ROLE OF LEUKOCYTES, COMPLEMENT SYSTEM AND ENDOTHELIUM IN RAT RENAL ISCHAEMIA-REPERFUSION INJURY

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By

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Who has grown up wondering why his dad spends so much time on the laptop

Abstract

Introduction:

Renal Ischaemia-Reperfusion injury (IRI) is a complex mechanism involving the interplay between endothelium, leukocytes and the complement system. To evaluate the role of these three key mediators, a rat model was used to evaluate changes seen in renal IRI. Two interventional agents: Anti-Thymocyte Globulin (ATG) and Soluble Complement Receptor-1 (sCR1) were used to modulate leukocyte and complement response in this IRI model with a view to assess, and define IRI mechanism.

Methods:

The Ischaemia-Reperfusion (IR) model involved unilateral left renal ischemia (n=10) for 40 minutes, followed by 48 hours of reperfusion. ATG (n=8), ATG Isotype (n=8) and sCR1 (n=8) were given IV prior to the laparotomy followed by IR model. The sham group (n=6) served as controls. Blood CD3 lymphocyte counts and CH50 complement assay were used to check efficacy of ATG and sCR1 respectively. The kidneys retrieved at 48 hours were analysed for histology, immunohistochemistry and RT-qPCR studies.

Results:

The IR group showed significant injury compared to the sham group. ATG treatment offered significant histological protection mainly via decreased leukocyte infiltrate and endothelial protection compared to the IR and Isotype controls. CH50 assay showed complete ablation of complement activity at the time of reperfusion, with return to normality at 24 hours. sCR1 treatment conferred protection from IRI predominantly via suppression of the complement cascade (C3, C9), reduced leukocyte infiltrates and

endothelial protection. RT-qPCR showed down-regulation of injury molecules – KIM-1 and NGAL in both the intervention groups.

Conclusion:

Modulation of leukocytes and complement system using single dose ATG and sCR1 led to significant endothelial protection, resulting in amelioration of renal ischaemia-reperfusion injury. The complement system was ablated at the time of reperfusion and was reconstituted by 24 hours, thus indicating that suppression of complement system during the phase of IR provides an avenue for mitigating IRI.

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Publications

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3. Soluble Complement Receptor 1 attenuates complement and inflammatory response in rat renal ischemia-reperfusion injury. *British Transplantation Society,* Glasgow, Feb 2014.

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List of Abbreviations

ADP	Adenosine Diphosphate
AKI	Acute Kidney Injury
APC	Activated Protein C
ARF	Acute Renal Failure
ASPA	Animals Scientific Procedures Act
ATG	Anti-Thymocyte Globulin
ATN	Acute Tubular Necrosis
ATP	Adenosine Triphosphate
CBS	Central Biotechnology Services
CIT	Cold Ischaemic Time
Ct	Cycle Threshold
DAA	Decay Acceleration Activity
DBD	Donation after Brain Death
DC	Dendritic Cells
DCD	Donation after Cardiac Death
DGF	Delayed Graft Function
ECD	Extended Criteria Donors
ECMO	Extracorporeal Membrane Oxygenation
E-Selectin	Endothelium- Selectin
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase

GBM	Glomerular Basement Membrane
GFR	Glomerular Filtration Rate
GMCSF	Granulocyte-Macrophage Colony Stimulating Factor
H&E	Hematoxylin & Eosinophil
HMP	Hypothermic Machine Perfusion
ICAM	Inter-Cellular Adhesion Molecule
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IR	Ischaemia-Reperfusion
IRI	Ischaemia-Reperfusion Injury
JBIOS	Joint Biological Services Cardiff Unit
KIM	Kidney Injury Molecule
LCP	Lectin Complement Pathway
MAC	Membrane Attack Complex
MASP	MBL-Associated Serine Protease
MBL	Mannose-Binding Lectin
MIP	Macrophage Inflammatory Protein
NGAL	Neutrophil Gelatinase-assosciated Lipocalin
NK	Natural Killer
NKT	Natural Killer T-cells

NMP	Normothermic Machine Perfusion
NO	Nitric Oxide
OPN	Osteopontin
PBS	Phosphate Buffered Saline
PDGF	Platelet-derived growth factor
PCR	Polymerase Chain Reaction
PNF	Primary Non Function
P-Selectin	Platelet- Selectin
PTC	Peritubular Capillary
PTEC	Proximal Tubular Epithelial Cells
qPCR	Real Time Polymerase Chain Reaction
RAG	Recombination-activating gene
RIN	RNA Integrity Number
RIPC	Remote Ischaemia Pre-Conditioning
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
sCR1	Soluble Complement Receptor Type 1
SD	Standard Deviation
SEM	Standard Errors of Mean
TBV	Total Blood Volume
TCR	T-cell Receptor

TF	Tissue Factor
TLR	Toll-like Receptors
TGF	Transforming growth factor
TM	Thrombomodulin
TNF	Tumor Necrosis Factor
VBS	Veronal Buffered Saline
VEGF	Vascular Endothelial Growth Factor
VCAM	Vascular Cell Adhesion Molecule
vWF	von Willebrand factor
WIT	Warm Ischaemic Time
WT	Wild Type

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CHAPTER I:

RENAL ISCHAEMIA- REPERFUSION INJURY

Ischaemia refers to deprivation of blood, oxygen and essential nutrients to an organ. Reperfusion injury refers to the damage that ensues in the tissue following the restoration of the blood supply after a period of ischaemia. The absence of oxygen and nutrients from blood creates a condition whereby the restoration of circulation results in oxidative damage, inflammation, and immune mediated damage to the organ. Ischaemia-Reperfusion injury (IRI) involves multiple mediators, and the focus of this research project is to evaluate the role of three mediators: Endothelium, Complement System and Leukocytes.

1.1. Clinical Overview

In clinical settings, many organs are exposed to the harmful effects of IRI. For example, when a patient suffers a myocardial infarction, the myocardium is deprived of essential oxygen, and subsequent reperfusion leads to morbidity and mortality depending on the size of the infarct. The primary goal of treatment for an acute myocardial event is to establish early reperfusion, and current clinical trials are looking into ways of reducing the effects of the 'lethal reperfusion injury' (1). Similarly, in acute embolic stroke, current therapies are based on principles of reducing the ischaemic event, and controlling the reperfusion injury phase. Intravenous thrombolysis and intra-arterial therapies aim to recanalise the occluded arteries at the earliest, thus improving clinical outcomes (2). Cardio-pulmonary arrest, trauma, vascular surgery and many other clinical scenarios are inherently linked with ischaemia and reperfusion. Solid organ transplantation is another clinical domain where the transplanted organ is invariably exposed to the sequelae of IRI. Kidney is one of the more common organs exposed to IR, and can occur following ischaemia, transplantation and secondary to sepsis and medications.

Renal ischaemia- reperfusion injury is an invariable component of renal transplantation and is shown to be responsible for delayed graft function, primary non-function (total lack of function) or long term malfunction of a proportion of solid organ grafts (3, 4). DGF is usually defined as patients requiring dialysis in the first week post transplantation (apart from treating hyperkalemia). Reported incidence of DGF varies between 2% to 50% of recipients of cadaveric donations in the first week following transplantation, while in recipients of live donor kidneys, the reported incidence is 2% -5% (5-8). DGF and dependence on dialysis in the initial post-transplant period can increase dialysis-related morbidity, prolong hospital stay and increase costs. DGF increases the risk of acute rejection, and in the setting of DGF and AR, the long term graft survival is reduced (9).

Renal IRI in the transplant setting is complex as many variables affect the outcomes of the transplanted kidney. This includes the donor, the organ (retrieval and preservation) and the recipient. Donor treatment prior to transplantation has many ethical issues, and the focus of current research has been towards attenuating the IRI in the transplant organ. IRI involves complex interactions of innate intrinsic mechanisms and immune mediators. Understanding the molecular and immunological consequences of IR will pave way for innovative therapeutic strategies aimed at attenuating ischaemia and reperfusion-associated tissue inflammation and organ dysfunction (10).

1.2. Pathophysiology of IRI

The pathophysiology of IRI has been defined predominantly thorough animal studies, which has been supplemented by study of the changes observed in biopsies of native and transplant kidneys exposed to IR. The proposed mechanisms for the cellular, biochemical and the molecular changes in a kidney following IR are discussed in this section.

1.2.1. Ischaemia- Reperfusion sequel overview

Ischaemia-reperfusion is an inflammatory state where complex molecular and cellular processes take place. Ischaemic insult damages the endothelial barrier leading to partial disappearance of the cell-cell borders and interruption of the cell-cell contacts, leading to endothelial disintegration and increased vascular permeability (11). Damaged endothelial cells release cytokines and chemokines that facilitate endothelial interaction with leukocytes and platelets. Upregulation of cytokines like TNF-alpha, Interferon-gamma, IL-1, IL-2 and IL-8 in turn upregulate adhesion molecules such as E-Selectin, P-Selectin, ICAM-1 and VCAM-1, thus triggering the infiltration of inflammatory cells (12). IRI also leads to upregulation of neutrophil chemotactic mediators including PAF (Platelet activating factor) and leukotrienes. Neutrophils in turn release chemotactic substances like IL-8 which promote neutrophil migration and activation, leading to glomerular injury (13). Leukocytes accumulate in the reperfused organ and release proteolytic enzymes that further damage the endothelium leading to oedema and capillary plugging (14, 15). The tubular epithelium, which is metabolically very active, maintains a critical oxygen tension is often the site of IR injury. It secretes proinflammatory cytokines (TNF alpha, IL-8, IL-6, TGF-beta etc.) that recruit and activate inflammatory cells. It is also involved in T-cell

recruitment via expression of receptors for complement, toll-like receptors (TLR), MHC-II and co-stimulatory molecules that regulate T-cell activity (16).

Reperfusion leads to initiation of blood flow, however due to endothelial cell swelling, interstitial oedema and capillary vasoconstriction, a 'no-reflow' phenomenon occurs that further potentiates tissue damage (17). Reactive oxygen free radicals and pro-inflammatory cytokines/ chemokines further augment endothelial damage. Endothelial damage leads to glycocalyx loss, disruption of the cytoskeleton and alteration in endothelial cell-cell contacts leading to breakdown of perivascular matrix, leading to increased vascular permeability (16, 18). Neutrophils are believed to be the early mediators of parenchymal damage, while macrophages and monocytes are considered late mediators, and prolong the injury phase (17). The complement system is also activated following IR, and the complement cascade further aids the inflammatory process. C3a and C5a are potent chemo-attractants drawing the inflammatory cells to the site of injury. Membrane Attack Complex (MAC) causes cellular damage by inserting into the cell membrane, and in kidneys, the tubular epithelial cells are a major target for complement-mediated attack (19). IR leads to several biochemical changes at a cellular level, and they are discussed below.

Ischaemic injury

Ischaemia deprives an organ of essential nutrients, and leads to accumulation of metabolic products. This leads to anoxic injury of renal tubular cells, leading to a decrease in mitochondrial energy production (20). The anoxic environment contributes to anaerobic glycolysis and inhibition of the NA/ATPase pump (5). In states of normal perfusion and oxygen delivery, adenosine triphosphate is eventually broken down into hypoxanthine, but

in the presence of anaerobic glycolysis, further degradation of hypoxanthine is not possible and this leads to accumulation of hypoxanthine, and reduction in ATP content (21). This increase in hypoxanthine triggers the conversion of nicotinamide-adenine-dinucleotide reducing xanthine dehydrogenase to the oxygen radical producing xanthine oxidase, leading to formation of oxygen free radicals that contribute to tissue injury (22-24). Dephosphorylation of ATP to ADP leads to a sequence of events that leads to altered cellular permeability leading to loss of substrates that could have been used for synthesis of high energy phosphates on reperfusion (25).

The ATP synthetase activity can be restored after 1 hour of cold ischaemia, but periods greater than 24-hours leads to irreversible loss of synthetase activity with lethal cell injury (26). Combination of anaerobic glycolysis and accumulation of lactic acid lowers intracellular pH, culminating in the failure of the sodium-potassium pump and deregulation of sodium and water homeostasis (22). Failure of the pump leads to accumulation of Na+ ions leading to termination of the Na+ /Ca^2+ antiporter pump activity. This leads to rise in intracellular calcium levels, which is further augmented by ATP depletion (27). Excess intracellular calcium is linked to opening of the mitochondrial transition pore (mPTP) leading to necrotic cell death (28). Failure of the Na+ /Ca^2+ antiporter pump leads to mitochondrial and nuclear swelling, and eventual cell rupture causing loss of electrolyte homeostasis. Subsequent increased protease and phospholipase activity are also damaging to neighboring cells (5, 29, 30).

Intracellular binding of iron to its carrier protein is also inhibited due to ischaemic insult, leading to increase in free iron that acts a catalyst for generation of oxygen radicals and reactive oxygen species (ROS) (31). Oxidative stress is also further accentuated by the

reduction in anti-oxidant enzymes (e.g.: catalase, superoxide dismutase glutathione peroxidase etc.) which leads to inhibition of the expression of cytochromeioxidase subunits that further increase ROS production upon reperfusion (32). Oxygen free radicals also contribute to production of nitric oxide (NO) that can transform into peroxynitrite, a potent oxidant (21). However, the role of nitric oxide in ischaemic injury is controversial. NO acts as a potent vasodilator, and so augments renal medullary blood flow during ischaemia contributing to renal protection (33, 34). This action must be weighed against the capacity of NO to disrupt the cytoskeleton and cellular adherence, leading to proximal tubular cell detachment and tubular obstruction (5, 35, 36).

In response to renal ischaemia, cytoprotective mechanisms are also invariably activated. The immediate response to ischaemia is an overall decrease in the cellular metabolic activity (37). At the molecular level, transcription of genes whose products are cytoprotective (38) or linked to cellular regeneration (39) (for example, Heme oxygenase-1 and VEGF) are also activated (40). In summary, the net effect of these processes is that at the end of the ischaemic phase, proximal tubular cells are ATP depleted, and show evidence of mitochondrial injury and down-regulated antioxidant defenses (27).

Reperfusion injury

Reinstitution of blood flow to an ischaemic kidney leads to a sequence of pathological processes together referred to as reperfusion injury. Reperfusion injury depends on the severity and the length of the ischaemic insult, and can take hours to days to develop. Reperfusion leads to rewarming, reoxygenation and a return to aerobic metabolism (5). Intracellular pH returns to baseline leading to massive sodium influx with mitochondrial

calcium overloads that lead to the activation of the calpains. Calpains hydrolyze target proteins leading to mitochondrial dysfunction and cell death (41). Increase in perfusion pressure causes direct endothelial damage, and also triggers an inflammatory response that exacerbates pre-existing renal injury (42).

Following reintroduction of oxygen, Reactive Oxygen Species (ROS) are generated in high concentration. ROS are formed by incomplete reduction of molecular oxygen and include superoxide anion (O2-.), hydrogen peroxide (H2O2), hydroxyl radical (OH.), and singlet oxygen. Extensive evidence links ROS to the tissue damage associated with reperfusion injury (43, 44). ROS causes direct oxidative damage to nucleic acids and proteins. ROS can also modify glomerular permeability to proteins (through activation of proteases and reduction of proteoglycan synthesis), and cause reduction in glomerular blood flow and filtration rate through liberation of vasoconstrictory lipids (prostaglandins and platelet activating factor) (45). In the normal kidney, the actions of ROS are mitigated by antioxidant enzymes, but reperfusion injury subdues the 'scavenging effect 'of these protective enzymes (21, 46). The combination of ROS and high intracellular calcium leads to mitochondrial matrix changes, causing rupture of cellular membranes and activation of pro-apoptotic caspases (25). Reperfusion also activates repair and regeneration processes, and the fate of an organ either as recovery or death will be characterized by various cellular events characterized by necrosis (traumatic cell death due to acute cellular injury), apoptosis (process of programmed cell death) or autophagy (catabolic mechanism that involves degradation of unnecessary or dysfunctional cellular components) (25, 27).

1.2.2. Pathological changes

The pathophysiological changes following IR involve complex interactions between the inflammatory mediators and the renal architecture. *Tubular injury* is a characteristic feature of IRI, usually presenting as acute tubular necrosis (ATN). The tubular cells in the proximal tubules (straight portion) and the ascending loop of Henle are more susceptible to hypoxic insult due to ischaemia, and these cells show changes in the form of apical blebs and loss of the brush border membrane, eventually leading to loss of polarity and integrity of the tight junctions (47). Hemodynamic alterations lead to reduction in the glomerular filtration rate (GFR), which is compounded by formation of casts and debris that obstruct the tubule lumen, leading to back-leak of filtrate through the damaged epithelium (48). The inhibition of the NA/ATPase pump accelerates cell death by both necrosis and apoptosis, and the sloughing of cells leads to cast formation and obstruction of the tubular lumen. Tubular and interstitial damage leads to inflammation and haemorrhage, with widespread necrosis indicating a more severe injury (49).

Tubulointerstitial inflammation is also a hallmark of renal IRI. The inflammatory response following IRI leads to synthesis and release of multiple cytokines including TGF-beta, a profibrotic cytokine with pleiotropic actions that together drive tubulointerstitial fibrosis and atrophy (50). Fibrosis and atrophy in the tubulointerstitial compartment of the kidney is a pathological hallmark of chronic kidney disease, and is a predictor of progression to end-stage renal disease (51).

Glomerular damage is also seen following IRI. The glomerular barrier is composed of three layers: the fenestrated endothelium with its glycocalyx, the glomerular basement membrane

(GBM), and the podocytes with their interdigitating foot processes (52). Following IR, ROS and inflammatory mediators cause alterations in the endothelial glycocalyx layer, flattening and spreading of the podocyte foot processes, and creation of large pores in the glomerular basement membrane, that collectively cause proteinuria (53). IRI has also shown to be contribute towards degradation of the GBM (54). Glomerular damage following IRI can be histologically seen as thickening of the glomerular capsule and retraction of glomerular tuft, but in severe IRI, glomerular fibrosis can be seen (55). Ischaemia leads to endothelial cell damage and smooth muscle damage. The endothelial damage can be visualised as endothelial cell swelling in mild IRI, with disruption and loss seen in severe IRI (56).

Recovery from renal IRI requires *tubular regeneration and repair*. Experimental models indicate that repair is promoted by growth factors, integrins and extracellular matrix proteins that promote tubular cell survival and proliferation. This aids in the recovery and reversal of AKI (57). Recent studies have focused on using stem cells and other interventions to improve outcomes after renal injury, mainly by providing factors that promote renal tubular repair. This avenue offers promise for future interventions.

1.3. Mediators of Renal IRI

In addition to the direct injury from ischaemia and hypoxia, the inflammatory response in IRI also plays a significant role. The mediators discussed in this section are linked in modulating both the inflammatory and the immune response.

1.3.1. Inflammatory Mediators

1.3.1.1. Cytokines

The cytokines are cell signaling proteins that aid cell to cell communication, predominantly in immune responses and inflammatory states. In renal IRI, cytokines are produced mainly by the leukocytes and renal tubular cells, and mediate and propagate AKI by attracting leukocytes and other inflammatory mediators to the site of injury. Cytokines such as TNF-alpha, IL-1, MCP-1, IL-8, PDGF and VEGF have been shown to be produced by the PTC (58). Pro-inflammatory cytokines IFN-gamma, TNF-alpha, IL-1, IL-2, IL-6, GMCSF and TGF-Beta 1 are increased in the ischaemic AKI and renal injury rodent models (59, 60). IL-18 and IL-6 are also released from the PTEC and have been used as urinary biomarkers for AKI, and significant elevations of IL-6 and IL-8 were associated with mortality in an ITU setting (61, 62).

In an injury state, the pro-inflammatory cytokine response is also tempered by protective anti-inflammatory cytokines like IL-10, IL-11, IL-13 and specific cytokine receptors (for IL-1, TNF, IL-18) (63). In renal IRI, there is also secretion of protective cytokines such as IL-4, IL-10, IL-13, and VEGF that aid in reducing renal injury by inhibiting inflammatory and cytotoxic pathways of AKI (64). IL-10 was shown to protect against ischaemic and

cisplatin-induced AKI partly through its effect of inhibiting genes responsible for leukocyte activation and adhesion (65). IL-6 was shown to simultaneously promote a pro-inflammatory injury response and a renal protective response via a mechanism of trans-signaling (66).

Mitigating the effect of cytokines with interventional agents seems attractive, however the literature evidence remains equivocal. Blocking IL-1 receptor with antagonist (IL-1Ra) or using IL-1 knock out mice did not offer protection from renal injury (67). Interleukin-18 binding protein transgenic mice were shown to be protected against ischaemic AKI with IL-18 dependent cytokines (IL-3, IL-6, IL-15, macrophage colony-stimulating factor, MIP-2, GMCSF, MCP-1) shown to be increased in the AKI group compared to the sham group (68). In summary, there are hosts of cytokines that are released following AKI that have both a pro-inflammatory and anti-inflammatory properties. It is likely that these contradictory results with cytokine blockade reflect the complexity of responses seen, and that the therapeutic efficacy of targeting specific mediators to improve renal outcome in IRI will depend on precise clinical/cellular context.

1.3.1.2. Chemokines

Chemokines are cytokine-like molecules that are subdivided into four subfamilies: CXC, CC, C and CX3C – based on spacing and number of cysteine residues in their sequences(69). They are induced by cytokines (like TNF-alpha, IL-1), complement system, ROS, and TLR related pathway in an inflammatory setting and mediate leukocyte recruitment and immune responses (11). In renal IRI, CXC chemokines have been shown to promote neutrophil infiltration, CXCR3 ligands are involved in recruiting Th1 cells and CC

chemokines induce mononuclear cell infiltration and macrophage activation (70). CCR1 (chemokine receptor) has been shown to regulate macrophage and neutrophil movement into kidney in a murine IRI model, and treatment with CCR1 antagonist or using CCR1 deficient mice showed decreased inflammatory infiltrates (71).

CXCL1 has been proposed to be one of the mediators of ischaemic AKI as it is increased in post IRI kidneys, and a neutralizing antibody to CXCL1 has attenuated renal injury in rodent models through decreased neutrophil and inflammatory infiltrates (72, 73). CX3CL1 (also known as fractalkine) acts as a chemo attractant for NK cells and monocytes in renal injury, and fractalkine expression is increased in endothelial cells following ischaemic AKI (74, 75). Though blockade of CX3CR1 with specific antibody did not reduce the injury in cisplatin induced AKI (76), inhibition of CXCR2 inhibitor (using repertaxin) attenuated injury in a syngenic transplantation model in rats (77). In summary, chemokines regulate cytokine and adhesion molecule expression, mediate leukocyte recruitment and contribute to angiogenesis and fibrosis in the reperfusion phase (78).

1.3.1.3. Adhesion molecules

Adhesion molecules aid leukocyte adhesion to the endothelium during the inflammatory process that follows IRI. The adhesion molecules are broadly classified into calcium independent (Immunoglobulin superfamily) or calcium dependent (selectins, integrins, cadherins). The role of adhesion molecules in renal IRI is well known, with rodent models undergoing interventions prior to IR showing suppression of adhesion molecules, and reduction of inflammatory infiltrates. A brief synopses of various adhesion molecules is provided below:

- **ICAM-1** (Inter-cellular adhesion molecule: CD54) enhances leukocyte endothelial interaction, and guides leukocyte extravasation by binding to LFA-1 (79). In a study exploring ICAM-1 expression in rat kidney between IR and toxic AKI, increased expression of ICAM-1 along with monocyte and T-cell accumulation was seen in the inner strip of outer medulla very early after IR, compared to the toxic group where ICAM-1 expression was delayed with no leukocyte accumulation (80). Antibody to ICAM-1 administered prior to renal IR, and mutant mice genetically deficient in ICAM-1, were both protected against renal ischaemic injury (81, 82).

- **P-Selectin** (platelets, endothelial cells), E-Selectin (endothelium) and L-Selectin (leukocytes, lymphocytes) have all been implicated in the IRI process. P-Selectin has been shown to be involved in leukocyte recruitment to the kidneys in a murine renal IRI model (83), and its inhibition attenuated IR injury in rodent models (84, 85). P-Selectin is readily available within the endothelial cells in cytoplasmic deposits called Weibel-Palade bodies. Ultrasound imaging of P-Selectin-targeted contrast agent showed a significant increase in vascular P-Selectin expression 1 hour post reperfusion in murine renal IRI model, predominantly in the cortex of the post-ischaemic kidney compared to the contralateral kidney (86). Blockade of ligands for all three selectins using a novel small molecule (TBC-1269) showed functional and structural protection from IRI in moderate (30 minutes IR), but minimal protection in severe IR (45 minutes) (85).

- **L-Selectin** mediates leukocyte rolling on endothelium and has been upregulated in experimental and human renal ischaemia/reperfusion models. IRI leads to severe endothelial damage that modifies heparan sulfate proteoglycans (HSPGs) in basement membrane, enabling the endothelium to bind L-Selectin (87). Though L-Selectin blockade attenuated IRI in cardiac and skeletal muscles, in renal rodent models, it was shown not to confer any structural or functional protection from IR injury (88).

