fully formed podoplanin\(^+\) lymphatic vessels per high power field (x400 magnification) (D).
Figure 4.13 – The number of interstitial lymphatic vessels correlates with renal fibrosis.

The number of interstitial vessels in allografts exhibits a positive correlation with the extent of renal fibrosis quantified by picrosirius red staining ($p<0.05$).
Figure 5. Schema depicting the protocol for the experiments involving unilateral ureteric obstruction (UUO)

Rats underwent UUO on day 0 with animals culled on day 7, day 14 and day 21. Tissue was harvested for immunohistochemical analysis and gene expression studies.
Figure 5. 2 Obstructed kidneys rapidly develop hydronephrosis

The obstructed kidney exhibits significant hydronephrosis and hydroureter (A) compared to the contralateral kidney. When bissected (B) there is dilation of the renal calyces and atrophy with thinning of the renal parenchyma.
Figure 5.3 Obstructed kidney exhibits prominent tubular dilation and a mononuclear cell infiltrate.

Tissue sections from a control and obstructed kidney were stained with H&E. Representative image of a normal rat kidney (A). At 2 weeks following ureteric obstruction there is tubular dilation (TD) and infiltrate of mononuclear cell (arrows) (B).
Figure 5.4– Obstruction results in significant macrophage accumulation in the kidney.

Photomicrographs of rat kidney tissue sections from a normal control rat (A) and rat 2 weeks following UUO (B) stained with the macrophage marker ED1. Occasional ED1+ cells (arrowed) are apparent in normal kidneys. In contrast, obstructed kidney exhibits a significant macrophage infiltrate in the interstitium (arrowed).
Figure 5.4 cont

The accumulation in macrophages in the obstructed kidney was quantified by counting the number of positive cells per high power field (x400 magnification). There is a sharp increase in the number of ED1\(^+\) cells in which reaches a maximum at 2 week of obstruction (\(p<0.05\) vs control).
Figure 5.5  T-cells traffic to the interstitium of the obstructed kidney

Tissue sections from normal control kidney and kidney removed after 2 weeks of obstruction were stained with the T-cell marker CD3. Representative images of T cell immnostaining in the cortex in UUO reveals a diffuse infiltrate in the interstitium (A) and absence of CD3 cells in control kidney (B).
Figure 5.5 (cont.)

The accumulation in T-cells in the obstructed kidney was quantified by counting the number of positive cells per high power field (x400 magnification). There is a sharp increase in the number of CD3 positive cells in both cortex and medulla which increases with duration of obstruction ($p<0.001$ vs control).
Figure 5.6. B cells are predominantly concentrated in peri-arterial infiltrate

In the obstructed kidney B-cells were confined to perivascular infiltrates. Typical appearance of B cells in the cortex lying within periarterial infiltrates in close proximity to an intra-lobular artery (Art.).
Figure 5.7 In obstructed kidney there is striking intra-renal lymphangiogenesis.
Tissue sections from normal control kidney and kidney removed 2 weeks after obstruction were stained for the lymphatic marker podoplanin. In the normal kidney the lymphatic vessels are located adjacent to arterial structures (A) and absent in the interstitum (B). Podoplanin is also expressed by podocytes located within the glomeruli (G).
Figure 5.7 cont

Interstitial lymphatic vessels (arrows) were identified in the kidney 2 weeks following ureteric obstruction. They are remote from any arterial structure (A) and at high power they are observed to form vessels with obvious podoplanin positive lumina (B).

A

![Image A](image1)

B

![Image B](image2)
Figure 5.7 cont.

Kidney from normal and obstructed kidney were removed and stained with the lymphatic marker podoplanin. This was quantified by counting the number of podoplanin positive vessels per medium power field (x200). The number of lymphatic vessels increased with the duration of obstruction reaching a maximum at 3 weeks. P=0.0002
Figure 5.8 Confocal microscopy demonstrates macrophages within the lymphatic lumen.

Formalin fixed tissue from kidney removed 2 weeks after obstruction were stained for ED1 and podoplanin. Sections were examined by confocal microscopy (x1000 magnification) which revealed an ED-1 positive cell (green arrow) within a podoplanin positive vessel (red arrows).
Figure 5.9 Staining of serial sections from Prox-1 and podoplanin indicates the presence of interstitial lymphatic vessels in the obstructed kidney.