- **E-Selectin (CD62E)** and its ligands are essential for recruitment of leukocytes in inflammation, and its role in neutrophil recruitment was shown in a study where basigin/CD147 (ligand for E-Selectin that promotes renal inflammation in ischaemia/reperfusion) deficient mice demonstrated striking suppression of neutrophil infiltration in the kidney after renal IR (89). Preventing leukocyte adhesion and infiltration confer protection from IR injuries (90-92). Inhibition of ICAM-1 and E-Selectin expression by a radical scavenger (lecithinized superoxide dismutase) in a experimental model led to decreased neutrophil adhesion to the endothelium (93).

- **Platelet endothelial cell adhesion molecule** (PECAM-1: CD31) PECAM-1 is a cell adhesion and signalling receptor expressed on hematopoietic and endothelial cells with pro-inflammatory and anti-inflammatory functions. PECAM-1 is known to inhibit platelet function and thrombus formation. Pro-inflammatory functions of PECAM-1 include the facilitation of leukocyte transendothelial migration, and anti-

inflammatory functions include the dampening of leukocyte activation, suppression of pro-inflammatory cytokine production and the maintenance of vascular barrier integrity (94). Increased expression of PECAM-1 has been seen following renal IR in mice (95). NO-dependent vasodilator molsidomine injected 15-minutes before reperfusion prevented the IR induced renal dysfunction, with reduced inflammatory cell infiltration along with decreased expression of adhesion molecules (ICAM-1, PECAM-1, VCAM-1 and P-Selectin) (96).

- **Vascular cell adhesion molecule** (VCAM-1: CD106): VCAM-1 is found in very low levels on the cell surface of resting endothelial cells and other vascular cells such as smooth muscle cells and fibroblasts. It binds to very-late antigen-4 (VLA-4) integrin on the cell surface of leukocytes, mediating its adhesion to the vascular endothelium (97). IL-1 and TNF-alpha increase expression of VCAM-1, P-Selectin and other cell adhesion molecules on the vascular endothelial cells, however, knockout mice deficient in both IL-1 and TNF-alpha receptors were also shown to increase expression of ICAM and VCAM in renal IR implicating other pathways of activation (98, 99). Endothelial VCAM-1 expression contributed to T-cell infiltration at sites of inflammation(100), and was also shown to mediate mononuclear cell infiltration into the perivasculature after kidney transplantation in a rat model (101).

Adhesion molecules play a key role in the process of IRI. Rodent models using antibodies to adhesion molecules have shown promise, but whether they can be translated into clinical practice remains to be seen.

1.3.1.4. Cells mediating inflammatory response

Leukocytes are derived from a multi-potent hematopoietic stem cell, and are often classified into two broad categories depending on the granules in their cytoplasm:

- Granulocytes – neutrophils, basophils, eosinophils
- Agrnaulocytes - lymphocytes, monocytes, macrophages

Dendritic cells are also considered part of leukocytes and are cells of myeloid/lymphoid lineage. They are predominantly involved in the immune pathways, and have more recently been considered to have a role in mediating inflammation.

Ischaemia-reperfusion injury is a complex interrelated sequence of events that classically involves the vascular endothelium and activated leucocytes (102). Leukocytes adhere, marginate and infiltrate through into the interstitium leading to microvascular congestion and 'no-reflow' phenomenon. Tissue injury is further augmented with the release of pro-inflammatory cytokines. Leukocyte adhesion to endothelium and rolling have been visualised in primate limb IR studies (103). Experimental models employing perfusion of kidneys with leukocyte and platelet depleted blood (using white cell filters) have shown some promising results in relation to improved renal blood flow and creatinine clearance (104, 105). The relative importance of different leukocyte subtypes mediating IR is difficult to pin-point as all the individual cells have been shown to be recruited into the IR process at

various time intervals, and the contribution of each cell in the ischaemic or the reperfusion process varies (106, 107). In renal IRI, the individual cells have been evaluated in isolation to determine the effect on the IR, and have shown mixed results. The following paragraphs succinctly review the role of the leukocytes involved in the IRI process.

Neutrophils

Following IR, neutrophils attach to the activated vascular endothelium and release reactive oxygen species that can damage the tubular cells and accentuate IRI (102, 108). The recruitment of neutrophils to the inflammatory site is governed by a variety of adhesion molecules. Selectins are involved in mediating the initial rolling of cells followed by integrins that enable neutrophils to attach firmly to the endothelium. The final migration throughout the endothelial wall is mediated by adhesion molecules like PECAM and ICAM (13). Chemokines are potent neutrophil chemo-attractants, and studies have shown the primary mediators of IRI injury to be the neutrophils (109-111). Ischaemic, nephrotoxic and endotoxemia induced acute kidney injury have consistently showed enhanced neutrophil infiltration, but the precise kinetics of neutrophil trafficking is not fully defined (11, 112). CXC chemokines are known to direct neutrophils to tissue sites of inflammation, and pre-treatment with CXCL1 neutralizing antibody showed reduced neutrophil infiltration into the ischaemic kidneys with attenuation of the IR injury (113).

Mutant mice genetically deficient in ICAM-1 were protected from renal IRI mainly via attenuation of neutrophil-endothelial interactions, leading to decreased inflammatory infiltrates (81). In the early reperfusion phase following IR, kidney IFN-gamma-producing cells were comprised largely of CD11b neutrophils and CD1d-restricted NKT cells, and

depletion of NKT cells led to decrease in IFN-gamma-producing neutrophils indicating a role for NKT cells driving neutrophil migration (114). CD44 is expressed by neutrophils and is rapidly upregulated by capillary endothelial cells after IR injury, and CD44 deficiency/ administration of anti-CD44 to mice lead to reduced influx of neutrophils into the post ischaemic tissue, along with preservation of renal function and morphology (115). Inhibitors of proinflammatory factors (P-Selectin, TNF alpha, ICAM 1), and other interventional agents used in renal IR models have consistently showed reduced neutrophil infiltration in the post ischaemic kidney, although the 'protective effects' of these agents were not exclusively attributed to neutropenia alone (60, 116-118).

Studies have shown that reperfusion of ischaemic kidneys with neutrophils can worsen ischaemic injury (107, 119). Rodents rendered neutropenic by using antineutropil serum did not confer protection from IRI (120, 121). Administering a monoclonal antibody (MoAb 60.3- blocks neutrophil adherence to rabbit endothelial cells) in rabbits or anti-neutrophil serum administration in rats did not offer functional or morphological protection from renal IRI (122). In summary, the role for neutrophils in renal IRI still needs to be accurately elucidated, as it's seen in IRI kidneys, however mitigating the function or reducing the influx did not show benefits.

Macrophages

Macrophages are major players in the innate and adaptive immune response of renal IRI. The macrophages reside in the interstitial extracellular compartment of the kidneys, and interact with substances transported from the tubule lumen into peritubular capillaries. Apart from being a cellular mediator of immunity, they are also involved in the

inflammatory process that takes place in a IRI setting, and is involved in the injury/repair of post-ischaemic kidneys (64, 123). In the kidney, macrophages are well-known sources of pro-inflammatory caspases and cytokines (IL-18, IL-1, TNF-alpha, MCP-1). Osteopontin (macrophage chemoattractant) knockout mice showed reduced macrophage infiltration, enhanced apoptosis in the injury phase and decreased collagen I and IV expression in the regeneration phase of post-ischaemic AKI (124). In a mice model, CCR2- and CX3CR1-dependent mechanisms were shown to be involved in the migration of inflamed monocytes to the renal IRI site, and the injury was further accentuated by secretion of cytokines like TNF-alpha, IL-6, IL-1 and IL-12 by the inflamed monocytes (125). In this study, macrophage numbers in the ischaemic kidney increased at 1-hour post reperfusion and persisted for 7 days.

Macrophage depletion using clodronate prior to renal IR demonstrated protective benefits from IRI (90, 126, 127). This effect was attributed to the depletion of CXCL1 (neutrophil and T-lymphocytes chemo attractant) chemokine, and macrophages are the main source of CXCL1 in the ischaemic kidney (11). Different types of macrophages have been described that promote (M1) and inhibit inflammation (M2) (128). Lee et.al showed that depletion of macrophages before IR diminished mice kidney injury, with pro-inflammatory (M1) macrophages recruited into the kidney in the early phase (48 hours) after IR, and non-inflammatory (M2) macrophages seen at later time points, suggesting that macrophages underwent a switch from a pro-inflammatory to a trophic phenotype that supported kidney repair (129). Macrophages are known to have various subsets, and targeting only 'activated macrophages' has been shown to be beneficial in the IRI setting (130).

T-Lymphocytes

Presence of lymphocytes in the post-ischaemic human kidney biopsies alerted to its possible role in IRI (131). T-cells and macrophages were localized predominantly in outer stripe of outer medulla, the zone of most severe injury (112). Proximal tubular epithelium and dendritic cells (presenting antigen to native T-cell and expressing co-stimulatory molecules) have been implicated in the activation and regulation of T-cells in IRI (132). Ascon et.al showed increased trafficking of CD3+ T cells and CD19+ B cells in both sham-operated and IRI mice 3-hours after renal IRI, thus alluding to the possibility that even abdominal surgery itself can lead to lymphocyte changes in kidney (133). Blocking of chemotaxis factors CXCR3 and CCR5 in a rat renal IRI model showed decreased infiltration of T cells and NKT cells in the kidneys, and had lower tubular injury scores compared to the controls (134). Upregulation of lymphocyte-related cytokines and adhesion molecules (CD11/CD18, ICAM-1) in experimental IRI models have further supported the role of T-lymphocytes in IRI (135).

Takada et.al showed that blockade of T cell CD28-B7 co-stimulation with CTLA4Ig prevented the early and late consequences of IRI, and lead to significant inhibition of T cell and macrophage infiltration and activation in situ (136). Though the exact role of T-lymphocytes in the IRI has to be ascertained, it has been hypothesised that T-lymphocytes may mediate neutrophil infiltration into ischaemic kidneys as evident by decreased neutrophil infiltration and decreased tubular necrosis in CD4+ and CD8+ deficient mice undergoing IRI compared to their WT controls (135). T-cell deficient (nu/nu mice) and CD4+ deficient mice were functionally and structurally protected from post ischaemic renal injury, compared to the WT control and CD8 deficient mice (99). Attenuation of IR effects

in other organs has also been observed with T- cell deficiency, or via T-cell depletion (137, 138). Similarly, T-cell receptor (TCR) deficient mice showed reduced renal tubular injury, reduced TNF-alpha and IL-6 protein expression in kidney tissue compared to WT littermates after IR (139). Yokoto et.al demonstrated that T cell depletion [Antibodies to CD4 (GK1.5), CD8 (2.43) and pan-T cells (30.H12)] offered protection from renal IRI in mice, though the antibody combinations did not significantly alter other leukocyte populations (140).

On the contrary, there have been studies that have shown that T-lymphocytes have a limited role in IRI. Peripheral CD4+ T-cell depletion did not confer functional or histological protection against AKI in a murine renal IRI model (141). Recombination-activating gene (RAG)-1-deficient mice (deficient in Ig and T-lymphocytes) when subjected to bilateral renal IR, showed ATN and neutrophil infiltration in the kidney compared to the WT mice (142). RAG-1 deficient mice lacking both B and T cells were not protected from renal IRI, and adoptive transfer of either B or T cells into RAG-1 deficient mice led to a significant protection of renal injury(143). Overall, T-cells are considered to have a more important role in the early inflammatory process than the late adaptive immune response (144), but a definitive role from these studies still needs to clearly defined.

B-Lymphocytes

B cells are involved in adaptive immune response and contribute to tissue pathology in immune-mediated disorders, not only by producing antibody, but also through other mechanisms which may involve antigen presentation, cytokine production and complement system activation (145). Knockout mice deficient in B-cells and models using complement

components that interact with B-cell receptor have shown protection from IRI in selected organs (heart, intestine, skeletal muscles) (17). The precise role and kinetics of B-cell recruitment in renal IRI models are yet to be elucidated. Infiltration of CD19+ B-cells were seen in both sham-operated and IRI mice 3-hours after renal IRI (133). B cell-deficient (μMT- mutation of the heavy chain of IgM (μ chain) that doesn't allow differentiation of premature B cells to mature B cells) mice were conferred structural and functional renal protection compared to their WT in a 30-minute renal ischaemia model, and was attributed to lack of circulating natural antibodies (146). Depletion of peritoneal B-1 cells prior to renal IR was shown to confer protection via reduced mesangial IgM, along with increased production of the anti-inflammatory cytokine IL-10, but, μMT mice in this study suffered worse renal IR injury than the WT mice (147). RAG-1 deficient mice (lacking both B and T cells) subjected to IR failed to confer protection from IR injuries (143, 148). Hence, the precise role of B-cells in renal IRI is still to be ascertained.

Regulatory T-Cells (Tregs)

Tregs have anti-inflammatory and immunosuppressive properties, and are identified by the expression of CD4 and CD25 on cell surface, and up regulation of transcription factor FOXP3. Tregs can traffic to areas of inflammation to mitigate immune reactions, and can inhibit neutrophil ROS generation and pro-inflammatory cytokine production (149, 150). They reside in the kidney under normal conditions, and its role in IRI has been the focus of recent research. Partial depletion of Tregs using anti-CD25 antibody worsened ischaemic kidney injury, and adoptive transfer of Tregs into RAG-1 knockout mice prevented kidney IRI (151). In the same study, FoxP3(+) Treg-deficient mice was shown to accumulate a greater number of inflammatory leukocytes after renal IRI than mice containing Tregs, and

transfer of Tregs attenuated renal injury. The authors concluded that Tregs modulated injury through IL-10-mediated suppression of the innate immune system.

Treg depletion starting a day after after ischaemic injury using anti-CD25 antibodies worsened renal tubular damage, and increased infiltrating T lymphocyte cytokine production at 3 days, which was reversed with the infusion of CD4(+)CD25(+), thus suggesting a role for Tregs in the healing process promoting repair (152). A pharmacological agent- sphingosine N,N-dimethylsphingosine (DMS) was shown to recruit Tregs to IRI kidneys and confer protection against IRI (153). Based on these studies, Tregs seem to have a beneficial effect in both the initiation and the repair phases of renal IRI, but further studies aimed at identifying mechanisms by which Tregs facilitate and promote renal recovery can hold immense potential in treating acute kidney injury (154).

Natural killers cells (NK cells)

NK cells interact with various components of the immune system and function as regulatory cells, from assisting in dendritic cell maturation to T-cell polarization (155). RAG-1 mice deficient in T and B cells were not protected from IRI, suggesting a role for NK cells or other innate immune responses (143). Rae-1, a cell surface receptor recognized by NKG2D on NK cells, is upregulated following kidney IRI (156). In an experimental renal IRI model, the PTEC were shown to upregulate the expression Rae-1 by activating the NKG2D receptor on NK cells, and furthermore, NK cell depletion in WT mice conferred protection (157). C57BL/6 OPN-deficient mice had reduced NK cell infiltration and lesser tissue injury compared to WT after IR, suggesting OPN expression by PTEC as an important factor in initial inflammatory responses involving NK cells in kidney IRI

(158). NK cells were rapidly recruited into kidneys 4-hours after IRI, which in turn recruited neutrophils by stimulating the CD137L signalling pathway in PTEC, leading to production of CXCL1 and CXCL2 (159).

Natural Killer T cells (NK T cells)

NKT cells are unique subset of T-lymphocytes with surface receptors and functional properties shared in common with conventional T-cells and NK cells (NK cell-associated marker NK1.1/ NKR-P1.9), and exist in the thymus and peripheral lymphoid organs. NKT cells have the ability to rapidly produce large amounts of cytokines, including Th1-type (IFN, TNF) and Th2-type (IL-4, IL-13), and can amplify and regulate functions of DCs, Tregs, B-cells, T-cells and NK cells (123). Conventional CD4+ T cells play a role in antigen-specific adaptive immunity that requires several days to be active, and as such cannot be part of the rapid immune response seen in IRI, hinting at a possible role of NKT cells as an early mediator (160). In an experimental model aimed at characterizing lymphocytic infiltrates, increased infiltration of NK1.1+ and CD4+ NK1.1+ cells compared with normal and sham-operated mice was observed 3 and 24-hours after renal IRI respectively (133). In a murine IR model, GR-1(+)CD11b(+) neutrophils, and CD1d-restricted NKT cells were seen within three hours of reperfusion, and antibodies to block CD1d/deplete NKT cells/ mice deficient of NKT cells (Jalpha18(-/-)) markedly reduced IR injury (114). Chemotaxis factors CXCR3 and CCR5 are also expressed on CD4T cells and NKT cells. Blocking of chemotaxis factors CXCR3 and CCR5 by an antagonist (TAK) significantly reduced the infiltration of CD4+ T cells and CD8+ NKT cells, and showed functional and structural protection compared to the control and sham groups (134).

Dendritic cells

Dendritic cells (DC) are primarily involved in adaptive immune response and possess surface receptors that recognize pathogen-associated molecular patterns, thus enabling them to respond to infection and inflammation (161). DCs originate from the bone marrow and remain in an immature form until stimulated by a focus. DCs are present throughout the renal interstitial space, and are interposed between the tubular epithelium and the peritubular capillaries in the healthy kidney (162). CD11c+ MHC class II+ DCs are the most abundant leukocyte subset in the normal mouse kidney, and upon stimulation can activate T-cells (123). Increase in the differentiation of peripheral monocytes into DCs with increased IL-12 and IFN-gamma production by DC-stimulated T cells, were seen two days after IRI compared to the sham group (163). Studies have shown that endothelial activation following ischaemia leads to DC adhesion which is partially mediated by ICAM-1, and DCs in turn activate alloreactive T-cells leading to allotransplant rejection (164). Cell suspensions from mouse IRI kidneys showed increased secretion of TNF, IL-6, MCP-1 and RANTES from resident DCs, and in vivo depletion of intrarenal DCs (Clordonate liposome-mediated depletion) substantially attenuated TNF secretion, there by attributing DCs as the predominant secretors of TNF within 24 hours of IRI (165).

The role of DCs in renal IRI has been the focus of research in recent years. Paucity of DC-specific cell surface markers and difficulty in distinguishing DC from other members of the monocyte/macrophage lineage probably explains the reason. The role of DCs in regulating balance between regulatory T-cells and effector T-cells at IR site can be a potential target for treating IRI (161, 166), and future IRI models using genetically engineered mouse with specific DC depletion can shed more light.

1.3.2. The Complement System

The complement system is an integral part of the innate immune response and acts as a bridge between innate and acquired immunity. It consists of a series of proteins that are mostly (although not exclusively) synthesised in the liver, and exist in the plasma and on cell surfaces as inactive precursors (zymogens). The complement proteins are activated by, and work with IgG and IgM antibodies, hence the name "complement". The complement system is more complex than many enzymatic cascades as it requires the formation of sequential non-covalently associated activated protein fragments. These in turn become convertases and cleave components for the next enzymatic complex in the cascade, and the rapid dissociation of these complexes (and loss of enzymatic activity) forms an integral part of the elegant regulation of complement activity.

Complement mediates responses to inflammatory triggers through a co-ordinated sequential enzyme cascade leading to clearance of foreign cells through pathogen recognition, opsonisation and lysis (167). Complement binds to immune complexes and apoptotic cells, and assists in their removal from the circulation and damaged tissues (168, 169). A comprehensive review of the complement system can accessed via our publication: 'The complement system: history, pathways, cascade and inhibitors' (170), and only relevant sections are covered in this section.

1.3.2.1. Pathways of Activation and Cascade

There are three known pathways for complement activation: Classical, Alternative and Lectin pathway.

Classical pathway

The classical pathway is initiated by IgM or IgG antigen/antibody complexes binding to C1q (first protein of the cascade) leading to activation of C1r, which in turn cleaves C1s. This in turn activates the serine proteases that lead to cleaving of C4 and C2, leading to formation of C4b2a (C3 convertase), which in turn cleaves C3 into C3a and C3b (171). While C3a acts as a recruiter of inflammatory cells (anaphylatoxin), C3b binds to the C4b2a complex to form C5convertase (C4b2a3b). The C5 convertase initiates the formation of the Membrane Attack Complex (MAC), that inserts into cells creating functional pores leading to its lysis (172). The classical pathway can also be activated by other danger signals like C-reactive protein, viral proteins, apoptotic cells and amyloid, thus providing evidence that classical pathway could be activated independent of antibodies (173-176).

Alternative Pathway

50 years after the discovery of the classical activation pathway, Pillemer et al (177, 178), proposed a highly controversial alternative activation pathway. The alternative pathway is not so much an activation pathway, as it is a failure to regulate the low level continuous formation of a soluble C3 convertase. The internal thioester bond of C3 is highly reactive and undergoes spontaneous hydrolysis resulting in a molecule known as C3 (H2O), which resembles C3b. This can then bind to factor B, and be processed into a short-lived soluble C3 convertase that can generate more C3b. If this C3b binds to a nearby surface that is incapable of inactivating it (such as bacteria/yeast cells or damaged host tissues), this then leads to amplification of the alternative pathway (179-181). The presence of complement regulators (Factor I and Factor H) in healthy cells ensures the spontaneous hydrolysis of C3

is kept in check. C3 activation takes place when C3b binds to Factor B, and is then cleaved by Factor D (182). The enzymatic action of Factor D acts as the rate limiting step of the alternative pathway and cleaves Factor B, the larger fragment of which remains bound to C3b to form the alternative pathway C3 convertase – C3bBb (171, 183). C3b is able to create new C3 convertase in the presence of Factor B and D, thus acting as an 'amplification loop' for the classical and lectin pathways, in addition to the alternative pathway (175). The alternative pathway omits the components: C1, C2 and C4.

Lectin Pathway

40 years after the proposal of the alternative pathway, the MBL (Mannose-binding lectin)/ MASP (MBL associated Serine Protease) pathway was discovered. This pathway was characterised by using proteins isolated from rabbit liver and serum, but its function remained unclear initially (184, 185). Two forms of MBL (MBL-A and -C) are present in rodents compared to a single form in the humans. Studies linking the deficiency of MBL protein to immunodeficiencies in children led to its recognition as an important activator of the complement system (186, 187). The initiating molecules for this pathway are collectins (MBL and ficolin), which are multimeric lectin complexes. These bind to specific carbohydrate patterns leading to activation of the pathway through enzymatic activity of MASP (182). There are structural similarities shared between MBL and C1 complexes (MBL- with C1q-associated serine proteases, MASP-1 and MASP-2 with C1r and C1s respectively), leading to the belief that complement activation by MBL and C1 complexes are similar (188). MASP-2 cleaves C4 and C2 to form C3 convertase, while MASP-1 may cleave C3 directly bypassing the C4b2a complex, albeit at a very slow rate (189, 190).

Another serine protease, MASP-3 was shown to down-regulate the C4 and C2 cleaving activity of MASP-2 (191). Following the initial characterisation of MBL, 3 other lectins (known as ficolins) have been shown be associated with MASP: ficolin-1 (or M-ficolin), ficolin-2 (or L-ficolin) and ficolin-3 (or H-ficolin or Hakata antigen). The ficolins activate the lectin pathway by forming active complexes with MASP (192, 193). More recently, a new C-type lectin (CL-11) was shown to interact with MASP-1 and/or MASP-3 and could activate the lectin pathway(194).

Complement cascade

All three pathways lead to the generation of C3 convertase that cleaves the C3 protein into C3a and C3b. While C3a acts as an anaphylatoxin, C3b covalently binds to the activating surface and participates in the self-activation loop of complement activation via the alternative pathway. C3b also associates with C3 convertases (C4b2a or C3bBb) to form the C5 convertase, which cleaves C5 complement into C5a and C5b (195). Interaction of C5b with C6, C7, C8 and C9 leads to formation of C5b-9 / MAC, a multimolecular structure that inserts into the membrane creating a functional pore leading to cell lysis (172). MAC can cause lysis of some cells (e.g. erythrocytes) with a single hit, but some nucleated cells required multiple hits, or rather, multiple channel formation to cause cell lysis (196, 197). However, studies have shown that when the number of channels assembled on the cells is limited, sublytic C5b-9 can activate transcription factors and signal transduction, leading to inhibition of apoptosis and cell homeostasis (198, 199). The complement cascade with the inherent inhibitors is shown in Figure 1. 1.

The anaphylatoxins (C3a and C5a) are key players in the recruitment of inflammatory cells, and release of mediators that amplify the inflammatory response. C5a binds to C5a receptor (C5aR or CD88) that is widely present on inflammatory and non-inflammatory cells, and increases neutrophil adhesiveness and aggregation (200, 201). C5a also lead to secretion of pro-inflammatory cytokines and lysosomal enzymes from the macrophages and monocytes, thus leading to chemotaxis (171, 202, 203). C5a was shown to be an important inflammatory mediator for the early adhesive interactions between neutrophils and endothelial cells in the acute inflammatory response, and upregulates various adhesion molecules (171, 204, 205). C3a does not act as a chemoattractant for neutrophils, but aids migration of eosinophils and mast cells (206, 207). C3a and C5a also act on their receptors expressed on innate immune cells such as dendritic cells, thus playing a role in initiating and regulating T-cell responses (208). In the IRI setting, MAC has been shown to mediate IR injury, and its inhibition was shown to attenuate the IRI effect (209, 210).

Inherent regulation of pathways

To prevent inadvertent injury by activated complement, the host tissues have developed intricate and elaborate mechanisms in the form of soluble and membrane bound complement regulators that inhibit complement activation. The two main regulation mechanisms are: Decay-acceleration activity (DAA) which increases the rate of dissociation of (C4b2a and C3bBb) C3 convertases, and Factor I cofactor activity (CA), which results in the factor I-mediated cleavage of covalently bound C3b and C4b into inactive fragments incapable of reforming the C3 convertases (211, 212).

Membrane Bound Complement Regulators

The membrane bound regulators - DAF, CR1 and MCP belong to a gene super family called as regulators of complement activation' (RCA) / Complement control proteins (CCP) and share a common structural motif called short consensus repeat (SCR). The SCR structure consists of around 60 amino acids held together by two disulfide bridges formed by cysteine residues (213). The structural moiety of the membrane bound complement regulators are depicted in Figure 2.

1. *CD 35 - Complement Receptor 1/ CR1:* CD35 is a transmembrane glycoprotein that facilitates the decay of C3/C5 convertase in both the classical and alternate pathways, and acts as a co-factor for factor I in the degradation of C3b and C4b (214).
2. *CD46:* CD46 acts as a cofactor for factor I mediated cleavage of C3b and C4b. Deficiency of CD46 is a predisposing factor for numerous diseases due to complement-mediated 'self-attack' (215)
3. *CD55:* CD55 is a glycosyl-phosphatidyl-inositol (GPI) anchored membrane protein that is widely expressed on vascular and non-vascular tissue cells. The main role of CD55 is the inhibition and acceleration of the decay of classical and alterative pathways C3 convertase (216).
4. *CD59 (Protectin):* CD59 is a GPI-anchored membrane complement that is expressed on almost all cells in the body (217). CD59 is the only well-characterized membrane inhibitor acting at the terminal step, and prevents the assembly of the MAC by inhibiting the C5b-8 catalysed insertion of C9 into the lipid bilayer (218).
5. *CrrY- Complement receptor 1-related gene/protein Y:* CrrY is a transmembrane protein specific to rodents, and possesses both DAA and CA properties, and mimics the

activities of the human DAF and MCP which regulate C3 deposition on host cells (219).

Fluid phase or 'soluble' regulators

1. *C1 – Inhibitor (C1-INH):* C1-Inhibitor (C1-INH) forms an irreversible complex with the serine proteases C1r and C1s and inactivates them. This leads to the disassociation of C1r and C1s from C1q in the complex. C1-INH can also bind to MASP-1 and MASP-2, and inactivate them leading to disruption of the lectin pathway (220, 221). Various animal IRI models have shown that C1-INH can protect liver, intestine, heart, and brain tissue from ischaemia-reperfusion damage (222).
2. *Factor I:* Factor I cleaves C3b and C4b to form C3 and C4 fragments, thus blocking the formation of C3 and C5 convertase enzymes (220). The cofactors supporting factor I cleavage are factor H, CD35, CD46 and C4b-binding protein (223).
3. *Factor H:* Factor H possesses multiple binding sites for C3b and accelerates the decay of the alternative C3 convertase through 'competitive binding' for factor B (224). It also facilitates the cleavage of C3b by supporting factor I activity. Impaired recognition of Factor H by host cell surfaces due to mutations and polymorphisms can lead to complement-mediated tissue damage and disease (225).
4. *C4bp (C4b-Binding protein):* C4bp binds to C4b and accelerates the decay of the C3 convertase (226, 227). It also acts as a cofactor for the cleavage of C4b by Factor I.
5. *Carboxypeptidase-N:* Carboxypeptidase-N was found to abolish the activity of the anaphylatoxins C3a and C5a (228). It cleaves carboxy-terminal arginines and

lysines from C3a and C5a, resulting in the formation of C3a (desArg) and C5a (desArg), both of which have markedly lower ability to signal through receptor binding (229). Carboxypeptidase-N plays in important role in protecting the body from excessive build-up of potentially deleterious peptides that can act as local autocrine or paracrine hormones (230).

Figure 1.1: Pathways of complement activation - Classical, Alternative and Lectin pathway

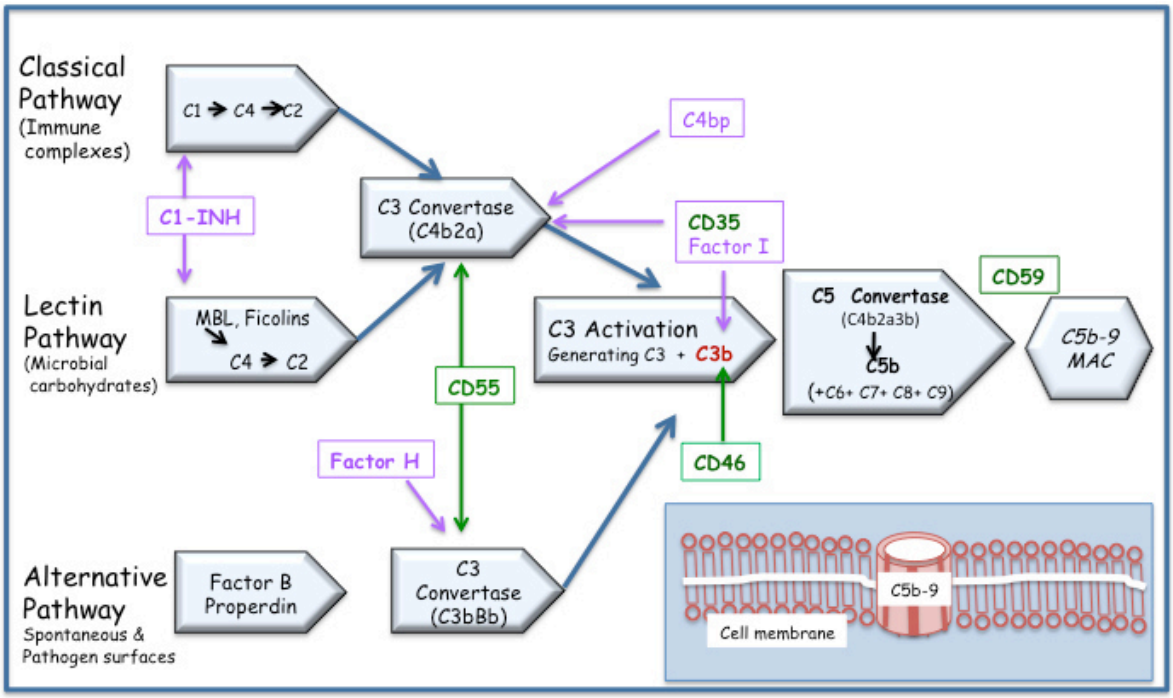


Figure 1.1: Pathways of complement activation - Classical, Alternative and Lectin pathway: *IgM or IgG antigen/antibody complexes binding to C1q, the first protein of the cascade, initiate the classical pathway. The alternative pathway is not so much an activation pathway, as it is a failure to regulate the low level continuous formation of a soluble C3 convertase. The third pathway is known as MBL (Mannose-binding lectin)/MASP (MBL associated Serine Protease) pathway. The initiating molecules for the MBL pathway are multimeric protein-lectin complexes that bind to specific carbohydrate patterns uncommon in the host, leading to activation of the pathway through*

enzymatic activity of MASP. The sites of action of the ***membrane bound complement regulators*** *- CD35, CD46, CD55 & CD59 (green boxes) and the* ***fluid phase regulators****- C1-INH, Factor H, Factor I & C4bp (violet boxes) are represented with arrows.*

Insert: *Membrane Attack Complex (MAC). The interaction of C5b with C6, C7, C8 and C9 leads to formation of C5b-9 or Membrane Attack Complex (MAC), a multimolecular structure that inserts into the membrane creating a functional pore leading to cell lysis.*

Figure 1.2: Membrane Bound Complement Regulators

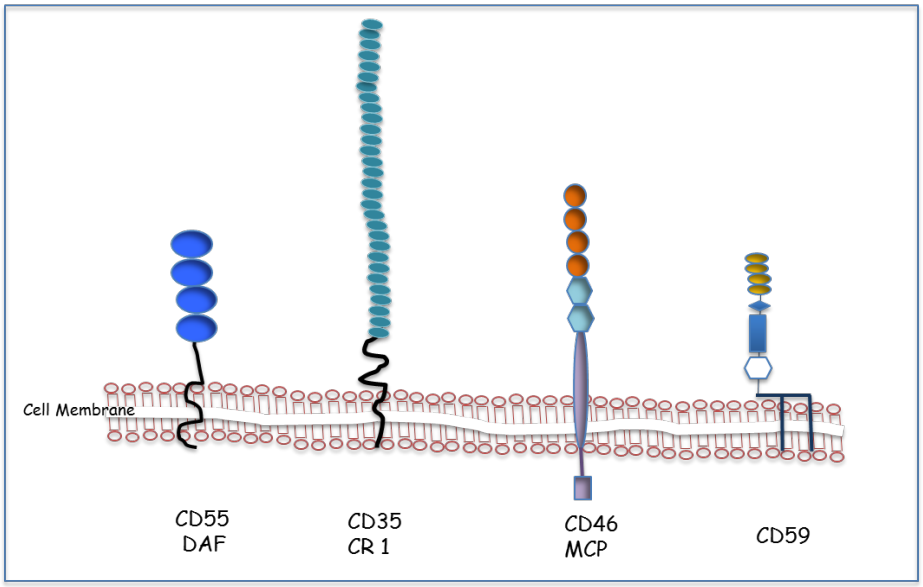


Figure 1.2: Membrane Bound Complement Regulators*: DAF, CR1 and MCP belong to a gene super family called as regulators of complement activation' (RCA) / Complement control proteins (CCP) and share a common structural motif called short consensus repeat (SCR). The SCR structure (circles) consists of around 60 amino acids held together by two disulfide bridges formed by cysteine residues. CD59 is a GPI-anchored membrane complement that is expressed on almost all cells in the body. CD59 is the only well-characterized membrane inhibitor acting at the terminal step, and prevents the assembly of the MAC*

1.3.2.2. Complement and Renal Ischaemia Reperfusion

Complement system plays an active role in mediating IRI. IRI studies have shown that each organ (heart, intestine, skeletal muscle, liver and brain) has its own complement activation pathway (classical/alternative/lectin) and various triggers mediate the IRI damage. The earliest evidence for its involvement was provided by Hill et.al who suggested a non-immunologic role for C3 in the mediation of the acute inflammatory response in nonspecific tissue injury (231). IgM antibody mediated complement activation has been shown in intestine (232), myocardium (233) and skeletal muscle (234) studies, but its role in renal IRI is equivocal (148).

Complement activation in ischaemic kidney

Renal endothelial and epithelial cells are considered to be the targets for complement activation in IRI, with proximal tubular epithelial cells (PTEC) located at the corticomedullary zone being an important target (19, 235). The complement system is activated on ischaemic PTEC and induces the cells to produce pro-inflammatory chemokines. Locally produced complement is critical as large complement molecules cannot pass through the basement membrane, and circulating complement leads to vascular injury. Endothelial cells, mesangial cells, epithelial cells, tubular epithelial cells can all produce complement including C3, C4 and Factor B. Renal glomerulus possesses four membrane bound complement regulators – CD35, CD55 (DAF), CD46 and CD59, while PTEC has only CD 59 (236), which all play a role in regulation.

Kidney transplantation into complement-inhibited rats reduced inflammation of the graft, and authors felt that locally synthesised complement proteins by the renal tubule had a

more important effect than circulating components (237). Local epithelial production of C3 can be stimulated by inflammation and ischaemia, and this has been shown to promote local complement activation leading to acute kidney injury, raising the possibility that local production may contribute to the IR induced injury (238).

Complement Pathways in Ischaemia-Reperfusion Injury

The evidence for complement activation in renal IRI has shown involvement of all three activation pathways, either in combination or as 'single pathway' mediation. Complement activation is an early event in the process of reperfusion injury. Various pathways have been implicated in the renal IRI, with alternative pathway playing a predominant role compared to the other pathways. This was proved by Zhou et.al who showed that C3-, C5-, and C6-deficient mice were protected from renal IR injury, whereas C4-deficient mice were not protected (19). This was further supported by Thurman et.al who showed that Factor B deficient mice showed less functional and morphologic renal injury with minimal tubulointerstitial complement C3 deposition compared to their WT control after renal IRI (239). The same author substantiated his findings by using an inhibitory mAb to mouse Factor B effectively that prevented complement activation in kidney, and protected the mice from necrotic and apoptotic injury of the tubules (240). It is well known that Factor B plays a critical role in the alternative pathway, and deposits of factor B were seen at interstitial and tubular level following IRI (241).

The role of Classical pathway mediated IRI is very limited. In a mice model of renal IRI, Park et.al showed that recombination-activating gene (RAG)-1-deficient mice were not protected from IRI, and immunoglobulins were not found in kidneys 30-hours post

reperfusion indicating that antibody mediated classical pathway was not involved in the pathogenesis (148). An experimental model evaluating complement activation by inducing hypoxia-reoxygenation in human and mouse PTEC showed that hypoxic human PTEC induced the classical pathway of complement (dependent on both IgM antibodies and C1q) while hypoxia-induced mouse PTEC activated the alternative pathway (242). This study highlights the important species-specific differences in complement activation by PTEC. In a swine IRI model, kidney biopsies examined at 15-minutes after reperfusion showed increased deposits of MBL and Factor B compared to C1q, implicating a predominant role for lectin and alternative pathways compared to classical pathway in the early reperfusion phase (241). However, in the same study when they analysed DGF transplant biopsies (7-15 days), deposition of both C1q/C4d (classical pathway) and MBL/C4d (lectin pathway) were seen in the peritubular capillaries, interstitium and on the glomerular endothelium, implicating both the classical and lectin pathways. Using classical and lectin pathway inhibitor C1-INH pre ischaemia, they also showed reduction in C4d and C5b-9 deposition, along with reduction in infiltration of inflammatory cells.

Lectin complement pathway's (LCP) role was initially shown in cardiac IRI models that showed LCP mediated complement activation after tissue oxidative stress (243). Role of LCP in renal IRI was shown in a study using transgenic MBL deficient mice. Following renal IR, MBL deficient mice showed significant protection from IRI along with decrease in nephric C3 deposition, and decreased C3a (desArg) levels indicating less complement activation (244). In an earlier study, mice undergoing warm renal IR showed MBL (MBL-A and MBL-C) deposits in tubular/interstitial cells and peritubular capillaries (245). This was more pronounced at 24-hours post-reperfusion and co-localised with C6 deposits. In a

study comparing allograft biopsies, it was shown that C4d-positive biopsies demonstrated diffuse H-ficolin and IgM deposition in the PTEC, compared to C4d-negative group, thus implying a role for lectin pathway activation by H-ficolin (246). MBL was shown to be critically involved in the early pathophysiology of renal IRI, and therapeutic inhibition of MBL was protective against kidney dysfunction, tubular damage, neutrophil and macrophage accumulation (247).

In summary, renal IRI complement mediated damage is primarily via activation of the alternative and partly via lectin and classical pathways. Local synthesis of complement production also contributes to the pathogenesis, in addition to the systemic attack.

1.3.3. Endothelium

The endothelium forms the interface between the vessel wall and the circulating blood/lymph. Under optimal conditions, the endothelium dictates the vascular tone and perfusion, regulates the influx of inflammatory cells into tissue, and prevents coagulation (248).

1.3.3.1. Endothelium in Renal IRI

Following IR, regulatory function of endothelium is altered leading to a detrimental impact upon the renal function. In IR conditions, the endothelium changes from an anti-adhesive to a pro-adhesive state, usually due to the action of inflammatory cytokines (249). Following ischaemia, the small arterioles are prone to vasoconstriction due to the increase in vasoconstrictive mediators (thromboxane A2, angiotensin 2, prostaglandins etc.), coupled with decrease in vasodilatory mediators (bradykinin, acetylcholine, nitric oxide) (16).

Vasoactive cytokines (TNF-alpha, IL-beta, IL-6, IL-12, IL-15, IL-18, IL-32) and endothelin generated due to endothelium-leukocyte interaction further augment the vasoconstrictive effect (250). The ischaemic insult damages the endothelial barrier leading to partial disappearance of the cell-cell borders and interruption of the cell-cell contacts, leading to endothelial disintegration and increased vascular permeability (11). Damaged endothelial cells release cytokines and chemokines that facilitate endothelial interaction with leukocytes and platelets (12). Reperfusion leads to the 'no-reflow' phenomenon, which occurs due to endothelial cell swelling, interstitial oedema and capillary vasoconstriction that further potentiates tissue damage (17).

Endothelial damage also leads to glycocalyx loss, disruption of the cytoskeleton, alteration in endothelial cell-cell contacts leading to breakdown of perivascular matrix and increased vascular permeability (16, 18). The reduction of renal blood flow to outer medulla leads to occlusion of vulnerable capillaries and damage to epithelial cells, especially in the pars recta (16). The ischaemic insult also leads to a inhibition of the VEGF pathway during the early injury and repair phase of renal ischaemia, leading to tubular injury and tubulointerstitial fibrosis (251, 252). Vascular endothelial damage coupled with vasoconstrictive response leads to reduction in renal blood flow and glomerular filtration, and eventual AKI (253, 254) .

1.3.3.2. Markers of endothelial damage

Endothelial cell injury can be evaluated by a variety of local and serum markers: thrombomodulin, P-selectin, E-selectin, intracellular adhesion molecule, vascular cell adhesion molecule and others, all of which have been used in animal and clinical studies

(255). The selectins and other adhesion molecules have been covered in the previous section.

- **Thrombomodulin (**TM) is a transmembrane glycoprotein expressed on the surface of all vascular-endothelial cells with multidomain structure which is able to interact with thrombin, Protein-C and complement components, thus contributing to localized haemostatic and inflammatory response following injury (256). In the presence of cytokines and activated neutrophils/ macrophages, endothelial TM is enzymatically cleaved releasing soluble fragments which circulate in the blood, and plasma TM level is regarded as a molecular marker reflecting injury of endothelial cells (257). Soluble thrombomodulin administered pre-ischemia or 2-hours after reperfusion in a rat renal IRI model markedly reduced IR related renal dysfunction and tubular injury scores (258, 259).
- **Tissue Factor (TF) is** a transmembrane glycoprotein present in the subendothelial cells and forms high affinity complex with coagulation factors VII/activated factor VIIa (FVII/VIIa), playing an essential role in essential role in the clotting system by activating the extrinsic coagulation pathway following vascular injury (260, 261). In renal IRI model, TF was stained on the GBM and endothelial cells, and was credited to play an important role in IRI as the microcirculatory incompetence due to TF mediated microthrombus causing necrosis was prevented by using TFPI (Tissue factor pathway inhibitor)(262). Similar protective effects on renal IRI have also been reported by using TF antisense nucleotides, thus confirming its presence as a marker for renal ischaemic injury (263).

- Shed components of **glycocalyx** (covers the luminal side and prevents leukocyte and thrombocyte adhesion) are probably the earliest sign of endothelial damage (18). Endothelial glycocalyx is a complex network of soluble components consisting of proteoglycans and glycoproteins (264). Many endothelial cell adhesion molecules (Selectins and ICAMS) are the major glycoproteins of the glycocalyx, and prevent erythrocytes and leukocytes from interacting with the vessel wall (265). Modifications of the glycocalyx appeared to be an early step in the inflammatory cascade of reperfusion injury, and inhibition of the glycocalyx modification using a adenosine A (2A) receptor agonist showed protection from IR injury (258).
- Sutton et.al showed that following renal IRI, **von Willebrand factor (vWF)** was elevated along with microscopic evidence of disruption of the actin cytoskeleton of renal microvascular endothelium (266). Similar findings of disarray of the actin cytoskeleton in vascular smooth muscle cells and disappearance of vWF from vascular endothelial cells were found in cadaveric renal biopsies post reperfusion (252).
- P-Selectin and E-Selectin have also been used as markers of endothelial activation, and have been discussed in the previous section on adhesion molecules.

1.4. Therapeutic approaches to minimise IRI

As evident by the earlier chapters, there are many players in an ischaemia-reperfusion injury (IRI) in kidneys. Clinically, there are only a few avenues where interventions can be feasible. The following section provides a succinct overview of various strategies' adopted to minimise IRI with special emphasis on complement and leukocyte modulation, which is the focus of my research project.

1.4.1. Clinical Strategies

In clinical studies, there are only a few studies looking at preventing ischaemic AKI prophylactically. One group are vascular patients undergoing aortic surgery, where a few patients need to have their renal vessels clamped leading to IRI. Though many patients recover, there is fraction of patients who required renal support post operatively (267). The other group where IRI could be targeted are renal transplantation group where the interventions could be broadly classified into optimisations aimed at the donor, the recipient and organ preservation and storage.

1.4.1.1. Donor optimisation

Following brain death, there are hormonal changes within the body leading to hemodynamic alterations including hypotension and organ hypoperfusion, which is modulated by circulating catecholamines levels and cytokine responses, and extensive complement activation (268). Optimising these physiological derangements prior to planned donation has been the focus in the intensive units across the globe. For example, in a retrospective matched-paired study, donor resuscitation with a rapidly degradable HES

preparation (with a molecular weight of 130 kDa, HES 130/0.4) had a significantly reduced DGF and lower creatinine levels at 1 year compared to resuscitation with a HES 200/0.6 (269). Optimising the respiratory, cardiovascular, coagulatory and the systemic effects of brain death remain the basic principles of managing a DBD (Donation after brain death) donor.

Unlike DBD kidneys, DCD (Donation after Circulatory Death) kidneys undergo warm ischaemia (systolic blood pressure less than 50 mm Hg and finishing with cold arterial flush) that coupled with cold ischaemia increases the risk of DGF and primary non-function. Though the length of time from the onset of warm ischaemia (WIT) to retrieval does appear crucial, however recent evidence suggests that the hemodynamic profile of the patient after withdrawal is more critical than the time from withdrawal to retrieval (270). The focus of optimization in DCD donors currently revolves around reducing the warm ischaemic insults that the kidney suffers. Regional perfusion (RP) of intra-abdominal organs can be achieved by placing a double-lumen catheter in the aorta, and perfusing the kidneys with cold or normothermic circulation. ECMO (extracorporeal membrane oxygenation) has been advocated for reduction of WIT and IRI. A systematic review showed that RP-DCD kidneys at 1 year had better patient and graft survival rates than standard DCDs, and were comparable to DBD outcomes. The author's concluded that RP/ECMO could lead to significant reductions in DGF however ethical and legal issues prevented from having a worldwide consensus (271).

In a study comparing three different techniques [in-situ perfusion (ISP), total body cooling (TBC) and normothermic recirculation (NR)] to reduce DGF and PNF (primary non function) in DCD kidneys, the incidence of DGF and PNF was significantly lower in

kidneys perfused with NR than those with ISP or TBC, and this has been observed in other clinical studies (272, 273). However, further critical clinical studies are required to confirm the short and long term effect on grafts with NR. With extended criteria being used for more donor recruitment, further clinical studies will be aimed at optimizing the DCD kidneys prior to retrieval.

1.4.1.2. Ischaemic preconditioning

The concept of ischaemic preconditioning originated in 1986 when Murray et.al demonstrated reduction in the size of the myocardial infarct by repeated short spells (5 minutes) of ischaemia-reperfusion prior to 40-minutes of ischaemia, and hypothesized the role of triggers, mediators and effectors in reducing the injury via pre-conditioning mechanism (274). The various mechanisms involved the preconditioning mechanism has been reviewed by Selzner et.al and can be referred for understanding the mechanisms (275). Animal based experiments have shown that preconditioned tissues exhibited better electrolyte homeostasis and genetic reorganization with decreased levels of oxygen-free radicals and activated neutrophils (276). Furthermore, rodent models showed reduced apoptosis and better microcirculatory perfusion compared to the controls.

Preconditioning can be carried out either directly at the renal pedicle or remotely. Remote ischaemic preconditioning (RIPC) can be performed by accessing peripheral arteries, and noninvasively by using BP cuffs. Though the animal models have shown reduction in injury with RIPC, no obvious benefits have been shown in clinical transplantation. RIPC have shown some benefits in cardiac and aortic aneurysm surgery, but further clinical studies are needed to prove its efficacy.

1.4.1.3. Organ preservation

Following retrieval of organs, the donor cells and tissue are exposed to hypoxia and ischaemia until the time they are reperfused in the recipient. Hypothermic protection in the form of static cold storage or hypothermic machine perfusion (HMP) and normothermic machine perfusion (NMP) can to a certain extent combat the deleterious effects of ischaemia, but this has to be balanced between the benefits and the detrimental effects of cooling.