Photomicrographs of alternatively staining serial sections with prox-1 a transcription factor specific for LECs (A) and the membrane mucoprotein podoplanin (B). Arrows of the same colour are likely to correspond to the same lymphatic vessel seen in serial sections.
Figure 5.10  Dual immunofluorescence for podoplanin and PCNA. confirms the active proliferation of de novo lymphatic vessels.

Formalin fixed tissue from kidney removed 3 weeks after obstruction were stained for podoplanin and PCNA. Fluorescence microscopy revealed co-localisation of PCNA and interstitial podoplanin positive lymphatic vessels confirm that these cells undergoing active proliferation.
Figure 5.11 Obstructed kidney exhibits increased expression of VEGF-C mRNA.

Gene expression studies performed on mRNA extracted from whole kidney removed from control and weeks 1, 2 and 3 following obstruction. RT-PCR for VEGF-C revealed an increase in mRNA expression relative to control mRNA. The highest level of expression was seen at week 2 ($p<0.001$).
Figure 5.12 Obstructed kidney exhibits increased expression of TonEBP mRNA.

Gene expression studies performed on mRNA extracted from whole kidney removed from control and weeks 1, 2 and 3 following obstruction. RT-PCR for TonEBP revealed an increase in mRNA expression relative to control mRNA. The highest level of expression was seen at week 2 and sharply declines in week 3 (p <0.005)
Figure 6. 1 Schema depicting the protocol for the second experiment involving unilateral ureteric obstruction (UUO) and liposomal clodronate.

Rats underwent UUO on day 0. 1.5ml of liposomes containing clodronate (n=7) or PBS (n=5) was administered intravenously on days 6,9,13,16 and 20. Rats were culled on day 21 with kidney, liver and spleen harvested for histological analysis and gene expression studies.
Figure 6.2  Liposomal clodronate fails to deplete ED1 positive cells in the kidney

Rats underwent UUO (d0) with 1.5mls of liposomes containing clodronate or PBS administered intravenously on days 6, 9, 13, 16, and 20. Rats were culled on day 21 with kidney, liver and spleen harvested. Kidney sections were stained with ED-1. Representative photomicrographs from animals treated with PBS (A) and Clodronate (B).
Figure 6.2 (cont.)

Kidney sections immunostained with antibody against ED-1 were quantified using computerised image analysis. In the renal cortex there is no depletion of the ED1 positive cells in the obstructed kidney $p=0.085$, ns. (C) No depletion was seen in the medulla $p=0.0956$, ns (D).
Figure 6.3  Liposomal clodronate depletes resident Kupffer cells in the liver.
Rats underwent UUO (d0) with 1.5mls of liposomes containing clodronate or PBS administered intravenously on days 6, 9, 13, 16, and 20. Rats were culled on day 21 with kidney, liver and spleen harvested. Liver sections were stained with ED-1. Representative photomicrographs from animals treated with PBS (A) and Clodronate (B)
ED1\(^+\) cells were quantified by counting the number of positive cells per high power field. This revealed significant depletion of ED1\(^+\) cells in the liver following administration of clodronate \(* * * p < 0.001\) (C).
Figure 6.4 ED-1 populations within the spleen are depleted with liposomal clodronate

Rats underwent UUO (d0) with 1.5mls of liposomes containing clodronate or PBS administered intravenously on days 6, 9, 13, 16, and 20. Rats were culled on day 21 with kidney, liver and spleen harvested. Spleen sections were stained with ED-1. Representative photomicrographs from animals treated with PBS (A) and Clodronate (B).
Figure 6.4 cont
Spleen sections stained with antibody against ED-1 were quantified using computerised image analysis (C), 7.58± 0.5% of the surface was positive in PBS treated controls. Clodronate treated animals demonstrated significant depletion of the ED-1+ macrophages, 0.73± 0.174%. ***P<0.001.
Figure 6.5 Liposomal clodronate depletes ED 2 population in both cortex and medulla in the obstructed kidney.

Rats underwent UUO (d0) with 1.5mls of liposomes containing clodronate or PBS administered intravenously on days 6, 9, 13, 16, and 20. Rats were culled on day 21 with kidney, liver and spleen harvested. Spleen sections were stained with ED-2. Representative photomicrographs from animals treated with PBS (A) and Clodronate (B).
Figure 6.5 (cont.)