Static cold storage:

Static cold storage is the most widely used form of organ preservation around the world, and the machine perfusion kits have challenged its use in developed countries. The characteristics of various types of solutions (Intercellular/ Intermediate/ Extracellular) and there uses have been reviewed Bon et.al (277), and can be referred for further reading. Randomized trials of histidine-tryptophan-ketoglutarate (HTK), Celsior, and University of Wisconsin solutions showed equivalent risk of delayed graft function after kidney transplantation, but opined that more clinical trials were needed to justify a use of one solution over the other (278). Similarly, a retrospective study comparing 610 cadaveric renal transplants showed no difference in outcomes between UW and Celsior solutions (279). A systematic review and meta-analysis comparing preservation solutions for static cold storage of kidney allografts showed no difference between existing solutions (280). Intracellular and intermediate solutions are the current preferred choice for storage, however experimental data in animals have shown polythene glycols in storage solution have aided organ function recovery and reduced inflammation and fibrosis development in

the models (281). Additives into preservation solutions that aim at reducing inflammatory mechanisms and minimize the cold ischaemia effects offer an attractive option, and currently being evaluated in experimental and some human models. The various pharmacological and genetic interventions that are being currently trialed can be found in an article by Chatauret et.al. (282).

Machine perfusion (MP):

Machine perfusion kits have facilitated change from an emergency setting for transplants to an elective with reduced costs and improved outcome. It also provides access for administration of cytoprotective agents and/or immunomodulatory drugs (146). It also aids in assessments of quality of organ by analyzing perfusates and resistance pressures (283, 284). Machine perfusion can be either with hypothermic or normotheric solutions, or blood. Hypothermic Machine Perfusion (HMP) maintains patency of the vascular bed and the cooling effects reduces the metabolic rate leading to decreased oxygen demands (285). A recent meta-analysis compared HMP versus static cold storage of kidney allografts on transplant outcomes. Seven RCTs (1475 kidneys) and 11 non-RCTs (728 kidneys) were included in the analysis. The study showed that HMP group had lower incidence of DGF, but no difference was observed in the PNF, acute rejection or patient survival (286). Similar findings of HMP affecting only DGF were observed in other systematic reviews (287, 288).

Normothermic Machine perfusion (NMP) carries the benefit of avoiding cold ischaemic injury and is based on the principle that the organ can be maintained in a stable state close to its physiological state (289). There have been clinical reports of improved outcomes following normothermic perfusion in both kidney and liver transplants, and there is

ongoing research in this field. The ability to add interventional agents to the perfusion offers an attractive avenue for future research, but its review is beyond the perspective of my thesis. MP use is still hindered due to economical constraints compared to static storage, but the ability to predict graft quality and ability for intervention may change its outlook. Further clinical trials are needed to evaluate its effect in the current context of ECD donors.

1.4.1.4. Recipient Optimisation

Recipients offer another therapeutic option to minimize the IRI, however any interventions are still in very experimental stage. Neonephrogenesis (capacity of renal tissue to regenerate) involves giving recipients agents that promote cell repair and regeneration in the IRI kidney. Stem cells have shown potential in other organs, but its role in renal neonephrogenesis is still in very early experimental stage. In a recent review of role of mesenchymal stem cells (MSC) in renal IRI, majority of the studies in the showed substantial benefits in cell recovery/repair, but they were mostly in small animal models and called for larger animal/pre-clinical studies to evaluate the efficacy of MSC (290). Further research will be aimed to assessing the safety profile, mechanisms of action, reproducibility, drug delivery and short and long-term effects.

1.4.2. Leukocyte modulation

Leukocytes play an important role in the IR process, and suppressing the activity of each leukocyte cell has shown protective effects as discussed in the previous section (1.3.1.4). Anti-thymocyte globulin is one of the intervention agents being used in my study, and hence I have reviewed its effect on IRI in depth.

1.4.2.1. Leukocyte depletion and IRI

Leukocyte depletion can be achieved either by using filters or by using drugs/molecules that cause leukopenia or inhibit leukocyte function. Activated protein-C (APC) is a major physiological anticoagulant with anti-inflammatory and anti-apoptotic activities in endothelial cells, has been shown to reduce leukocyte rolling and leukocyte adhesion in systemic endotoxemia (291). APC conferred protection in spinal cord IRI (292) and hepatic IRI models (293). Vinblastine induced leucopoenia in a liver IRI model showed significantly better recovery of blood flow and haemoglobin saturation than the control group after 45-minutes of ischaemia (294). APC was shown to inhibit the activation of leukocytes and confer protection against renal IRI, and its effects were similar to leukopenic (using nitrogen mustard) rats (295). Experimental models using leukocytes depleted blood for perfusing the kidneys in a swine model showed improved renal blood flow and creatinine clearance (104, 105). Though the decrease in neutrophil infiltration was a consistent finding, this methodology was unable to define which specific leukocyte subset was the main contributory factor.

1.4.2.2. Anti-Thymocyte Globulin (ATG; Thymoglobulin)

ATG is a lymphocyte depleting polyclonal antibody that is raised in rabbits/equines against human thymocytes. It binds primarily to peripheral blood lymphocytes, and displays differential specificity to antigens present on a variety of cells (including leukocytes) involved in immune responses, apoptosis, signal transduction, cell adhesion and trafficking (249, 296, 297). ATG shows differential binding to these cell populations, hence its varied effects. The immunosuppressive potential of polyclonal globulins was first demonstrated by

Woodruff et.al in the early 1950s (298, 299), and since then, has been extensively used in transplantation (induction, and in treatment of acute rejection), treatment of aplastic anaemia and in hematopoietic stem cell transplantation, and in other autoimmune diseases (300, 301). ATG has various modes of action due to effects on various cells, and these are not completely understood yet.

1.4.2.2.1. Mechanism of action

Leukocytes

In a limb IRI model, ATG treated primates completely inhibited leukocyte rolling and vascular adherence compared to the controls (302). Similar findings of reduced microvascular leukocyte adhesion, decreased leukocyte count and better blood flow velocity between ATG-treated and ATG-free groups was shown by the same German research group in a later study (103). ATG possesses antibodies to intercellular adhesion molecules (Leukocyte function associated antigen-1 etc.) and also against chemokine receptors (CCR5, CCR7 and CXCR4), thus inhibiting the leukocyte response to chemo-attractants (303).

The predominant effect of ATG is through **T-cell** depletion. ATG treatment led to dose-dependent lymphocytopenia in the blood along with T-cell depletion in spleen and lymph nodes, and reduction in B-cell and NK cell counts (304). Peripheral depletion of T-cells and other leukocytes has been attributed to complement dependent-lysis and ADCC (apoptosis and antibody-dependent cellular) induced cytotoxicity (305). ATG was also shown to reduce the expression of adhesion molecules on neutrophils and lymphocytes which have been shown to be beneficial in IRI (306). IL-4 regulates the differentiation of T-helper

lymphocytes into Th2 phenotype, and is also involved in cellular activation, both of which are features of IRI. In a primate limb IRI model, ATG was shown to inhibit the production, and release of IL-4 by activated lymphocytes compared to the non-treatment group (307). ATGs can modulate immune response by affecting the function of different immune effectors such as B lymphocytes, regulatory T lymphocytes (Treg), natural killer (NK)-T lymphocytes and dendritic cells (DC) (249).

Endothelium and Adhesion molecules

A study looking at the effect of immunosuppressive drugs on endothelial cell activation in a mice skin IRI model, ATG was shown to significantly increase the plasma concentration of VCAM-1 and P-Selectin (308). An in-vitro study looking at the effects of ATG on TNF-alpha stimulated human umbilical endothelial cells showed a trend towards reduced percentage of gated cells in the ATG treated group (before TNF-alpha stimulation) for CD54 and CD62E, though it was not significant (309). In a study evaluating the effect of ATG on primate limb IRI, ATG caused reduction in the expression of adhesion (ICAM-1, VCAM, PECAM, CD11b, CD62E) and inflammatory (IL-1, IL-6, TNF-α) molecules, both in endothelium and reperfused tissue (310). This was confirmed in a recent study where ATG caused pronounced activation of both circulating platelets and stationery endothelial cells (308). However, no studies have specifically looked into effect of ATG on the endothelium, or its markers.

1.4.2.2.2. ATG and Renal IRI

Animal models

In a study evaluating the effects of mouse anti-thymocyte globulin (mATG) on warm kidney IRI in mice, three different renal IR models (30 min bilateral, 60 min bilateral and 45 min unilateral) with differing times of ATG administration (pre-treatment [3 days prior to IR] vs. post treatment [after reperfusion]) were used. Though there was consistent depletion of T-cells in blood, ATG did not confer functional or structural protection from the IR injury (311). ATG has also been used in transplant models, which differs from the IRI model. ATG given pre- (2 hours) or peri- (end of surgery, prior to reperfusion) transplant, showed that pre-transplant group offered IR protection through reduced graft infiltrating inflammatory cells (leukocytes, macrophages, T-cells, LFA-1+ cells), reduced tubular injury and better graft function compared to the isotype control (312). ATG treated Wistar donor kidneys were transplanted into bilaterally nephrectomised recipient rats, and the transplanted kidney was analysed 24 hours later. This rATG offered protection from renal IRI with functional and structural protection (313). Similar to previous study, there was no significant effect of ATG on ICAM-1 in the QPCR array.

Effect of ATG on IRI in clinical transplant studies

ATG has been routinely used in transplantation for induction, and treatment of rejection episodes. DGF (renal) is a clinical representation of IRI, and studies evaluating ATG with regards to DGF prevention have been only reviewed. A randomised study looking into the effects of intra-operative vs. post-operative ATG administration, intra-operative ATG administration was shown to significantly reduce the incidence of DGF (14.8% vs. 35.5%),

along with demonstration of better early graft function (314). A retrospective analysis of 40 DGF patients comparing a Cyclosporine-based (n = 17) regimen since the day of transplant vs. ATG and delayed CsA administration, the latter group had accelerated renal function recovery, decreased rejection rates and significantly reduced treatment costs compared to the cyclosporine only regimen group, with the effects partly attributed to ATG (315). Live related renal transplantation is associated with relatively lower incidence of DGF. A single centre experience of using ATG induction for 214 live-donor patients showed a five-year patient survival of 96% and graft survival of 82%. Interestingly, none of the patients experienced DGF compared to the national average of 5%, and only 5% developed acute rejection (316). In a clinical trial evaluating various immunosuppressive protocols on neutrophil sequestration, intra-operative ATG resulted in marked leucopoenia of all white blood cell lineages, with considerable systemic neutrophil and monocyte activation (increase in plasma lactoferrin concentration and neutrophil and monocyte CD11b expression) (317).

From the animal and clinical studies, it is evident that ATG can have a favourable response in attenuating the IR injury. However, the effects of ATG in renal IRI studies have not been consistent, with some studies showing no protection. Moreover, the effects on adhesion molecules and leukocytes have been summarised from different species (primate, murine, rat) and overall effects in one model has not been evaluated. Our study evaluates the effects of ATG on leukocytes and endothelium in a renal IRI model.

1.4.3. Complement system modulation

In this research project, two interventional agents are being used that can modulate complement system and these are reviewed below.

1.4.3.1. Soluble CR-1 (sCR1)

CD35/Complement Receptor-1 is a transmembrane glycoprotein that facilitates the decay of C3/C5 convertase in both the classical and alternative pathways, and acts as a co-factor for Factor I in the degradation of C3b and C4b (214). Expression of CD35 on erythrocytes is believed to be crucial in handling circulating immune complexes, and abating the development of autoimmunity. Wiesman et.al developed soluble CR1 as a complement inhibitor by preparing a truncated, soluble form of membrane-associated CR1 that lacked the transmembrane and cytoplasmic domains, but retained the C3b- and C4b-binding functions and Factor I cofactor activities (257). In the same study, sCR1was shown to inhibit the activation of classical and alternative pathways in vitro and suppressed complement activation in vivo, and reduced inflammatory tissue damage in a model of myocardial infarction associated with reperfusion injury.

sCR1 has since been used as a 'complement inhibitor' in various studies. sCR1 attenuated myocardial IRI via reduction in the accumulation of leukocytes within the infarct zone, along with the reduction of the generation of C5a, and suppression of C5b-9 attack complex (318). sCR1 inhibited inflammation and demyelination in antibody-mediated demyelinating experimental allergic encephalomyelitis (319). In hepatic IRI, sCR1 attenuated hepatic IRI with fewer C3 deposits, decreased leukocyte adherence and significantly better liver function tests compared to control groups (320-322). sCR1 has also been shown to

attenuate intestinal IRI via decreasing complement activity and reduced neutrophil infiltration into the injured tissues (323, 324). In swine models of lung transplantation, sCR1was shown to block complement activation in the allograft and reduced post-transplantation reperfusion oedema, improved ventilation but did not improve hemodynamic parameters (325, 326).

There is very limited evidence evaluating the role of sCR1 in renal IRI. In a rat renal transplant model, sCR1-treated recipients had marked reduction in tissue C3 and MAC deposition along with inhibition of plasma complement activity, in addition to reduced leukocytes infiltrate and decreased vascular injury at day 5 after transplant (327). In another report by the same group, sCR1 treated recipients (Lewis to DA rat renal allograft) showed reduction in vascular injury and cellular infiltration, with reduced complement deposition (328). Alternative and lectin pathways predominantly mediate renal IR, and some studies even favour the classical pathway. In theory, sCR1 should attenuate renal IR as the degradation of C3b and C4b is common to all pathways, but paucity of studies can either reflect unreported poor outcomes or lack of interest in exploring this avenue.

1.4.3.2. MASP pathway inhibition

Lectin pathway is activated by the binding of multimolecular lectin complex with a carbohydrate moiety on pathogens or aberrant glycosylation patterns on apoptotic, necrotic or oxygen-deprived cells (329). The three MBL-associated serine proteases MASP-1, MASP-2 and MASP-3, along with MAp19 (competes with MASP for binding with the carbohydrate moiety) are able to bind to the carbohydrate recognition subcomponents (ficolins, CL-11, MBL) and activate the lectin pathway. MASP-2 cleaves C4 and C2 to

form C3 convertase, while MASP-1 may cleave C3 directly bypassing the C4b2a complex, albeit at a very slow rate (189, 190). Another serine protease, MASP-3 was shown to down-regulate the C4 and C2 cleaving activity of MASP-2 (191).

MASP-2 was identified in 1997, where it was shown to have a structure similar to MASP-1 and the two C1q-associated serine proteases, C1r and C1s (330). Using a transient expression system, a recombinant form of MASP-2 that bound strongly to MBL was capable of activating complement, while an equivalent recombinant form of MASP-1 had no enzymatic activity when tested alone or in combination with rMASP-2 (188). Recombinant MASP-2 was shown to have a C1s-like esterolytic activity, and specifically cleaved complement proteins C2 and C4, while recombinant MASP-1 had a lower and more restricted esterolytic activity, suggesting that MASP-2 in association with MBL was able to activate the lectin pathway (331). sMAP (small MBL-associated protein (sMAP), truncated form of MASP-2 associated with MBL/ficolin-MASP complexes) deficient mice showed decreased expression of MASP-2, and injection of recombinant sMAP and MASP-2 reconstituted the MBL-MASP-sMAP complex in deficient serum, suggesting that MASP-2 was essential for the activation of C4 and sMAP played a regulatory role in the activation of the lectin pathway (332).

MASP-1 and MASP-3-deficient mouse showed significantly lower activity of both C4 and C3 deposition on mannan-agarose, and a significant delay for activation of MASP-2, and reconstitution of recombinant MASP-1 in MASP-1/3-deficient serum was able to promote the activation of MASP-2 (333). MASP-1 (and probably MASP-3) was shown to be required for conversion of the alternative pathway activation enzyme Factor D from its zymogen form into its enzymatically active form in a MASP-1- and MASP-3-deficient

mouse model, implying a role for MASP-1 in both the lectin and alternative complement pathways (334, 335). A recent study employing MASP-2 deficient mice showed protection from myocardial and intestinal IR injuries, and this was further supported by the use of an inhibitory monoclonal antibody (selectively binds to mouse and rat MASP-2) pre-ischemia that reduced intestinal tissue I/R damage more than twofold compared with mice treated with the isotype control antibody (329). Furthermore, this study also showed that lectin pathway could activate complement C3 in the absence of C4 via a unique MASP-2–Dependent C4-Bypass, and lectin pathway-mediated activation of C3 in the absence of C4 was demonstrated in vitro and shown to require MASP-2, C2, and MASP-1/3.

The role of MASP-2 in renal IRI has not been explored. In this thesis, we evaluated the role of MASP-2 in renal ischaemia and reperfusion by using the same antibody as described in the previous study (329).

1.4.4. Modulation of endothelium and IRI

Evidence that vascular protection benefits IRI was provided by Brodsky et.al who showed in a rat renal IRI model that transplanted endothelial cells (or surrogate cells expressing endothelial nitric oxide synthase) offered functional protection from IR injury (336). Experimental studies using molecules such as sphingosine 1-phospate (that maintains endothelial cell integrity and inhibits lymphocyte extravasations) or prostacyclin analogue iloprost have conferred protection against ischaemic acute renal failure (12, 337). Coating of renal endothelial cells with vaccinia virus complement control protein (VCP) via fusogenic lipid vesicle (FLVs) delivery system prior to renal IR in Fischer rats showed significantly better functional and structural protection with reduced C3 production

compared to controls (338). Interleukin 18-binding protein (IL-18BP), a natural inhibitor of IL-18, was shown to attenuate renal IRI by reducing apoptosis in the peritubular capillary endothelium, along with down regulation of inflammatory cytokines and decreased macrophage infiltration (339). Recent studies have also shown that bone marrow-derived endothelial progenitor cells and endothelial cells may contribute to endothelial repair in the kidney immediately after ischaemia-reperfusion (340).

Adhesion molecules P-Selectin, E-Selectin and ICAM-1 have shown to be upregulated on the on the surface of endothelial cells following renal ischaemia. The rearrangements in the actin cytoskeleton following IRI enables rapid delivery of P-Selectin from its storage in Weibel-Palade bodies to the surface of endothelial cells (341, 342). Inhibition of these adhesion molecules via antibodies prior to renal IR have conferred protection, partly through protection of the endothelium by reducing leukocyte rolling and adherence, and by decreased neutrophil infiltration (81, 85, 343, 344). Selectins bind to sialylated and fucosylated receptors, and two enzymes, fucosyltransferase IV (FucT-IV) and VII (FucT-VII), are important in the function of these receptors. Mice deficient in fucosyltransferases were protected from IRI via reduced Selectin expression and reduced neutrophil infiltration (345).

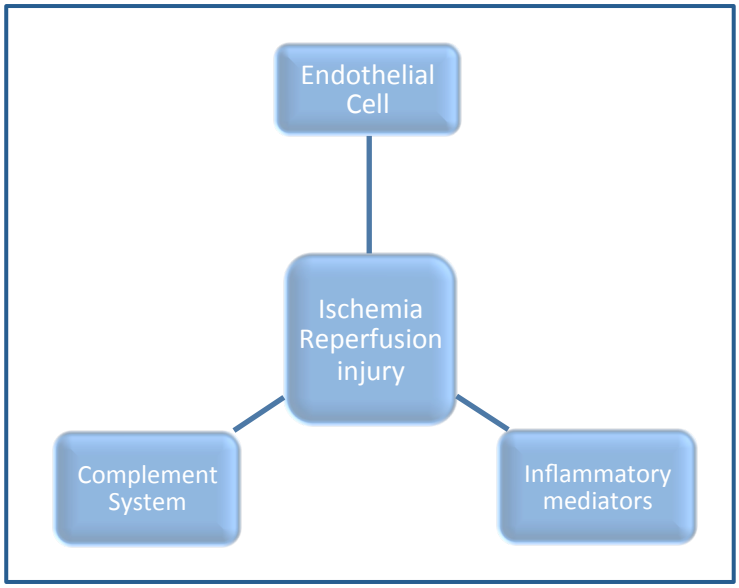
It is evident that endothelium is a highly reactive tissue, and is involved in the early phase of inflammation following IR by serving as an interface between immune/inflammatory cells and renal parenchyma (160). Manipulating endothelial dysfunction offers an attractive proposition for treating ischaemic AKI, but the pure complexities and intricate relations that endothelium shares with a variety of mediators may make it hard for ‘isolated

treatment target'. In this study, we investigated the endothelial changes associated to IR, including Tissue Factor, Thrombomodulin and P-Selectin.

1.5. Project Objectives

1.5.1. Hypothesis

Ischaemia-Reperfusion Injury remains an important clinical problem that influences outcomes following kidney transplantation. My hypothesis is that interplay between three key players determines the outcomes following IRI: leukocytes, renal endothelium and the complement system. In the introduction chapter, I have reviewed relevant previous studies in the scientific literature, to evaluate what is known already about these interacting mediators. Subsequently I have performed experimental work, described in the following chapters, to advance understanding of these processes. To do this, I have developed and used a unilateral IRI model in the rat to test the potential protective effects of manipulations including leukocyte modulation and complement system modulation, and evaluating the effects of these modulatory agents on inflammatory cells, endothelium and complement system.



1.5.2. Summary of aims and objectives

The aims of the project are:

- To determine the nature of ischaemia-reperfusion injury and classifying the leukocyte infiltrates, evaluate endothelial damage and discuss the role of the complement system

- To determine whether ATG can act as a 'leukocyte modulator' in this model, and evaluate whether it attenuates IR injury, as reports are inconclusive. We will also ascertain the effect of ATG on the leukocytes and the endothelium

- Using sCR-1 and Anti-MASP-2 antibodies, we aim to modulate the effects of the complement system on renal ischaemia reperfusion, along with evaluating the effects on leukocytes and the endothelium

CHAPTER II:

METHODOLOGY

Animal models to investigate the mechanisms of ischaemia reperfusion injury are well documented in literature. The methodology section discusses in detail the model and the outcome measures (Histology, immunohistochemistry and quantitative polymerase chain reaction).

2.1. Experimental subjects: Lewis Rats

The animals used in the project were adult male Lewis rats, weighing 180-220 g, 8-12 weeks old. The inbred lewis rats were supplied by Harlan Laboratories (UK). Lewis rats have a number of features that make them ideal for biological work. They are 'commercially viable' for the project and their physiological systems are well worked out from previous studies (346). The lifestyle and requirements are easily met, and protocols for maintaining these animals in JBIOS have been well established. Lewis rats have been used in renal studies, and hence was an ideal study animal for the project (80, 136, 347, 348). The rats were delivered one week before the experiments so as to enable acclimatisation. On arrival, the rats were checked by the experienced JBIOS staff and were housed in cages (maximum 4 per cage). They were kept in the conventional holding room in the JBIOS and had free access to standard rat chow and water. Following the experiments, the rats were housed in individual cages with free access to water (mixed with the analgesia) and food. At the end of 48 hours, the rats were sacrificed. The Lewis rats are generally very friendly and inquisitive in nature. They are easy to handle and are very playful. These characteristics were used to determine their post-operative recovery phase.

2.2. Operative model

This study was carried out at the Joint Biological Services Cardiff Unit (JBIOS, Heath) under Home Office License number 30/2506. The project licence was in the name of Mr R .C Chavez (Supervisor) and I was the Personal Licensee on this project. The care of animals was followed according to the Animals (Scientific Procedures) Act 1986 (ASPA).

2.2.1. Operating theatre and setup

The operative set up was based on the inputs from the Named Animal Care and Welfare Officer (NAWCO) and on guidelines issued by the Home Office, UK and Laboratory Animal Science Association (LASA), the emphasis being on aseptic surgery. Operations were carried out in designated operating rooms (Sterile Experimental Procedures – SEP) in the JBIOS unit. The operating table was placed under the 'laminar flow system' to aid sterility. The operating surgeon used sterile gown and gloves. Chlorhexidine spray was used to clean rat skin after shaving prior to making skin incision. An overhead lamp was used for lighting. The gas chamber for induction and the ventilator machine were on the main table itself enabling minimal transfer time to the operating board. A corkboard covered with a sterile drape acted as an operating table. This board enabled insertion of pins that enabled retraction of all four limbs (post anaesthetisation) using rubber bands, which aided surgery. Standard surgical instruments were used in the procedure, and a microvascular clip was used for clamping the renal vessels. Vicryl sutures were used for closure. Prior to each operation, the instruments were deep cleaned and were immersed in a solution of water mixed with Rely+On Virkon Tablets (Antec Internation, Du PONT, UK)

for 30 minutes. Following this, the instruments were washed off and sprayed with alcohol spray prior to usage. This cycle was repeated before each operation.

2.2.2. Anaesthesia

Anaesthetic mixture of Isoflurane (IVAX Pharmaceuticals, UK) and oxygen was used. The rat was initially placed in the anaesthetic chamber where Isoflurane (initially at 4 litres) with oxygen (2 litres) was administered. In a span of 4-6 minutes, the rat was deeply anesthetised (confirmed by tail pinch and side-side movement – no response). The rat was then moved to the operating table where the face of the rat was placed in a tunnel delivering the anaesthetic mixture. The rate of Isoflurane was altered in keeping with the heart rate and the breathing pattern of the rat (usually between 1.5-3 litres). At the end of procedure, anaesthesia was turned off and the rat was recovered on oxygen until awake.

2.2.3. Analgesia

Temgesic (Buprenorphine) mixed in water was used for analgesia. This was recommended by NAWCO (Named Animal Welfare officer) at JBIOS as it had an established track record with minimal side effects. Temgesic is an opioid analgesic, and has been proven to provide good analgesic cover in rat studies (349, 350). One tablet of temgesic was crushed and mixed in 500 millilitres of water. The analgesia was started 24 hours prior to surgery, and was continued until the day of retrieval. The rats did not demonstrate any visible side effects (sedation, pica effects) of the medication.

2.2.4. Blood collection from rats

The guidelines for blood collection are published by The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3RS), and were adhered to in this study (351). Tail tip under anaesthesia was used to collect blood while the rat was alive, and from the central abdominal veins during terminal anaesthesia. Rats are considered to have around 64 ml of blood per kg of bodyweight. A rat weighing 200 g would therefore have a total blood volume (TBV) of approximately 64 ml/kg x 0.2 kg = 12.8 ml. Other formulas suggested include: BV (ml) = 0.06 X BW + 0.77 (12.77 mls for 200 gm rat) (352). Suggested limit for blood withdrawal from a rat are:

- Suggested limit <10% TBV (= 1.28 ml) on any single occasion AND <25% TBV (= 3.2 ml) in 28 days
- For repeat bleeds at short intervals, suggested limit 1% TBV (= 0.1 ml) in 24 hours

2.2.5. Delivering Drugs

The penile vein was chosen for intravenous administration of drugs, and has been shown to be a reliable rote for injections (353, 354). In our study, the drug was always administered when the rat was under general anaesthetic, and no side effects were encountered.

2.2.6. Organ retrieval and storage

Both the kidneys were retrieved at 48 hours. Each kidney was initially divided into two halves and the kidney examined for IRI. The most consistent macroscopic finding in IR kidneys was:

- Left kidney generally oedematous compared to the right kidney
- Presence of reddish streaks in the outer cortex

Each half of kidney was then divided into 2 halves again, thus ending with four quarters. One quarter was preserved in formalin (10% Neutral-Buffered Formalin), and the rest three quarters were snap frozen. Rat spleen and liver were used as positive controls for immunohistochemistry, and these were also snap frozen.

2.3. Operating techniques

The methodology adopted to carry out the IRI studies was adapted from previous studies, and from the supervisor's experience (266, 294, 355). Once the rat was anaesthetised, the rats were weighed and then transferred to the operating table. The midline hair was shaved using an electric razor. The rat skin was then cleaned with chlorhexidine spray prior to the procedure.

2.3.1. Ischaemia- Reperfusion

10' blade scalpel was used to make the midline skin incision. The rectus sheath and the peritoneum were opened using scissors. The cecum was positioned into the right upper quadrant (usually a midline structure), and the stomach was pushed into the left upper quadrant (from the left lumbar region). This enabled the visualisation of the left kidney. The left kidney was gently elevated and the renal pedicle (both artery and vein) was clamped with a vascular clip for a pre-defined ischaemic time. Ischaemia was confirmed by noticing change in the colour of the kidney. The natural colour of the kidney is pinkish in nature, and this turned to dark brown in colour on application of the vascular clip, thus confirming ischaemia. Laser Doppler flowmetry was used to confirm ischaemia in the first few cases, but was subsequently abandoned given the obvious macroscopic changes in the kidney after clamping.

The laparotomy wound was then covered with saline soaked sterile gauze, with frequent observations at the colour of the kidney. The rat heart rate and breathing pattern were visually observed during the procedure, and the anaesthetic quantity delivered varied accordingly. Following the pre-defined time for ischaemia, the vascular clamp was

subsequently removed in order to establish reperfusion, which was again confirmed with the change in colour of the kidney. Normal saline drops equivalent to the interventional groups injected volume was then sprayed into the peritoneal cavity prior to closure. The laparotomy wound was then sutured in two layers using 3'0 vicryl. The rat was then transferred onto the cage (which was placed on a heating pad), and then transferred to the conventional holding room once mobile in the cage.

The rat was checked at the end of the day, and the next day post operation. Vital parameters like breathing pattern, skin texture (indicates hypothermia/hypotension) and passage urine and faeces (cage inspection) indicated satisfactory post-operative recovery. Wound was reviewed every day, and any incidences of infection or dehiscence recorded. On the day of retrieval (48 hours), under terminal maximal anaesthesia, the midline laparotomy was reopened. Both the kidneys were inspected to ensure adequate perfusion, confirmed by the colour of the kidneys. Any capsular hematoma or patchy perfused kidneys were recorded. The mediastinum was then opened, and the descending aorta/heart was punctured so as to ensure death by exsanguination. Once death was confirmed, both the kidneys were retrieved. The dead rat was then disposed in an orange bag and placed in the carcass freezer. Figure 2.1 represents pictorial representation of the operative technique including the observation of ischaemia following the application of vascular clip.

Figure 2.1: Pictorial representation of the operative technique

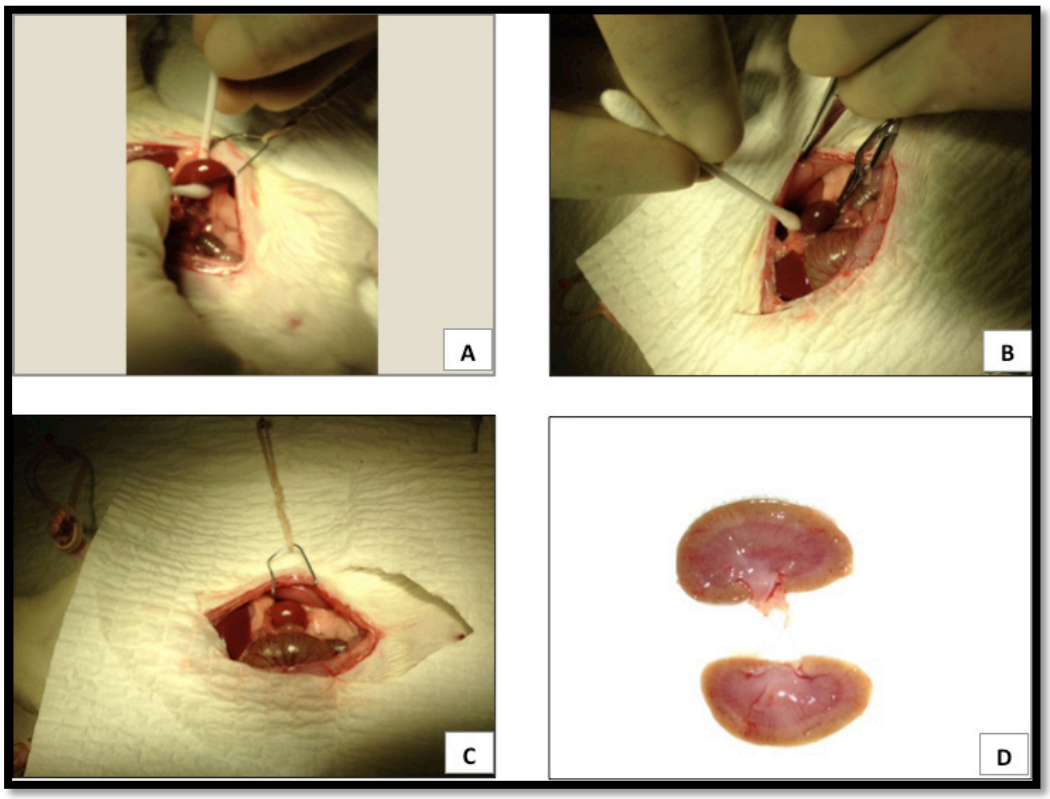


Figure 2.1: Pictorial representation of the operative technique.

Photo A: Shows the midline laparotomy wound with edges retracted by clips. Sterile cotton buds are used to elevate the left kidney into wound just prior to clamping. The kidney is normal pinkish colour indicating good perfusion.

Photo B: Shows the midline laparotomy wound with edges retracted by clips. The vascular clip is now clamped onto the left renal pedicle. The left kidney is now dark brown in colour, indicating ischaemia phase.

Photo C: Shows the reperfusion phase following the removal of the renal pedicle vascular clamp. Note the kidney is now back to its original pinkish colour, but is now oedematous following reperfusion.

Photo D: Two halves of left kidney post retrieval at 48 hours. Note the reddish streaks on the outer renal cortex indicating ischaemia-reperfusion injury, a phenomenon not observed on contra lateral right kidneys (picture not shown).

2.3.2. Sham Operation

All the steps were similar to the IRI group, except no vascular clip was placed on the left kidney. Normal saline drops were also placed in the abdominal cavity similar to the IR group. The laparotomy wound was covered with saline soaked gauze for 40 minutes, and wounds closed at end.

2.4. Intervention groups

The main hypothesis of this research project is to understand whether leukocyte and complement modulation affects renal ischaemia perfusion injury. To achieve this, we have used certain agents that are known to affect the leukocytes and complement system, and these are detailed below. The protocol sheets for all the groups are attached in Appendix 1.

2.4.1. Leukocyte modulation using ATG

As discussed in the previous section, ATG influences the function of a variety of leukocytes. The ATG used in our study was a 'rabbit anti-rat thymocyte immonuglobulin (rATG)' produced analogously to commercial ATG (Thymoglobulin®), kindly provided by Genzyme Corporation (Cambridge, MA, USA). Briefly, rabbits were immunized with a mixture of thymocytes of four strains of rats (Sprague Dawley,F344 Fisher, Lewis and Long Evans). Thymocyte suspensions were prepared using thymi from several donor rats. Fifty New Zealand White rabbits were immunized twice, two weeks apart, and terminally

bled two weeks after the last immunization. Total rabbit IgG obtained from the serum was pooled and purified similarly to Thymoglobulin® Genzyme, Cambridge, MA, USA). Control rabbit Ig was purified from whole normal rabbit serum (312).

ATG protocol:

The ATG dose used in this study was 10mg/kg body weight. Following general anaesthesia, a time zero blood sample (T0) was taken, and the required does of ATG was then injected into the penile vein. Following a period of 20 minutes, laparotomy and left renal pedicle clamping for 40 minutes was done as described earlier in the IR operative protocol. A second blood sample was taken at 48 hours under terminal anaesthesia. The protocol flowchart is attached in the Appendix.

2.4.2. Complement Modulation

Complement modulation was achieved by using two drugs: Soluble Complement Receptor -1 and a novel antibody to MASP-2 pathway.

2.4.2.1. Soluble Complement Receptor 1 (sCR1)

The sCR1 utilized in this study was a kind gift of SmithKline Beecham, Pharmaceuticals, Harlow, Essex, UK and has been used by previous investigators, including my supervisor (320, 327) This agent was soluble human complement receptor type 1, residues 42-1973 of human CR1, glycosylated, expression of plasmid pBsCR1c/pTCSgpt in Chinese hamster ovary cells (DUXB11). The dose used was similar as previous publications on sCR1 (5.3mg/ml): 25-mg/kg body weight. A time zero blood sample from tail tip (T0) was obtained followed by injection of sCR1 at the desired dose in the penile vein. Following 20

minutes, IR operative protocol was performed, and a second blood sample prior to reperfusion (T1) was obtained. Further blood samples were obtained at 24 (T2) and 48 (T3) hours. The protocol flowchart is attached in the Appendix.

2.4.2.2. Anti-MASP-2 antibody and Isotype Control

The anti-MASP-2 antibody was provided by Omeros Inc., Seattle, WA and was used to evaluate its efficacy in intestinal IR model(329). Its preparation is as follows: recombinant antibodies against MASP-2 (AbDH3 and AbD04211) were isolated from Human Combinatorial Antibody Libraries (Serotec; AbD) using recombinant human and rat MASP-2A as antigens. An anti-rat Fab2 fragment that potently inhibited lectin pathway-mediated activation of C4 and C3 in mouse plasma was converted to full-length IgG2a antibody. Polyclonal anti-murine MASP-2A antiserum was raised in rats. In this group similar protocol to sCR1 group was followed with T0, T1, T2 and T3 blood samples, and the protocol flowchart is attached in the Appendix.

2.5. Identification and Coding

Each rat had codes in this experimental set up that were numbered consecutively (01-77). Following anaesthesia, the rat-tail was marked with a permanent marker to identify it during the 48-hour period. Once the rats were in the cage, a tag was attached to each cage showing the rat identification details, operative details and contact number of the research fellow (in case of emergency). This tag also enabled to list the observations like wound check, rat behaviour etc which could be seen by the JBIOS staff as well.

Once the kidneys were retrieved, they were placed in formalin containers or cryotubes. These tubes were marked using a fine-tip permanent marker, either as R01 (R= right kidney) or L01 (L= Left Kidney). These codes were then subsequently used to for marking the H&E and Immunohistochemistry slides, and for QPCR experiments. For spleen and liver samples, the rat number and tissue were written on the cryotubes (64-spleen). Hence, each experimental rat was identified by a code for the experiment and analysis of results.

2.6. Blood and serum analysis

In the next few sections, the methodology adopted for analysing blood and serum samples will be discussed.

2.6.1. ATG efficacy, CD3 count analysis and Flow Cytometry

ATG has been routinely used in clinical transplantation for induction treatment, and treatment of rejection episodes. As discussed before, the predominant effect of ATG is via T-Lymphocyte depletion. Hence, peripheral T-lymphocyte CD3 count has been used as a reliable marker for testing the efficacy of ATG, and titrating the concentration of ATG dosage given to the patient (356-358). ATG has also been shown to reduce the peripheral T-cell CD3 count in rodent models (297, 311). Hence, we used peripheral CD3 count to check efficacy of ATG in lewis rats. Flow cytometry was used to analyse the CD3 count and was carried out by Dr.Brad Spiller according to pre-agreed protocol.

Flow Cytometry protocol for CD3 count analysis:

100 microliters of venous blood was collected into 50 mM EDTA (final concentration) collection tubes. Anti-coagulated blood was added to an equal volume of PBS prior to careful overlaying on 500 microlitres of histopaque (50% 1077 and 50% 1119 density) in a sterile 1.5 ml eppendorf microfuge tube to separate out the mononuclear leukocytes. Following 20-minute centrifugation at 450 x g (no with no brake on deceleration) erythrocytes and polymorphonuclear leukocytes passed through the histopaque and mononuclear leukocytes were collected and washed into flow cytometry buffer by pelleting (3 min at 3,000 xg) and resuspension to a density of 1 million cells/ml. One hundred

microlitres of these mononuclear cells were incubated with either 10ug/ml (final concentration) of FITC-conjugated IgG1 isotype control (Cat no: MG101, Invitrogen,), FITC conjugated mouse monoclonal anti-Rat CD45 (Cat no: 554877clone OX-1, BD Pharmingen) or FITC conjugated mouse monoclonal anti-rat CD3 (Cat No: 557354 clone 1F4, BD Pharmingen, 557354) diluted in flow cytometry buffer (PBS, containing 15 mM EDTA, 1% bovine serum albumin and 30 mM sodium azide).

Cells were incubated for 30 min at 4 C in the dark prior to removing unbound antibody by washing 3 times in flow cytometry buffer. Cells were analysed on a BD Facscalibur and mononuclear cells were gated on by forward and side scatter parameters to isolate from any minor contamination of erythrocytes. 10,000-gated cells were analysed for percent positive CD3 and CD45 (shift above isotype background control) and recorded for each time point for each rat. Figures 2.2, 2.3 and 2.4 represent the methodology used to analyse the CD3 count

Figure 2.2: Measuring B and T cell populations by flow cytometry

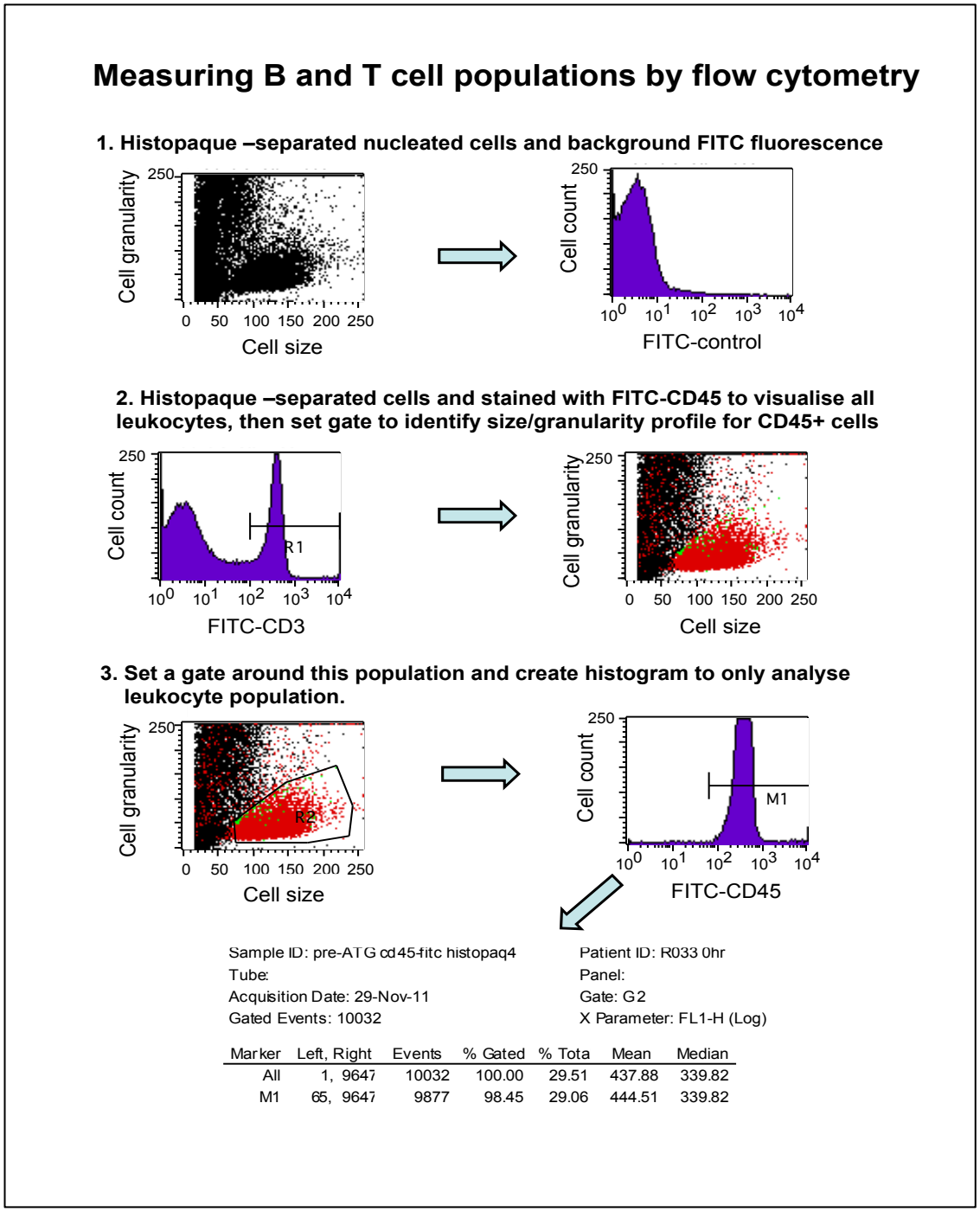


Figure 2.1: Graphic representation of measuring CD45 cells using flow cytometry

Figure 2.3: Examining T-cell depletion following ATG administration

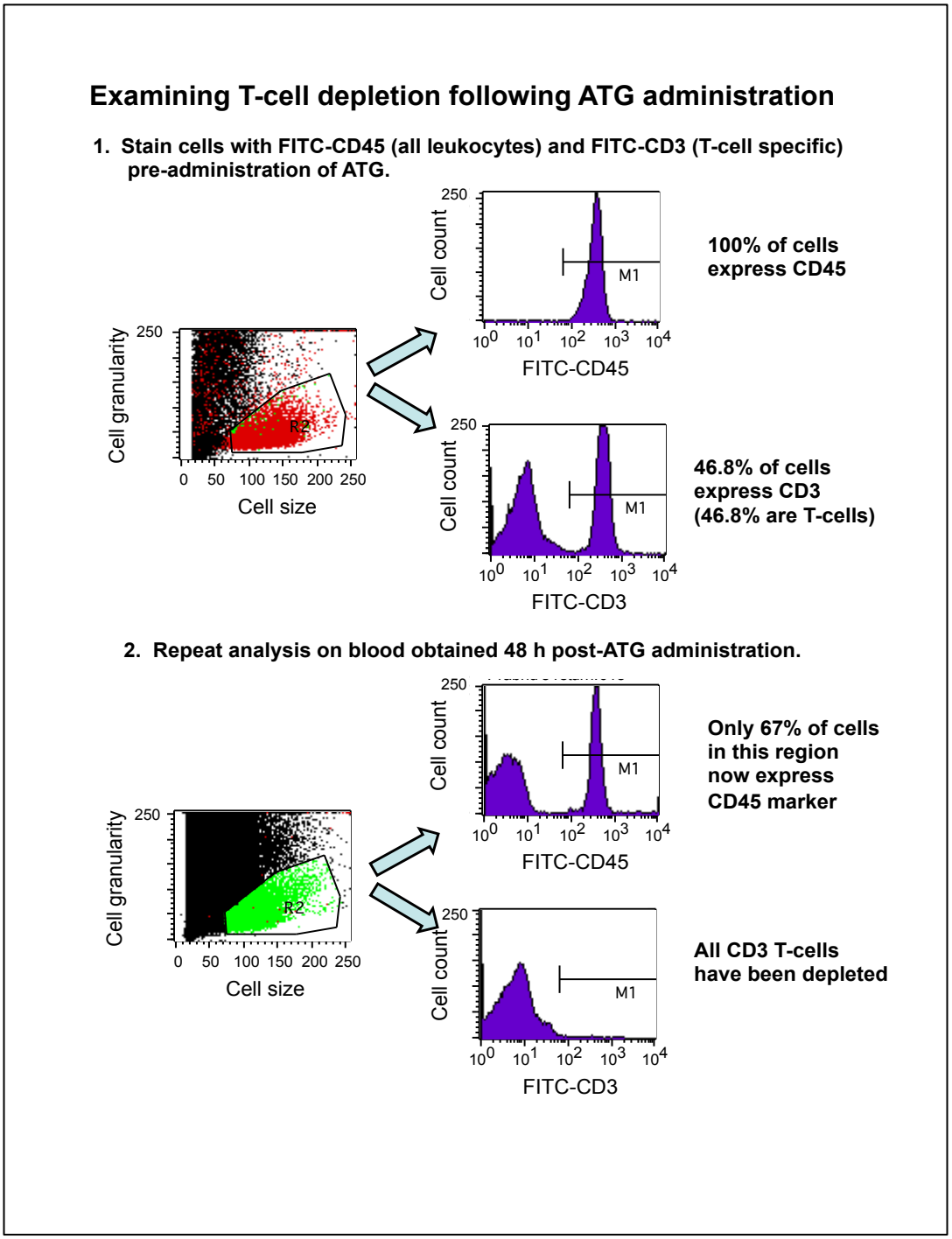


Figure 2.2: Graphic representation of CD45 and CD3 cells pre-ATG treatment, and depletion of CD3 cells evident post ATG administration.