Kidney sections stained with antibody against ED-2 were quantified using computerised image analysis. Expression was defined as the total surface area positive for staining. (C) In the cortex ED2 positive cells were significantly reduced *p=0.032. (D) This was reflected with similar depletion in the renal medulla *p=0.03
Figure 6.6  Liposomal clodronate depletes ED 2 population in the spleen.

Rats underwent UUO (d0) with 1.5mls of liposomes containing clodronate or PBS administered intravenously on days 6, 9, 13, 16, and 20. Rats were culled on day 21 with kidney, liver and spleen harvested. Spleen sections were stained with ED-2. Representative photomicrographs from animals treated with PBS (A) and Clodronate (B)
Spleen sections stained with antibody against ED-2 were quantified using computerised image analysis. Expression was defined as the total surface area positive for staining. (C) 7.58± 0.5% of the surface was positive in PBS treated controls. Clodronate treated animals demonstrated significant knock down in their populations 0.73± 0.174%. ***P<0.001.
Figure 6.7 Liposomal clodronate depletes ED 2 population in the liver.

Rats underwent UUO (d0) with 1.5mls of liposomes containing clodronate or PBS administered intravenously on days 6, 9, 13, 16, and 20. Rats were culled on day 21 with kidney, liver and spleen harvested. Spleen sections were stained with ED-2. Representative photomicrographs from animals treated with PBS (A) and Clodronate (B)
Figure 6.7

ED2$^+$ cells were quantified by counting the number of positive cells per high power field. This revealed significant depletion of ED2$^+$ cells in the liver following administration of clodronate **$p=0.001$ (C).
Figure 6.8  Liposomal clodronate did not affect lymphatic vessel numbers

Rats underwent UUO (d0) with 1.5mls of liposomes containing clodronate or PBS administered intravenously on days 6, 9, 13, 16, and 20. Rats were culled on day 21 with kidney, liver and spleen harvested. Kidney sections were stained with podoplanin and quantified by counting the number of fully formed podoplanin positive vessels. This revealed no difference between the two groups p=0.28,ns
Figure 6.9 Clodronate depletion had no effect on VEGF-C expression in whole kidney.

Rats underwent UUO (d0) with 1.5mls of liposomes containing clodronate or PBS administered intravenously on days 6, 9, 13, 16, and 20. Rats were culled on day 21 with kidney, liver and spleen harvested. Kidney sections snap frozen and mRNA extracted. RT-PCR failed to identify any difference between VEGF-C expression between PBS and clodronate treated groups. P=0.986,ns
Figure 6.10  PROX-1 expression was not abrogated following administration.

Rats underwent UUO (d0) with 1.5mls of liposomes containing clodronate or PBS administered intravenously on days 6, 9, 13, 16, and 20. Rats were culled on day 21 with kidney, liver and spleen harvested. Kidney sections snap frozen and mRNA extracted. RT-PCR studies of the nuclear transcription factor PROX-1 failed to identify any difference in expression between PBS or clodronate treated animals $p=0.6806$ which confirmed the histological assessment performed by podoplanin.
Figure 6.11 Flow cytometric cell sorting of CD11b-PE positive cells had a purity of 97%

Rats underwent UUO (d0) and culled on day 14. Kidney sections were harvested and enzymatically digested. The single cell suspension was labeled with anti-CD11b-PE and underwent flow cytometric cell sorting into CB11b⁺ and CD11b⁻ populations. Prior to analysis cells were stained with propidium iodide. FACS plots of (A) unstained samples and (B) CD11b+ve and PI negative cells which were selectively sorted and used for analysis.
Figure 6.12 CD68 is upregulated in CD11b positive populations.

Rats underwent UUO (d0) and culled on day 14. Kidney sections were harvested and enzymatically digested. The single cell suspension was labeled with anti-CD11b-PE and underwent flow cytometric cell sorting into CB11b+ and CD11b- populations. RT-PCR demonstrated significant upregulation of CD68 in CD11b positive populations, *p=0.013 when compared to CD11b negative populations.
Figure 6.13 RT-PCR studies between CD11b negative and positive populations reveal differences in expression of E-Cadherin and Alpha-SMA.