Figure 2.4: Examining T-cell effects following IgG control administration

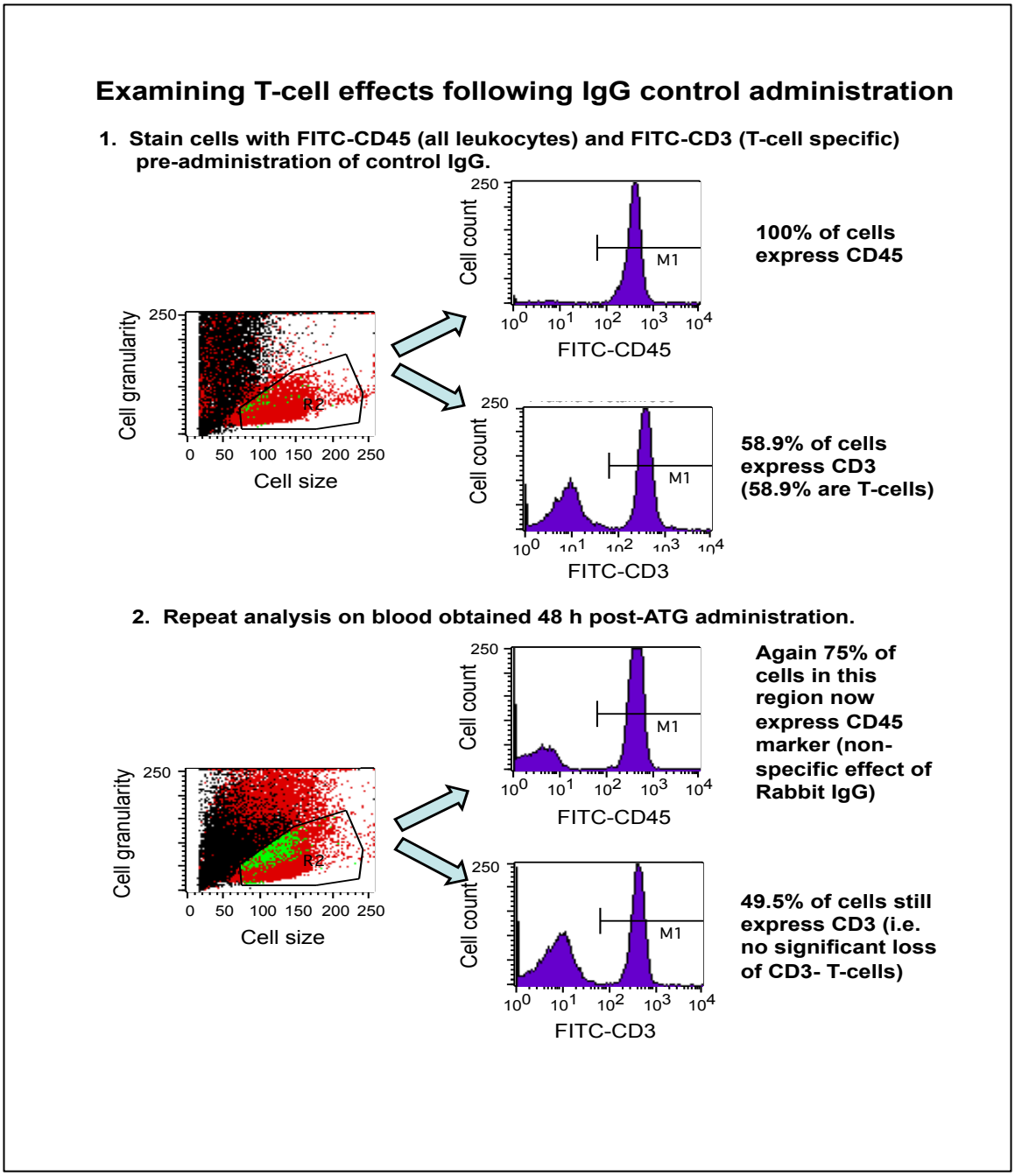


Figure 2.3: Graphic representation of CD45 and CD3 cells pre-ATG Isotype treatment. Compared to figure 2.2, no significant depletion of CD3 cells seen post ATG isotype administration.

2.6.2. sCR1 efficacy and CH50 Complement Assay

Complement haemolytic activity is a functional test of the classical complement pathway, lectin and alternative pathway of complement in plasma or serum. The classical pathway method (CH50) is based on the lysis of sensitised sheep erythrocytes in the presence of Ca++ and Mg++. The assay was carried out in collaboration with Dr. Spiller, who has published on this methodology (359). Rat blood was collected via tail tip into 500ul tubes, and placed on ice. The sample was then spun at 1300 G for 15 minutes at 5 degree centigrade, leading to separation of serum layer. The amber cells were then aspirated into fresh tubes, and the serum sample was then labelled and stored at -70 deg centigrade.

96 well CH50 assay:

Making the sheep EA:

- Sterile transfer 1 ml of sterile sheep RBC (Code: SB068: TCS Biosciences ltd; Lot-49657) into an eppendorf tube. Wash 3 times with 1 ml Phosphate Buffered SALINE (10X solution, BP 399-20, Fischer Scientific limited, Lot – 102355) in a bench top centrifuge(5000 rpm, 3 minutes) .

- Dilute 200 microlitres of packed RBC into 5 mls of PBS and add 5 ml of PBS which has 20 ul of Amboceptor (2 ml, Catalog Number: 9002; Institut Virion\Serion GmbH) added to it.

- Incubate for 15 minutes at 37^0 centigrade. Wash once in 10 mls of PBS (1000 g x 5 minutes), once in VBS (Veronal Buffered Saline, NC9460913; Fischer Scientific limited) resuspend to 20 ml in VBS, to give 1% final suspension.

Assay: in a 96-well round bottomed plate (MICR-TPF, Elkay Lab)

- Add 50 ul of VBS to 2x rows per sample of 96 well plate. Serially dilute 50 ul of serum sample through 12 dilutions for each row. Add 50 ul of sensitized sheep EA and incubate at 37 C.
- Dispense 50 ul of VBS to each of the wells in row A ie. A 1-12
- Check the plate at every 9 minutes, and if changes seen (haemolysis vs. no haemolysis macroscopically), then read in microplate reader (MRX TC Revelation, Dynex Magellan Biosciences) at every 9 minutes for 30 minutes at 415 nm.

2.6.3. Anti-MASP-2 antibody and Lectin Pathway assessment

Omeros Corporation (USA) provided the anti-MASP -2 antibody and its' isotype. They also provided guidance for methods to check the ablation of lectin pathway activity in serum, which was adapted in our lab under the guidance of Dr Brad Spiller. Functional activity of the lectin pathway in rat serum was assessed by detection of C4b deposition on mannan-coated plates. For obtaining serum samples, rat blood was collected via tail tip into 500ul tubes. The sample was then mixed well, and placed on ice. The sample was then spun at 1300 G for 15 minutes at 5 degree centigrade, leading to separation of serum layer. The amber cells were then aspirated into fresh tubes. The serum sample was then labelled and stored at -70 deg centigrade.

Ex-vivo assessment of Lectin pathway:

In a 96-well plate, 50 microlitres of mannan (from Saccharomyces cerevisiae -Cat: M7504, Sigma- Aldrich) solution [50ug/ml: 50 mgs of mannan in 1 ml of PBS. 1:1000 dilution] was added to each well prior to being sealed with an adhesive plate seal and incubated overnight at 37deg to enable coating of each well with mannan. Each well was washed with complement fixation buffer prior to addition of dilutions of rat serum (diluted in complement fixation buffer) and a further incubation at 37 deg for 1 hour. The plate was then washed with fresh complement fixation buffer to remove unbound serum proteins and the plate blocked. Blocking solution consisted of 10% w/vol of skim milk protein diluted in complement fixation buffer (10 grams of dried skimmed milk, Marvel, Premier International Foods (UK) Ltd in 100 mls of water with one Complement Fixation Tablet (Cat: BR0016, Oxoid Limited, UK)). After blocking non-specific binding sites, the bound rat C4b was detected with a 1/100 dilution of polyclonal anti-rat C4 (Hycult Technologies, Cat: HP8023) diluted in PBST (Phosphate Buffered Saline - Cat: BP 399-20, Lot – 102355, Fischer Scientific Ltd), with a further incubation of 1 hour at 37 degrees.

Following 1 hour, the wells were washed 3x with PBST 200 ul, and the bound anti-C4 antibody detected with 1/1000 dilution of Peroxidase AffiniPure Donkey Anti-Rabbit IgG (Cat: 711-035-152, Jackson Immuno-Research Lab) added to each well, incubated for 1 hour and then washed 3x with PBST, and final 1x wash with PBS. Peroxidase antibody (directly proportional to the amount of C4 deposited on the plate) was then developed by the addition of OPD-Easy substrate (Acros organics) and the reaction stopped with an equal volume of 2N H2SO4 when the maximum signal was achieved. The plate was then read in microplate reader at 490 nm (MRX TC Revelation, Dynex Magellan Biosciences).

2.7. Pathology

2.7.1. Histology

Paraffin embedded renal tissues were Hematoxylin & Eosinophil (H&E) stained by Central Biotechnology Services (CBS), Cardiff. These slides were then analysed by a pathologist (Dr Gilda Chavez) who was blinded to the treatment groups. Jablonski's acute tubular necrosis score has been used routinely to characterise renal injury, however, it was felt that a more comprehensive scoring was required that included endothelial, glomerular and tubulointerstitial damage as part of the scoring scale. The scoring scale was devised on literature evidence, and has been validated by our research group by using correlation histology scoring with the qPCR of kidney injury molecules (KIM-1 and NGAL) and has been presented at the European Transplant Congress (ESOT, Brussels 2015) and currently is being submitted for publication.

The histology scoring scale adopted for this study is detailed in Table 2.1. The main parameters of evaluation were changes seen in Tubular, Glomerular, Endothelial and Tubular-Interstitial. The relevant literature evidence used to arrive on formulating this score is also referenced in the table.

Table 2.1: Histology scoring system

Tissue type	Damage	Score
Tubular (52-53)	No damage	0
	Loss of Brush Border (BB) in less than 25% of tubular cells. Integrity of basal membrane	1
	Loss of BB in more than 25% of tubular cells, Thickened basal membrane.	2
	(Plus) Inflammation, Cast formation, Necrosis up to 60% of tubular cells	3
	(Plus) Necrosis in more than 60% of tubular cells.	4
Endothelial (55)	No damage	0
	Endothelial swelling	1
	Endothelial disruption	2
	Endothelial loss	3
Glomerular (56-58)	No damage	0
	Thickening of Bowman capsule	1
	Retraction of glomerular tuft	2
	Glomerular fibrosis	3
Tubulo/Interstitial(54)	No damage	0
	Inflammation, haemorrhage in less than 25% of tissue.	1
	(Plus) necrosis in less than 25% of tissue	2
	Necrosis up to 60%	3
	Necrosis more than 60%	4
	Overall score	**14**

Table 2.1: Histology scoring scale used to characterise renal ischaemia reperfusion injury. The marking scale range was from 0-14, and mean scores were used for statistical calculations. The references for arriving at this score are also listed.

2.7.2. Immunohistochemistry (IHC)

The IHC staining on the slides were carried out by CBS, Cardiff, and I assisted them in some of the staining methods in order to gain an understanding of the methodology. Frozen and paraffin section slides were used for IHC depending on the antibodies.

2.7.2.1.Primary and Secondary antibodies

Based on the resources at CBS, DAKO EnVision™ Detection Systems Peroxidase/DAB, Rabbit/Mouse (Cat no: Code K5007, DAKO) were used as secondary antibodies. The EnVision™ reagent of this kit is a peroxidase-conjugated polymer backbone, and carries secondary antibody molecules directed against rabbit and mouse immunoglobulins. The primary antibodies chosen were either rabbit or mouse in origin. The primary antibodies chosen in this study along with their source are detailed below.

Complement system markers:

As discussed in the introduction chapter, activated complement generates three major types of effectors *(360)*

1. Anaphylatoxins (C3a and C5a)
2. Opsonins (C3b, iC3b, and C3d) which bond to target cell surfaces and immune complexes to facilitate their removal
3. Terminal membrane attack complex (MAC, C5b-9)

In our study, we are using IHC markers for C3b and C9 to assess complement-mediated damage. CD59 is the only well-characterized membrane inhibitor acting at the terminal step,

and prevents the assembly of the MAC by inhibiting the C5b-8 catalysed insertion of C9 into the lipid bilayer (218), and this was also evaluated. The complement IHC markers were kindly gifted by Dr Paul Morgan, Cardiff University.

Endothelial markers:

1. Tissue factor Antibody (FL-294): Cat: SC-30201: Santa Cruz Biotechnology
2. Thrombomodulin antibody (H-300): Cat : SC-9162, Santa Cruz Biotechnology
3. P-Selectin: Cat: 3633R-100, BioVision, Cambridge Bioscience

CD (Cluster of Differentiation) markers for Leukocytes:

1. Neutrophils: CD15 (C3D-1) -Cat: sc-19648, Santa Cruz Biotechnology
2. Macrophage: CD68 - Cat: OASA04073, Aviva Systems, Cambridge Bioscience
3. T-Lymphocytes:

    CD 3 - Cat: 201401, BioLegend, Cambridge Bioscience

    CD4 - Cat: 201501, BioLegend, Cambridge Bioscience

    CD8 - Cat: OASA04086, Aviva Systems, Cambridge Bioscience

2.7.2.2. Positive and negative controls

Native rat tissue, which are considered abundant source of marker in question, served as positive controls. The right kidneys from the experimental groups served as negative controls. These are detailed below

- Complement system: Rat liver from Wistar rats served as positive control for complement system (C3, C9, CD59)

- Endothelium: Rat spleen and liver from Wistar rats served as positive control for Thrombomodulin, Tissue factor, P-selectin
- Leukoctyes: Rat spleen/liver served as positive control for leuckocyte markers

2.7.2.3. Scoring key

The scoring key for IHC slides were:

- C3, C9, CD59: scored 0-4 (vascular and stromal staining) based on intensity of staining
- P-Selectin, Tissue Factor, Thrombomodulin: scored 0 – 4 depending on intensity of staining
- Cellular markers (CD15, C68, CD3, CD4, CD8) were counted: n/ avg 5x HPF

2.8. Molecular Analysis

Real Time Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

In Real-time PCR (qPCR), specific sequences within a DNA or cDNA template can be amplified (thousand-to a million fold), thus enabling detection of target molecules that get doubled with each amplification cycle. The fluorescence-based real-time reverse transcription PCR is widely used for the quantification of steady-state mRNA levels, gene expression analysis, pathogen detection and analysis of chromosome aberrations (361). The starting point for PCR is good quality messenger RNA (mRNA). Reverse transcription of RNA leads to formation of cDNA, which in turn is used as a template in qPCR reactions to detect and quantitate gene expression products. Gene expression/ mRNA analysis refers to the process by which information from a gene is used in the synthesis of a functional gene product, and with qPCR, researchers are able to assess changes (increase/decrease) in the expression of a particular gene by measuring the abundance of the gene-specific transcript (362). Real time PCR incorporates fluorescent dyes in the reaction mixture enabling monitoring of increase in amplification products throughout the reaction, enabling measurements to be taken in the exponential phase of the reaction, before the reaction plateau (363).

In ischaemia-reperfusion injuries, qPCR is being increasingly used as a modality to report variations in cytokine/chemokine levels and changes in genetic expression between the treatment groups. The qPCR offers a powerful technique to analyse results, especially effect of new drugs/agents on modulating ischaemia reperfusion injury (313, 364, 365).

Considering qPCR offers an avenue to explore a host of genes that are involved in IRI, we have used qPCR in our studies to analyse specific genes of interest.

This experimental set up used a two-step quantitative reverse transcriptase PCR:

- The first step is extraction of RNA from the one quarter of frozen kidney tissue, followed by Reverse Transcriptase reaction to create cDNA copies.
- The final step involves the qPCR reaction using the SYBR Green mastermix and primers for the specific genes in questions.

The qPCR experiments were conducted in the Laboratory based in Institute of Nephrology, Cardiff under the guidance of Dr Rob Jenkins and Dr Donald Fraser. Good Laboratory practice was followed during these experiments.

(http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042557.pdf).

The steps in the qPCR experiments are described below:

2.8.1. Methodology: RNA extraction and Reverse Transcription reaction

2.8.1.1. RNA Extraction

The frozen sample of kidney was weighed in a petri dish, and then suspended in the TRIzol® Reagent (Cat No: 15596-026, Invitrogen). The RNA extraction was carried out according to protocol (http://products.invitrogen.com/ivgn/product/15596026).

The steps are briefly explained below:

- Homogenizing samples

  1 mL TRIzol® Reagent was used for 50–100 mg of tissue sample. The weight of the quarter piece of kidney varied from 100 to 300 mgs, and the TRIzol® Reagent was used accordingly (i.e. 1-3 ml). The sample was then homogenized using a power homogenizer, resulting in a clear pink solution. Following each sample, then homogenizer was cleaned by running it in three solvents sequentially: 1mmol Sodium Hydroxide followed by 100% ethanol, and finally in nuclease free water. These solvents were placed in individual 20 ml vials. The homogenizer was then wiped with a paper tissue. 1 ml of the resulting solution was used for the phase separation step, and the rest of the solution is stored at -70^0 to be used in the future. This step was carried out in clean conditions in the Astec Monoair Hood system

- Phase separation

  The homogenized sample was then moved into the qPCR bench and was incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. 0.2 mL of chloroform (Cat- C2432, Sigma-Aldrich) per 1 mL of TRIzol® Reagent was then added to the homogenized sample, and mixed by inverting the tubes a few times. This sample was then incubated for 3 minutes at room temperature. The sample was then centrifuged at 12,000 × g for 15 minutes at 4°C in the Eppendorf Centrifuge machine. Following centrifugation, the mixture separated into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The aqueous phase of the sample contained the RNA, and this

was then pipetted out into a new eppendorf tube (1.5 ml tube). The interphase and organic phenol-chloroform phase was saved for DNA and protein isolation.

- RNA Isolation Procedure

  0.5 mL of 2-Propanol (Cat: I9516, Sigma-Aldrich) was then added to the aqueous phase, per 1 mL of TRIzol® Reagent used for homogenization. This sample was then incubated at room temperature for 10 minutes. The sample is briefly vortexed, and then centrifuged at 12,000 × g for 10 minutes at 4°C. Following centrifugation, the RNA forms a gel-like pellet (whitish fleck) on the side and bottom of the tube.

- RNA wash

  The supernatant from the tube is pipetted out carefully, leaving only the RNA pellet at the bottom of the tube. The pellet is then washed with 1 mL of 75% ethanol (Ethanol absolute- catalog no. 1.08543.0250, Merck KGaA; diluted to 75% by adding 7.5ml + 2.5ml of RNAse free Water) per 1 mL of TRIzol® Reagent used in the initial homogenization. The sample is then vortexed briefly, followed by centrifugation at 7500 × g for 5 minutes at 4°C. The wash is then discarded and the above step repeated (two times). The wash is then discarded, and the RNA pellet is air dried for 5–10 minutes under a HOOD. The pellet is then resuspended in RNAse-free water (50 ul) to develop a RNA stock solution. This solution was then diluted in $1/10^{th}$ dilution (10 ul of RNA stock+ 90 ul of RNAse free water) to be used for the qPCR reactions, and the stock solution stored at -70^{0}c.

2.8.1.2. Assessing quality and quantity of RNA

RNA quality and quantity is important prior to the reverse transcription. Nanodrop Spectrophotometer (Thermo Fisher Scientific) based at CBS, Cardiff was used for this purpose. The procedure for carrying out the test and analysing the results is adopted from the manufacturer's protocol (http://www.nanodrop.com/library/nd-1000-v3.7-users-manual-8.5x11.pdf).

2.8.1.2.1. Nanodrop Spectrophotometer

The Nanodrop Spectrophotometer (Thermo Fisher Scientific) enables determination of the concentration and purity of nucleic acid. This is determined by the following outputs from the spectrophotometry, and the data is shown in the Appendix 3.

- ng/ul: sample concentration in ng/ul based on absorbance at 260 nm
- 260/280: The ratio of sample absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA in the given sample. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. If the If the ratio is lower than 1.8, it may indicate the presence of protein, phenol or other contaminants that may lower the qPCR reaction efficiency
- 260/230: ratio of sample absorbance at 260 and 230 nm acts as a secondary measure of nucleic acid purity. The ratio ranges commonly between 1.8-2.2. If the ratio is lower, this may indicate the presence of co-purified contaminants like phenol rings (salt guanidine isothiocyanate, phenol etc.), which can inhibit enzymatic reactions.

The methodology for carrying out Nanodrop Spectrophotometry is as follows.

- The receiving fibre of the machine is cleaned with a tissue and 1 ul of RNAse free water is pipetted onto it. On the computer desktop, the nanodrop icon is clicked (nuclei acid box chosen) and the sampling arm (second fibre optic cable) is then brought into contact with the liquid sample. Once this is done, the run logo is clicked on the computer to calibrate the machine. Following the dry run, the sampling arm is lifted, cleaned with a paper tissue and 1 ul of the RNA solution ($1/10^{th}$ dilution) is pipetted to the end the receiving fibre. The sampling arm is then gently positioned on the receiving fibre, over the solution. On the computed desktop, the relevant sample number is entered, RNA-40 is chosen as the sample type and the 'measure icon' is clicked to run the analysis. The results are stored on the computer, and this data was copied on to the memory stick, and analysed using MS Excel software.

2.8.1.2.2. Agilent 2100 Bioanalyzer: RNA Integrity (RIN):

The RIN analysis was carried out by Dr Megan Musson, CBS, Cardiff. Agilent Bioanalyzer is another methodology that helps in determining the quality of RNA by assigning a RIN (RNA Integrity Number) to it. It also helps determining to what extent the RNA in the sample is degraded, as RNA extracted from tissues exhibit varying degrees of degradation. The principle and the methodology of RIN analysis to assess integrity (as compared to traditional denaturing gel method) has been published by the Schroeder et.al from Agilent Technologies (366).

5 ul of 1/10th dilution of RNA stock was pipetted into a new 0.5 ml eppendorf for the analysis purposes. 1-4ul of the sample was loaded onto a chip (12 at a time) and inserted into the Bioanalyzer machine, which delivers results in 45 minutes. Further information about this procedure can be accessed at (http://www.genomics.agilent.com). The RIN values of all the samples are shown in Figure 1 at the end of this chapter.

2.8.1.3. Reverse Transcription

Reverse transcription involves formation of a complementary DNA (cDNA) from a messenger RNA (mRNA) template, and this reaction is catalysed by enzymes: reverse transcriptase and DNA polymerase. The methodology was as per protocol: http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042557.pdf

Reagents used:

1. High Capacity cDNA Reverse Transcription Kit (Cat No: 4368814, Invitrogen), consisting of the following reagents:
    - 10× RT Buffer
    - 10× RT Random Primers
    - 25× dNTP Mix (100 mM)
    - MultiScribe® Reverse Transcriptase
    - RNAse inhibitor (Cat No: M0307S, New England Biolabs)

Procedure:

The aim of this experiment is to create a single-stranded cDNA from total RNA. The first step is to create the 2xRT master mix (per 20ul reaction) in a 1 ml eppendorf tube.

Component	**Volume/Reaction (ul)**
10× RT Buffer	2
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2
MultiScribe® Reverse Transcriptase	1
RNase Inhibitor	1
Nuclease-free H2O	3.2
Total Per Reaction	**10**

In my experiments, the master mix was prepared for 30 samples at a time, and the above volumes were multiplied into 35x (5ul extra to avoid pipetting loss). The mastermix was then placed on ice. Nanodrop Spectrophotmeter provides quantity of RNA as ng/ul. This concentration is converted to ul/ug. The resulting volume is then made up to 10 ul by adding nuclease free water. This solution is made up in one single RNA tubes corresponding to individual ratcode. To this sample, 10 ul of mastermix is added, pipetting up and down at least 2xtimes to mix, leading to a final volume of 20 ul per reaction/tube. The eppendorfs are then placed on ice. The heat stable RNA tubes were then placed in the thermal cycler for the reverse transcription reaction to take place. The cDNA formed at the end of this reaction is then stored at -20^{0}centigrade in the freezer.

2.8.2. qPCR Primers and SYBR Green mastermix

The primers used in this study were designed by Dr.Rob Jenkins, and were ordered from Invitrogen, Life Technologies. Each gene of interest had a forward and reverse primer that was used in making the Power SYBR Green master mix. The primers used in this study are listed below in Table 2.2:

Table 2.2: qPCR Primers

Gene	Forward Primer	Reverse Primer
REFERENCE GENES		
PGK1	CGT GAT GAG GGT GGA CTT CAA	ACA ACC GAC TTG GCT CCA TT
HPRT1	ACA GGC CAG ACT TTG TTG GA	TCC ACT TTC GCT GAT GAC ACA
YWHAZ	TCT GCA ACG ACG TAC TGT CTC	CCT CAG CCA AGT AGC GGT AG
TBP1	TCC ACC GTG AAT CTT GGC TG	CAG CAA ACC GCT TGG GAT TA
ACTB	CGC GAG TAC AAC CTT CTT GC	CGT CAT CCA TGG CGA ACT GG
B2M	ACT GAA TTC ACA CCC ACC GA	TAC ATG TCT CGG TCC CAG GT
GAPDH	GCA TCT TCT TGT GCA GTG CC	TAC GGC CAA ATC CGT TCA CA
TARGET GENES		
NGAL	GGG CTG TCC GAT GAA CTG AA	CAT TGG TCG GTG GGA ACA GA
KIM-1	CGG CTA ACC AGA GTG ACT TGT	TAC AGA GCC TGG AAG CAG
ICAM-1	AGC GAC ATT GGG GAA GAC AG	CTC GCT CTG GGA ACG AAT ACA
VCAM-1	ACG AGT GAA TCT GGT TGG GAG	AAC AGT AAA TGG TTT CTC TTG AAC
IL-18	GAC CGA ACA GCC AAC GAA TC	ARA GGG TCA CAG CCA GTC CT
TNF-A	ATG GGC TCC CTC TCA TCA GT	GCT TGG TGG TTT GCT ACG AC

Table 2.2: Primers with the forward and reverse configuration for reference and target genes used in the RT-qPCR experiments.