Rats underwent UUO (d0) and culled on day 14. Kidney sections were harvested and enzymatically digested. The single cell suspension was labeled with anti-CD11b-PE and underwent flow cytometric cell sorting into CB11b+ and CD11b- populations.

(A) RT-PCR studies demonstrated a significant increase in mRNA expression of E-cadherin in CD11b-ve populations **p=0.0076. (B) Although there was an absolute increase in expression of alpha-sma mRNA this did not reach statistical significance p=0.13
Figure 6.14. Gene Expression studies between CD-11b sorted populations reveal no difference in VEGF-C expression between CB11b positive and negative populations.

Rats underwent UUO (d0) and culled on day 14. Kidney sections were harvested and enzymatically digested. The single cell suspension was labeled with anti-CD11b-PE and underwent flow cytometric cell sorting into CB11b$^+$ and CD11b$^-$ populations. (A). RT-PCR on CD11b positive populations revealed no statistical difference between VEGF-C expression between sham controls and UUO animals p=0.91 (ns) (B) Analysis of the CD11b negative populations failed to identify any difference between sham controls and obstructed kidneys p=0.0537
Figure 6.15 Flow cytometric analysis of the percentage purity in enriched and negative populations.

Rats underwent UUO (d0) and culled on day 14. Kidney sections were harvested and enzymatically digested. The single cell suspension was labeled with anti-CD11b-PE and underwent magnetic bead immunopurification into CB11b+ and CD11b− populations. (A) In UUO kidney 68.34±2.91% of cells were CD11b positive in the enriched populations, whilst in the negative fraction (B) 11.8±0.9% of the population were contaminated with CD11b positive cells.
Figure 6.16 Cytospins of cells following passage through MACS columns

Rats underwent UUO (d0) and culled on day 14. Kidney sections were harvested and enzymatically digested. The single cell suspension was labeled with anti-CD11b-PE and underwent magnetic bead immunopurification into CB11b\(^+\) and CD11b\(^-\) populations (A) Dif-Quick staining of cytospins demonstrating mononuclear cells from CD11b enriched populations and (B) epithelial cells from CD11b negative populations
Figure 6.17  VEGF-C expression is not increased in CD11b cells purified by MACS columns.

Rats underwent UUO (d0) and culled on day 14. Kidney sections were harvested and enzymatically digested. The single cell suspension was labeled with anti-CD11b-PE and underwent magnetic bead immunopurification into CB11b+ and CD11b- populations. (A) RT-PCR studies revealed no increase in VEGF-C mRNA expression between CD11b positive cell from sham vs UUO kidneys p= 0.38 and (B) CD11b negative cells p=0.34
Figure 6.18  CD68 expression in CD11b enriched populations.

Rats underwent UUO (d0) and culled on day 14. Kidney sections were harvested and enzymatically digested. The single cell suspension was labeled with anti-CD11b-PE and underwent magnetic bead immunopurification into CB11b+ and CD11b- populations. RT-PCR confirms increased expression of CD 68 in enriched populations when compared to negative populations. **p=0.0092
Figure 6.19 Expression of E-Cadherin and alpha-SMA is influenced by the method of cell separation.

Rats underwent UUO (d0) and culled on day 14. Kidney sections were harvested and enzymatically digested. The single cell suspension was labeled with anti-CD11b-PE and underwent magnetic bead immunopurification into CD11b+ and CD11b- populations. (A) RT-PCR studies revealed no difference in expression of E-cadherin $p=0.17$ ns or (B) in alpha-SMA, $p=0.53$ ns.
Figure 6.20 VEGF-C expression in CD11b sorted populations using β2 macroglobulin as a housekeeping gene.

Rats underwent UUO (d0) and culled on day 14. Kidney sections were harvested and enzymatically digested. The single cell suspension was labeled with anti-CD11b-PE and underwent flow cytometric cell sorting into CD11b+ and CD11b- populations. (A) RT-PCR studies using β2 macroglobulin as a housekeeping gene demonstrated increased expression of VEGF-C mRNA from CD11b +ve harvested from UUO tissue when compared to sham controls, however this is not statistically significant p=0.3 (B). There is no increase in VEGF-C expression in CD11b -ve cells from UUO kidneys.