SBYR Green:

For qPCR, either TaqMan® Chemistry or SYBR® Chemistry can be used. Though TaqMan has higher specificity and higher reproducibility, it is very expensive, and a different probe has to be used for each target reference gene. No probes are required for SYBR Green based assay, thus considerably reducing the cost of the assay. In our study, we have adopted SYBR® dye based platform, where the SYBR dye detects PCR products (formed through DNA polymerase amplifying the target sequence to create PCR products) by binding to the double-stranded DNA formed during PCR. As more PCR products are formed with each cycle, the SYBR dye binds to all the double-stranded DNA, which is reflected by an increase in the fluorescence intensity directly proportional to the amount of PCR product. One of the main disadvantages is generation of false positive signals as SYBR dye binds to even non-specific double-stranded DNA sequences, which we have negated by using well-designed primer and performing melt curve analysis as part of the qPCR.

Master Mix preparation:

Power SYBR® Green PCR Master Mix (5ml, Cat: 4367659, Invitrogen) was used. A mastermix was prepared per reaction to be used with the cDNA, and the composition is

MasterMix	**ul/Reaction**
Power SYBR® Green PCR Master Mix	10ul
Forward Primer	0.6ul
Reverse Primer	0.6ul
Nuclease free water	4.8ul

The primers were usually stored at -20^{0}centigrade. The primers were defrosted on ice, and centrifuged. Nuclease free water was added to this primer in the tube, vortexed and centrifuged. Based on the reaction numbers, then the desired quantity of forward and reverse primer was added to the SYBR Green master mix solution.

Preparing the plate:

For the qPCR reaction, MicroAmp Fast optical 96-well reaction plate (Cat: 4346906, Applied Biosystems, Life Technologies) was used. 16ul of SYBR master mix was then pipetted into each well according to the various groups. The cDNA tubes, which were preserved at -20^0 were defrosted on ice. Following defrosting, the samples were gently vortexed and placed in a centrifuge machine. The tubes were then placed on ice. 4ul was then drawn out from the RNA tubes using a pipette, and then mixed with master mix in one single well. In one well (usually the last row), 4 ul of water was added to 16ul of mastermix and this served as a no-template control (NTC). NTC contains all the reaction components apart from the cDNA, and any amplification detected in these wells can be due to primer-dimers or contaminants. Once all the desired cDNA samples were mixed with the SYBR green master mix, the plate was covered with MicroAmp Optical Adhesive Film (Cat – 4311971, Applied Biosystems, Life Technologies). A soft rubber plate was then used to gently press the adhesive film against the plate. The plate was then covered in a foil, and carried over to the qPCR machine on ice. Duplicates of each gene of interest were carried out to ensure no variations.

qPCR Machine:

The qPCR Machine used in these experiments was: 7900 HT Fast Real-Time PCR System, Applied Biosystems and was placed in the CBS unit. Prior to loading the plate in the machine, it was centrifuged at 9000G x 3 minutes. This machine was linked to a computer placed next to it that controlled the experiment. On the desktop, the analysis mode chosen was: Standard curve. The gene of interest was added onto the software, and SYBR chosen as the fluorescence method. The qPCR run was 'standard' in nature, and a dissociation stage was added to the cycle for melting curve analysis. As SYBR green binds to all double-stranded DNA, melting curve analysis reveals if any other contaminating DNA, primerdimers or PCR products from misannealed primer are present in the PCR reaction, usually evident by multiple peaks. If there is only a single peak on the melting curve analysis, it implies that the desired amplicon was only detected. The reaction lasted for 2 hours, and the results were reported as Ct (threshold cycle) values. The Ct value is used to calculate the initial DNA copy number, and the Ct value is inversely related to the starting amount of the target.

2.8.3. qPCR Analysis

Once the cT (cycle threshold) values are obtained, the determination of gene expression can be quantified either via absolute or relative quantification method. Absolute quantification is a methodology where samples of known quantity are serially diluted and amplified to generate a standard curve, and this in turn allows quantification of unknown samples against this curve. In relative quantification, the expression of gene of interest is compared between treated and untreated samples, and expressed as fold change (increase or decrease) using a reference gene as a control for experimental variability. For our experiments, I have chosen the relative quantification method of normalising to a reference gene.

2.8.3.1. Normalising to a reference gene:

It is critical in qPCR to minimise any experimental variations and inconsistencies. Sources of variability can arise at each step of the experiment, from amount of starting sample to RNA isolation and reverse transcriptase, to qPCR amplification. Normalisation aims at addressing these variabilities, and the three main approaches include (http://www.biotechniques.com/protocols/PCR/Real-time_PCR/Normalization-Methods-for-qPCR/biotechniques-115492.html):

- Normalising to a sample quantity
- Normalising to RNA or DNA quantity
- Normalising to a reference gene

Of all the methods, the most widely used and reliable technique is to normalize RNA levels to an internal reference gene (367). The assumption in this method is that the reference gene is abundantly present in the tissue, and levels of expression don't vary between the treated and untreated groups. In our study, we have adopted this method as there are multiple groups with different treatments and differing RNA concentration and normalising against reference gene would minimise variability.

There are a variety of genes that have been used as reference genes in renal IRI models. Based on the literature evidence (368-371) and opinion of Dr Rob Jenkins/ Dr D Fraser, the following genes were evaluated as reference genes, and the results are discussed in the next chapter.

- GAPDH
- rRNA
- PGKQ
- HPRT1
- YWHAZ
- TBP
- Beta -2 –Microglobulin

2.8.3.2. Genes of interest

Based on the aims of the study, the following genes of interest were identified and used in the study.

Kidney injury markers:

- **Kidney Injury Molecule (KIM-1):**

  KIM-1 is expressed in renal PTEC and facilitates apoptotic and necrotic cell clearance. KIM-1 is a membrane glycoprotein that contains both a six-cysteine immuno- globulin-like domain and a mucin domain in its extracellular portion, and has two spliced variants: KIM-1a expressed in the liver and Kim-1b, which is the predominant form in the kidneys (372). Following renal injury, KIM-1 is shed from the tubular cells into the urine and extracellular space, and this phenomenon has been consistently showed in literature leading to KIM-1 being used a biomarker for kidney injury (373, 374). KIM-1 has been shown to be elevated in urine within 12 hours following acute renal injury, and in biopsies of acute renal failure patients (375). KIM-1 has been shown to be elevated with ischaemia in rats and other species using qPCR studies (313, 376), and correlated well with kidney injury and recovery (with intervention agents), leading to its use in our study,

- **Neutrophil Gelatinase- associated Lipocalin (NGAL):**

  NGAL is produced by neutrophils, and is a stress response protein that is upregulated on injury, and released into urine and plasma from the kidney. The proximal tubule, thick ascending limb and collecting ducts appear to be sources of urinary NGAL and was identified in proximal tubules on both IHC and qPCR (377).

Evidence for NGAL as marker for kidney injury was initially from mice renal IRI studies where upregulation of NGAL was seen following IR, with administration of purified recombinant NGAL resulting in improvement of morphologic and functional parameters (378). In a recent meta-analysis, serum and urine NGAL levels were found to be independent predictors of AKI and outcomes (379). There is very limited studies evaluating NGAL in rat renal IRI (380), and hence was used as a second marker in our study.

Cell Adhesion Molecules markers:

- **Inter-cellular adhesion molecule (ICAM-1)**

  ICAM enhances leukocyte endothelial interaction, and was seen very early after IR in murine model, with antibody to ICAM-1 protecting from ischaemic injury in the renal IRI model (81, 82). qPCR studies in renal IRI have shown elevated levels of ICAM-1 following ischaemic insult, with certain intervention agents showing downregulation, but this is not consistently seen with all agents (313, 381).

- **Vascular cell adhesion molecule** (VCAM-1: CD106): VCAM-1 is found in very low levels on the cell surface of resting endothelial cells and mediates leukocyte adhesion to the vascular endothelium (97). Similar to ICAM, VCAM expression has also shown to be upregulated in murine 24-hour reperfusion model with intervention agents then showing downregulation of VCAM-1 (382). As with ICAM-1, there is limited evidence of VCAM-1 downregulation with intervention agents, and we hope to evaluate this further.

Cytokine markers:

- **TNF-alpha**
- **IL-18**

The role of TNF-alpha and IL-18 has been extensively discussed in the introduction chapter. In essence, they are pro-inflammatory cytokines that are upregulated following renal IRI, and qPCR is one of the methods to evaluate these two makers.

2.9. Analysis

2.9.1. Log book and excel database

A logbook was maintained which detailed all the operations carried out. Each rat experiment was recorded and the details included were:

- Rat identification
- Rat weight
- Date of operation
- Treatment arm
- Intraoperative findings and procedure
- Unexpected events
- Post operative follow up – wound, rat behaviour
- Retrieval findings

The logbook details were also maintained in a excel database, and was accessible to the supervisor also.

2.9.2. Statistical analysis

The size of experimental groups is estimated by power calculation (Dupont WD and Plummer WD: PS Power and Sample Size Calculation. This calculation was also based on published results of comparable experiments (202, 266). To ascertain changes between groups, the minimum number per group is 6, and the number of rats/experiments in each group is tabulated below:

- 40 minute unilateral left IR with 48 hours of reperfusion: n=10
- Sham group (48 hours): n=6
- ATG + 40 minute unilateral left IR with 48 hours of reperfusion: n=8
- ATG isotype +40 minute unilateral left IR with 48 hours of reperfusion: n=8
- sCR1 +40 minute unilateral left IR with 48 hours of reperfusion: n=8
- Anti-MASP2 Antibody +40 minute unilateral left IR with 48 hours of reperfusion: n=6
- aMASP-40 minute 2 antibody isotype +40 minute unilateral left IR with 48 hours of reperfusion: n=6

The statistical tests used were based on the advice from the biostatisticians at Cardiff University, and the literature evidence, and have been discussed in the methodology chapter. The article 'Principles for Valid Histopathologic Scoring in Research' by Gibson-Corley

et.al (383) also guided on the use of appropriate statistical tests. Based on these discussions, the following tests were used:

- Histology and Immunohistochemistry scorings were classed as non-parametric data, while qPCR results were classed as parametric data.

- Results were be analysed for significance by parametric (unpaired Student's t-tests, one-way ANOVA, paired Student's t-est) and non-parametric tests (Mann-Whitney U and Kruskal-Wallis) If differences were noted between three groups, then post hoc analysis using Neuman-Keuls (Anova) or Dunn's (Kruskal-Wallis) were used.

- Graphpad Prism 6 software was used for statistical analysis. Statistical significance was defined as $P < 0.05$, and 'star marks' as indicated in the table below depicted the level of significance seen

P value	**Wording**	**Summary**
< 0.0001	Extremely significant	****
0.0001 to 0.001	Extremely significant	***
0.001 to 0.01	Very significant	**
0.01 to 0.05	Significant	*
≥ 0.05	Not significant	ns

CHAPTER III:

RESULTS – BASELINE STUDIES

3.1. Introduction

Renal ischaemia-reperfusion injury is a complex mechanism involving numerous cellular and subcellular processes. Among those, the complement system, leukocytes and endothelium seem to have an important role in the inflammatory process that occurs following ischaemia and reperfusion. These components have an important role to play in the initiation and propagation of renal IRI. With my thesis focusing on these three systems, it was necessary to establish baseline studies to evaluate the participation of these elements prior to any interventional studies.

Model:

We chose a unilateral left renal ischaemia-reperfusion rat model. Safety profiling preferred a unilateral approach, as it would be less harmful for the subject. It would also enable us to compare the ischaemic left kidney with the right kidney, which served as an internal control. The drawback of this model is that we would not be able to obtain any physiological measurement such as serum creatinine. However, considering we are using comprehensive histology and immunohistochemistry scoring, we felt that it was justifiable to use the unilateral model. I have discussed the strengths and limitations of this model in the final chapter.

Ischaemia timing:

There is variability in literature regarding the length of ischaemia used in each model. The timings have varied between 20, 30, 40, 45, 60 and 120 minutes. The 40-minute group has also been used to been used by other researchers' to demonstrate renal IRI, and has been

valid across different species (384-387). All these studies were also able to show reversal of injury with interventional agents. 40-minute ischemia was shown to represent the 'fatal' turning point beyond which the autophagy process transformed cell survival into cell death stage, but was dependent on the species and strain (388, 389). There is also some early evidence that autophagy coordinates various extracellular and intracellular triggers that lead cell viability (390). Hence keeping in context that we needed a model where changes could be sufficiently seen, and any intervention would be able to reverse these changes, we proceeded with the 40-minute time interval.

Aims of this chapter:

- To evaluate safety profiling of unilateral model
- Evaluate histopathological differences between the sham and IR group using a comprehensive scoring scale
- Evaluate different subsets of leukocyte infiltration using immunohistochemistry
- Evaluate the role of complement system by measuring the activated complement products
- Comprehensive evaluation of the endothelial changes following ischaemia-reperfusion
- To determine differences in mRNA levels between sham and IR group

3.2. Methods

The sham group served as a control for the 40-minute ischaemia group. The sham group had exactly the same protocol as the IR group, only difference being no clamping of the left renal vessels. The baseline kidneys were from straight kill rats, and both the right and left kidneys were used for analysis.

Groups:

- The 40-minute IR group: n= 10 rats
- Sham group: n=6 rats
- Baseline group (2 rats) and both kidneys used for scoring: n=4

Analysis:

Kidneys retrieved at 48 hours were analysed for:

- Histology
- Immunohistochemistry
- RT-PCR

3.3. Results

3.3.1. Histology

Haemtoxylin and Eosinophil (H&E) stained slides were analysed by a blinded pathologist and scored for injury on a scale from 0-14. The results are depicted in the graphs below.

Figure 3.1: Histology Score – Sham and 40' IR

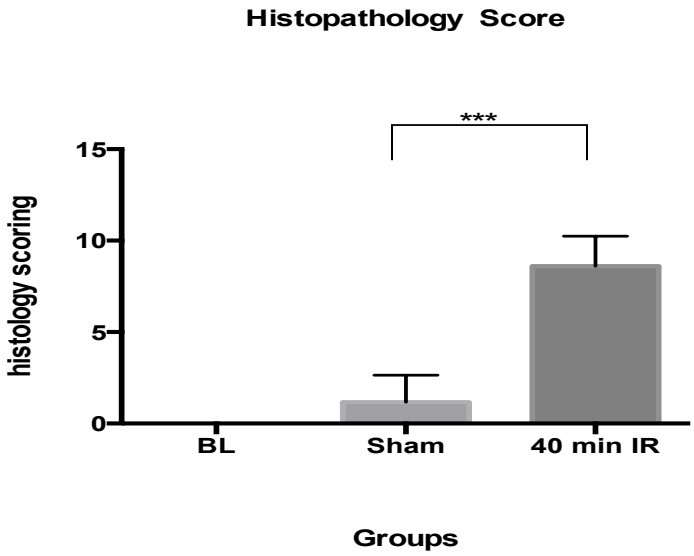


Figure 3.1: Scoring of H & E renal cortex sections from Baseline (BL) (n=4), sham (n=6) and 40-minute unilateral IRI (n=10) in rats at 48 hours after reperfusion. A comprehensive scoring system (0-14) evaluated tubular, endothelial, glomerular and tubulo-interstitial cell damage. Histology scores are plotted as Mean +/- SD, and Mann Whitney tests used to test significance [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05 (*)].

Figure 3.2: Renal cortex H&E sections – Sham and 40' IR

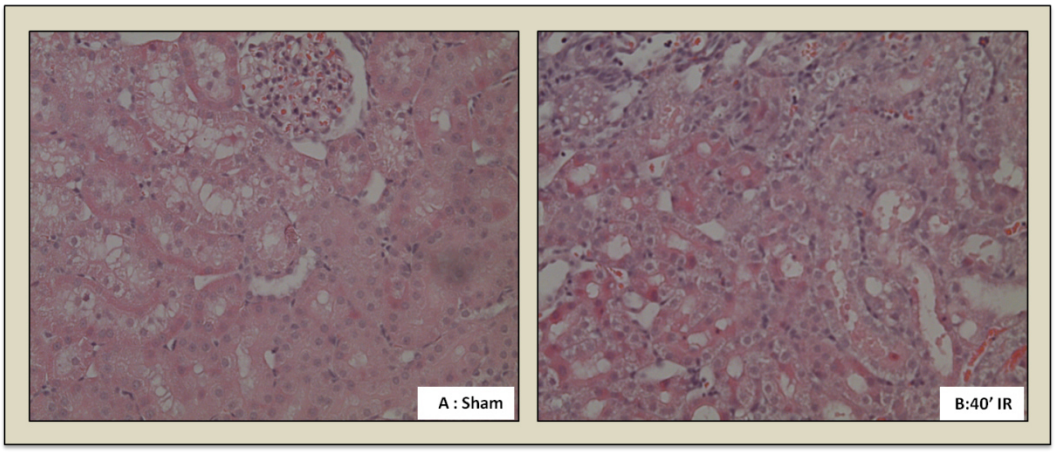


Figure 3.2: Representative rat renal cortex H&E (x200) paraffin sections, from sham and 40-minute IR group at 48 hours after reperfusion, assessed for tubular, endothelial, glomerular and tubulo-interstitial cell damage. (A) Normal appearance of renal cortex in a sham rat. The brush border of the tubular cells is intact with no thickening of the basal membrane. No inflammation or necrosis is seen (Tubular score 0). The endothelium is uniform with no swelling or disruption of the endothelial cells (Endothelial score 0). The glomeruli are intact with thin walled Bowman's capsules and no tuft retraction (Glomerular score 0). There is no visible interstitium signifying no damage/abnormality within the tubulo-interstitial compartment (Tubulo-interstitial score 0). Overall score =0

(B). Moderate to severe damage of the renal cortex in a 40-minute IRI rat. Glomeruli are seen with evidence of thickened Bowman's capsule, and glomerular tuft retraction (Glomerular score 1). Within the tubules, there is evidence of inflammation and cast formation. Upto 60% of the tubular cells show necrosis (Tubular score 3). There is endothelial disruption with no evidence of endothelial loss (Endothelial score 2). In addition to the inflammation and haemorrhage seen within the tubulo-interstitial compartment, there is necrosis in up to 60% of the cells (Tubulo-interstitial score 3). Overall score =9

3.3.2. Immunohistochemistry

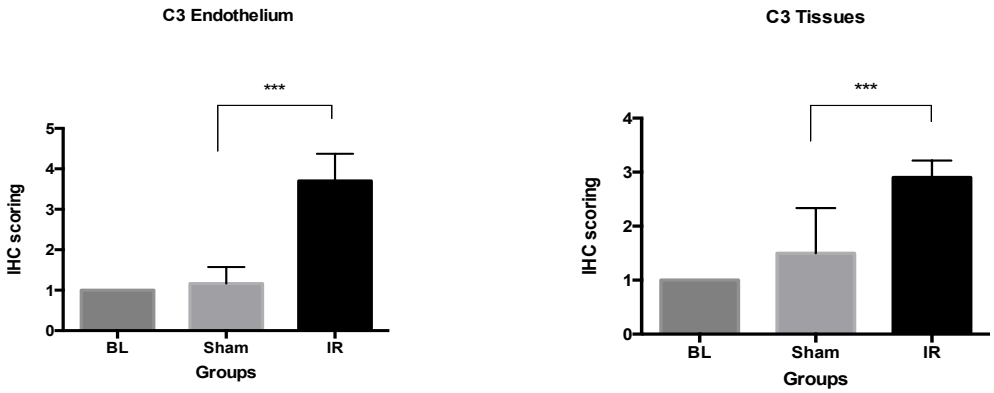
IHC was used to test a variety of markers encompassing the complement system, endothelium and leukocytes. The results are shown in summative graphs , with representative IHC pictures showing the changes.

3.3.2.1.Complement studies

The IHC markers used to assess complement system were:

- Opsonin **C3b** which binds to target cell surfaces and immune complexes to facilitate their removal
- C9: marker for terminal membrane attack complex
- CD59: membrane inhibitor acting at the terminal step, and prevents the assembly of the MAC by inhibiting the C5b-8 catalysed insertion of C9 into the lipid bilayer

Figure 3.3: Summative IHC scoring of complement markers – Sham and 40' IR



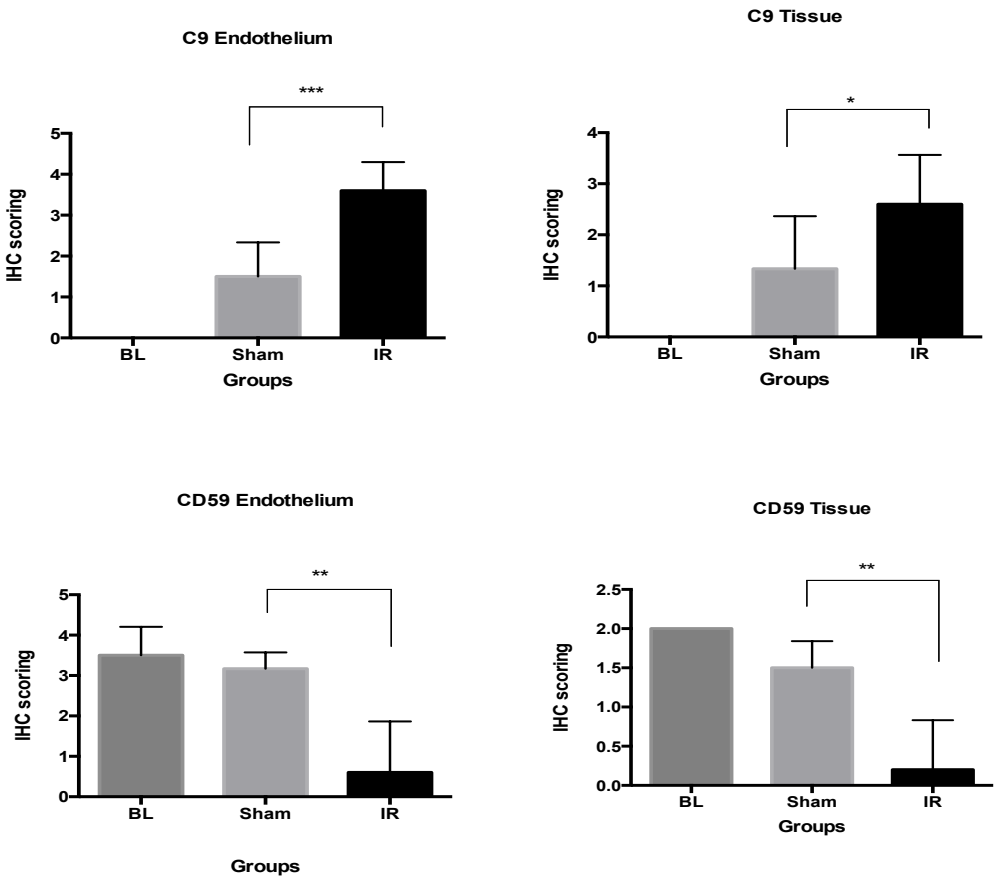


Figure 3.3: IHC scoring of renal cortex sections from baseline (n=4), sham (n=6) and 40-minute unilateral IR (n=10) in rats at 48 hours after reperfusion. Complement markers C3, C9 and CD59 stained as per protocol and scored 0-4 (vascular and stromal staining). IHC scores are plotted as Mean +/- SD, and Mann Whitney tests used to test significance [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05 (*)].

Figure 3.4: Renal cortex IHC sections of complement markers – Sham and 40' IR

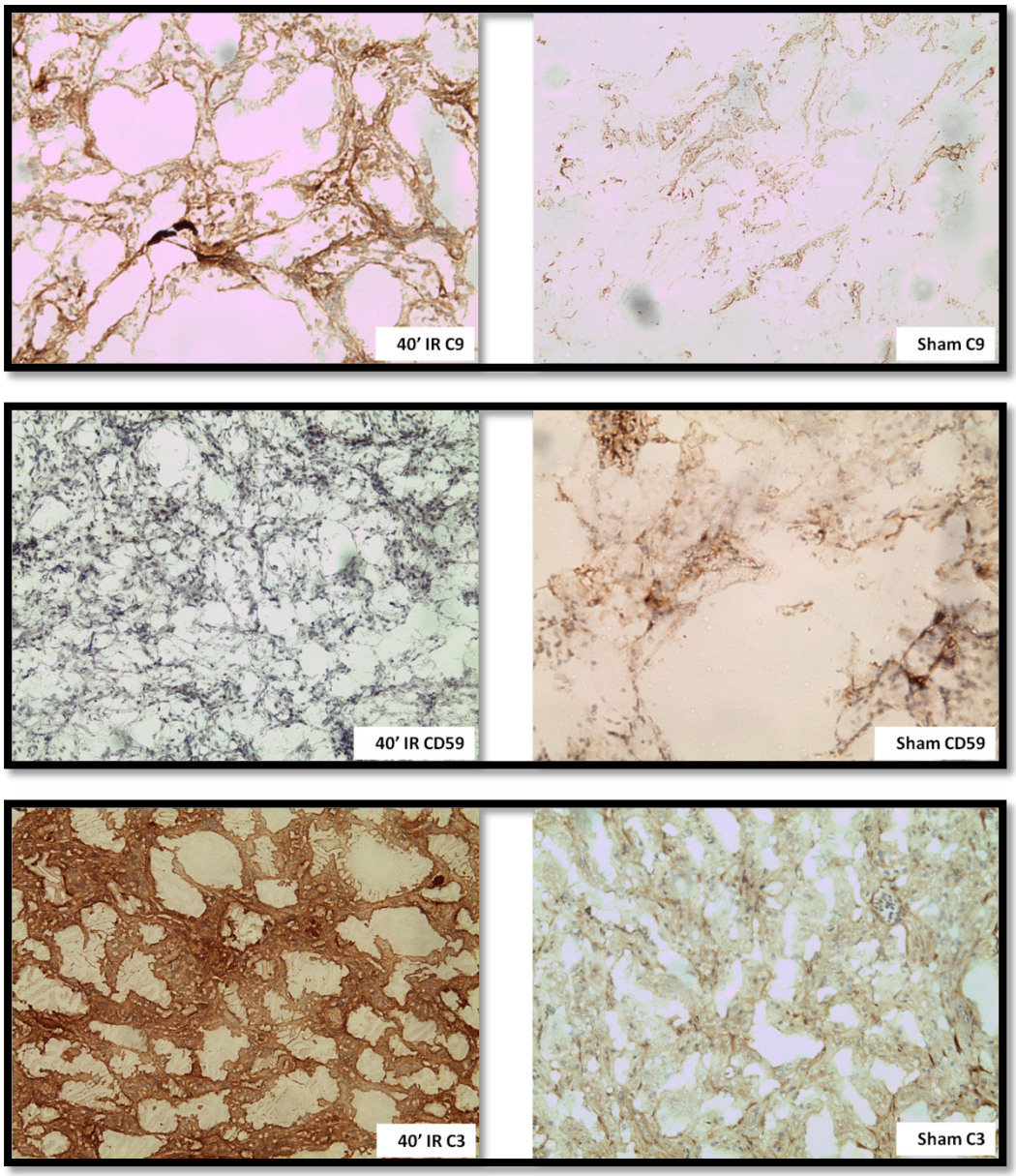


Figure 3.4: Representative rat renal cortex (x200) IHC sections, from sham and 40-minute IR, at 48 hours after reperfusion. Complement markers C3, C9 and CD59 stained as per protocol and scored 0-4 (vascular and stromal staining). The 40 minute-IR slides on the left depict increased deposits of C3 and C9 compared to the sham group indicating injury, along with decreased of CD59 staining in the IR group indicating loss of the inhibitory capacity of CD59 in forming membrane attack complex (C9).

3.3.2.2. Endothelial markers

Figure 3.5: Summative IHC scoring of endothelial markers – Sham and 40' IR

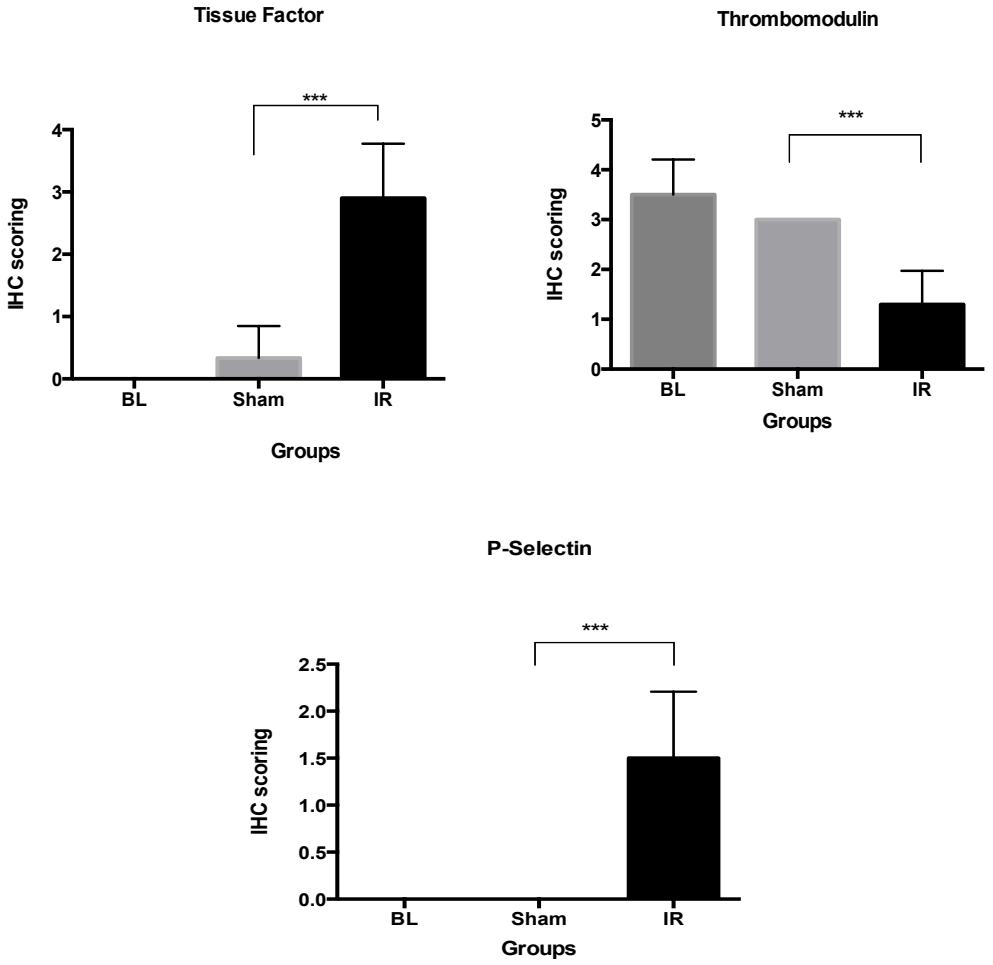


Figure 3.5: IHC scoring of renal cortex sections from baseline (n=4), sham (n=6) and 40-min left unilateral IRI (n=10) in rats at 48 hours after reperfusion. Endothelial markers: Tissue factor, Thrombomodulin and P-Selectin were stained as per protocol and scored 0-4 (vascular and stromal staining). IHC scores are plotted as Mean +/- SD, and Mann Whitney tests used to test significance [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05 (*)].

Figure 3.6: Renal cortex IHC sections of endothelial markers – Sham and 40' IR

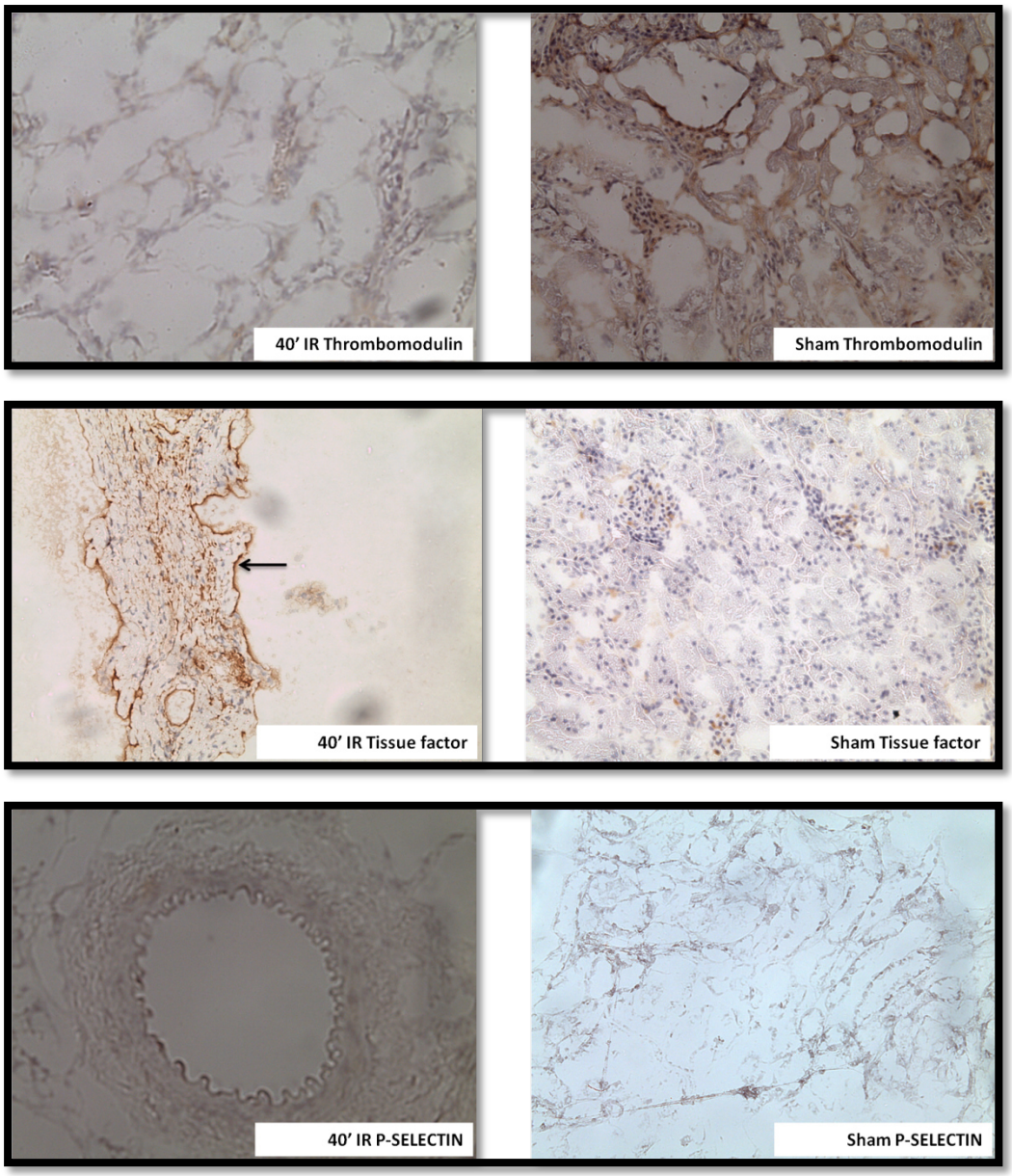
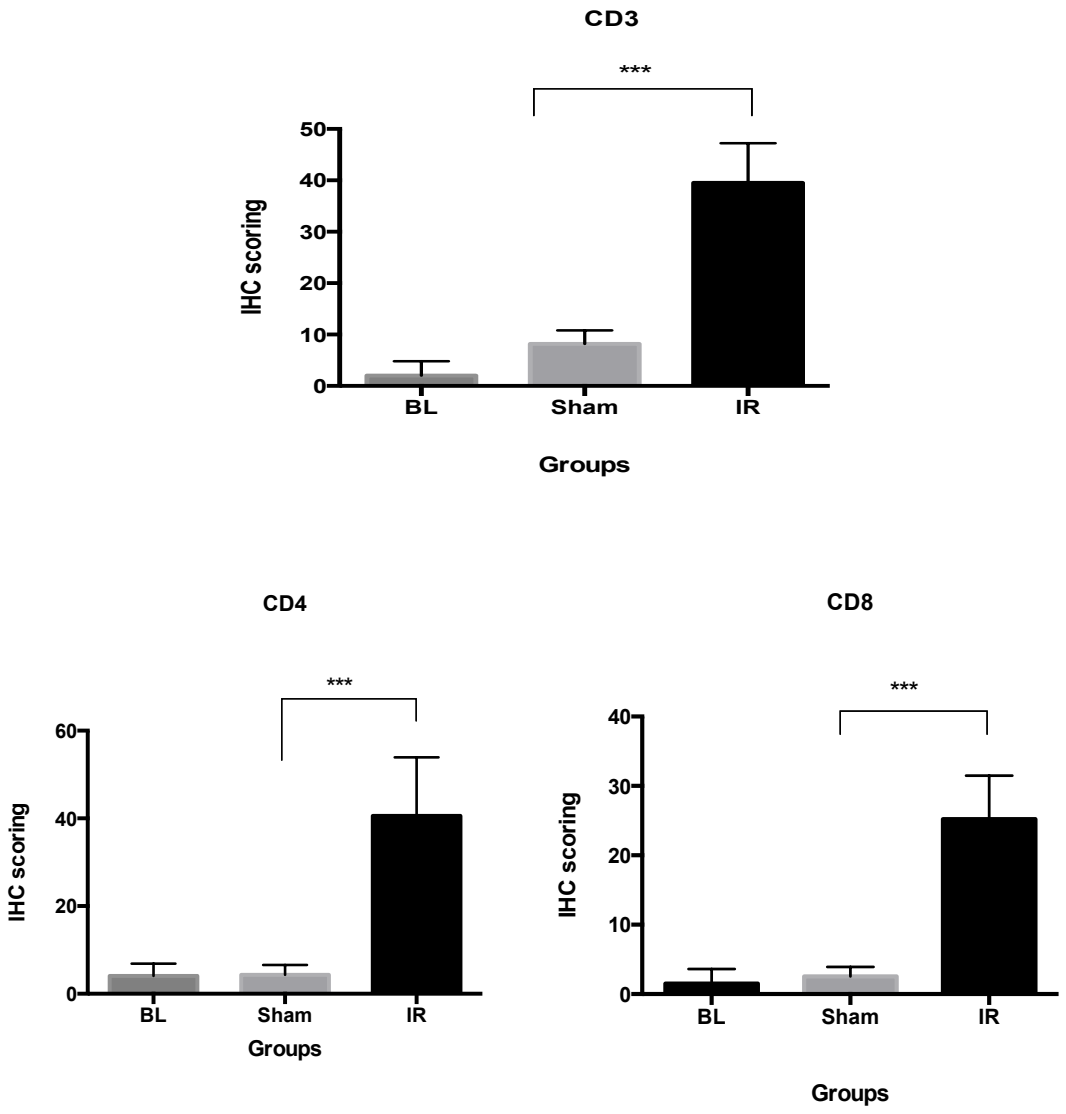


Figure 3.6: Renal cortex (x200) IHC sections from sham and 40-minute IRI in rats, at 48 hours after reperfusion. Endothelial markers: Tissue factor, Thrombomodulin and P-Selectin were stained as per protocol and scored 0-4 (vascular and stromal staining). Increased deposits of Tissue factor and P-Selectin were seen in the IR group, coupled with decrease deposits of thrombomodulin indicating endothelial damage in the IR group compared to the sham group.

3.3.2.3. Inflammatory Markers

Cellular markers for lymphocytes (CD3, CD4 and CD8), macrophages (CD68) and neutrophils (CD 15) were used to assess inflammatory infiltrate in IR setting.

Figure 3.7: IHC scoring of cellular markers for inflammation mediators – Sham and 40'IR



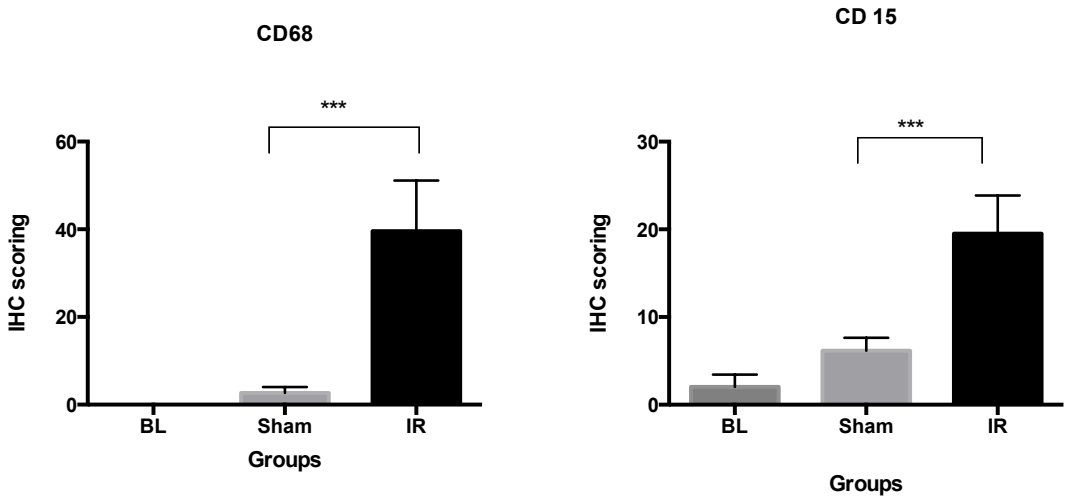
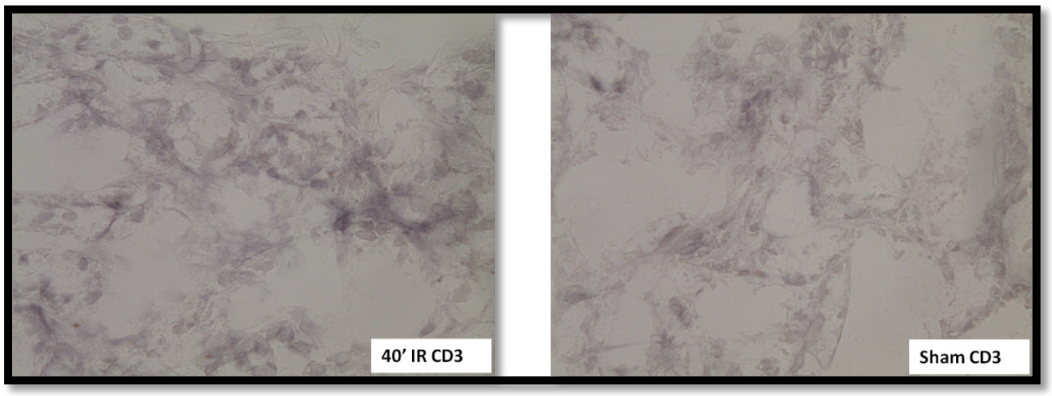


Figure 3.7: IHC scoring of renal cortex sections from baseline (n=4), sham (n=6) and 40-minute unilateral IRI (n=10) in rats at 48 hours after reperfusion. Cellular markers for inflammation mediators were stained as per protocol, and were counted: n/ avg 5x HPF (high power field). IHC scores are plotted as Mean +/- SD, and Mann Whitney tests used to test significance [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05 (*)].

IHC slides:

Figure 3.8: Renal cortex IHC sections of cellular markers for inflammation mediators – Sham and 40' IR



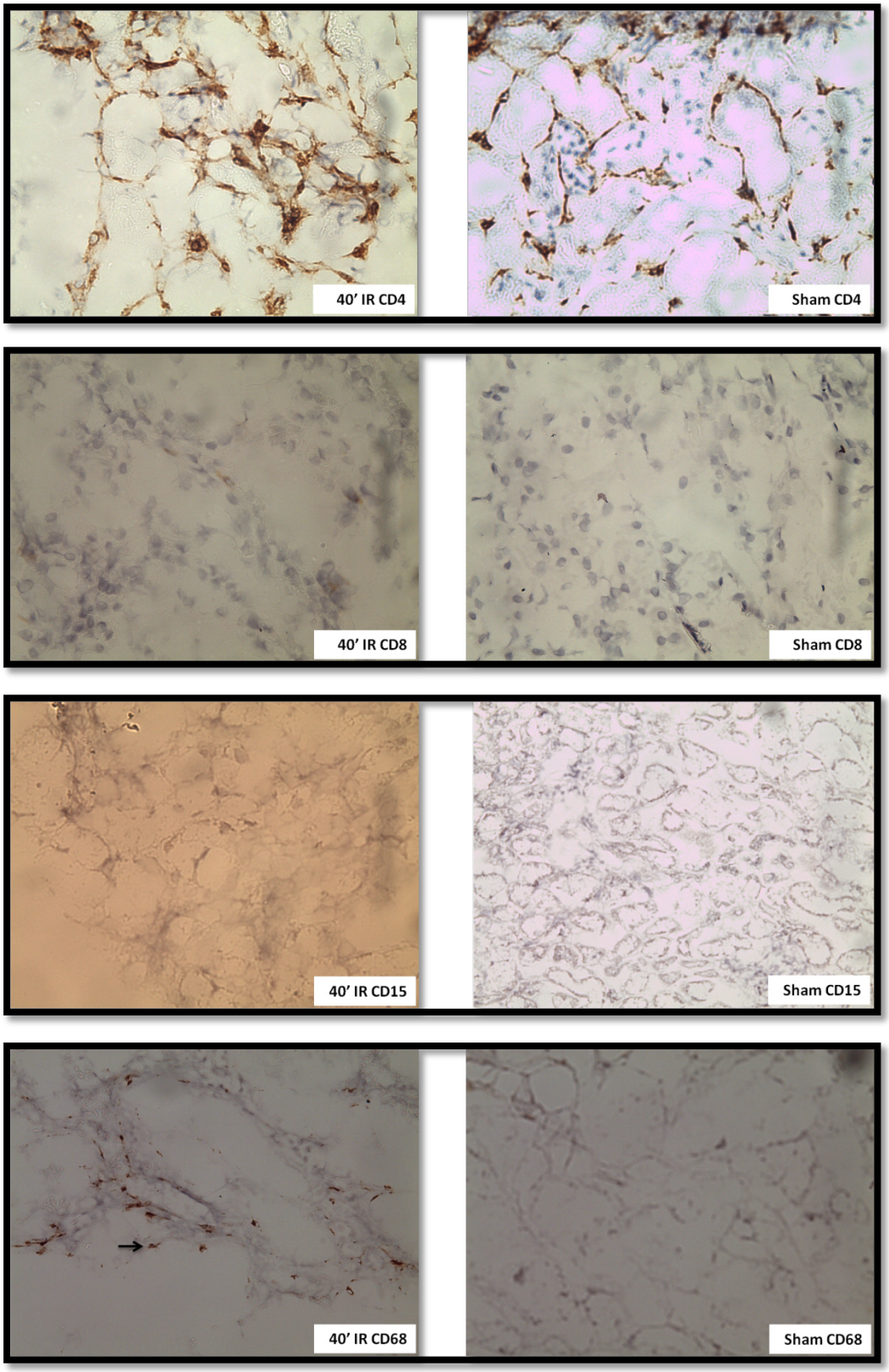

40' IR CD4
Sham CD4
40' IR CD8
Sham CD8
40' IR CD15
Sham CD15
40' IR CD68
Sham CD68

Figure 3.8: Representative rat renal cortex (x200) IHC sections from sham and 40-minute IRI group, at 48 hours after reperfusion. Cellular markers for inflammation mediators were stained as per protocol, and were counted: n/ avg 5x HPF (high power field). As evident by the earlier quantitative figures, there were increased infiltrate of CD3, CD4 and CD8 lymphocytes, CD15 (neutrophils) and CD68 (macrophages) in the IR kidneys compared to the sham group.

3.3.3. Real-time reverse transcription PCR (RT-qPCR)

Reverse Transcription–quantitative PCR (RT-qPCR) is widely used for the quantification of gene expression at the mRNA level (443). Gene expression (mRNA) analysis refers to the process by which information from a gene is used in the synthesis of a functional gene product, and hence able to assess changes (increase/decrease) in the expression of a particular gene by measuring the abundance of the gene-specific transcript.

As discussed in the methodology section, the process of RNA extraction needs to be stringent and devoid of any contaminants that may affect the reaction. The Nanodrop spectrophotometry provides data on the quantity and the purity of the RNA used. The RNA Integrity Number (RIN) values indicate the quality of the RNA and indicate any degradation seen at all in the samples.

Nanodrop Spectrophotometry:

Nanodrop is one of the methods used to interpret RNA purity and quantity. The purity of RNA is critical in ensuring that the process of obtaining the mRNA has been devoid of any contaminants that could hinder the results. The nanodrop results of all the groups are

attached as Appendix 3, with emphasis on ratio of sample absorbance at 260/280 (ratio of ~2.0 is generally accepted as "pure" for RNA) and at 260/230 (ratio ranges commonly between1.8-2.2). All the samples had absorbance characteristics of pure quality RNA and were devoid of evidence of contaminants that could affect the efficiency of the qPCR reaction.

RNA Integrity Number Values:

Agilent Bioanalyzer is another methodology that helps in determining the quality of RNA by assigning a RIN (RNA Integrity Number) to it. It also helps determining to what extent the RNA in the sample is degraded, as RNA extracted from tissues exhibit varying degrees of degradation. The RIN values of all groups are highlighted in Appendix 3. As evident by the RNA values, there was degradation seen which has been shown due to degradation by post-mortem processes and inadequate sample handling or storage (445). The eventual reflection of RNA degradation is mainly on cycle threshold (Ct) values and a minor effect on PCR efficiency (391). Further reading on the low RIN values and Ct values can be accessed at

(*http://www.invitrogen.com/site/us/en/home/References/Ambion-Tech-Support/rtpcr-analysis/general-articles/rna-isolation-for-qrt-pcr.html*).

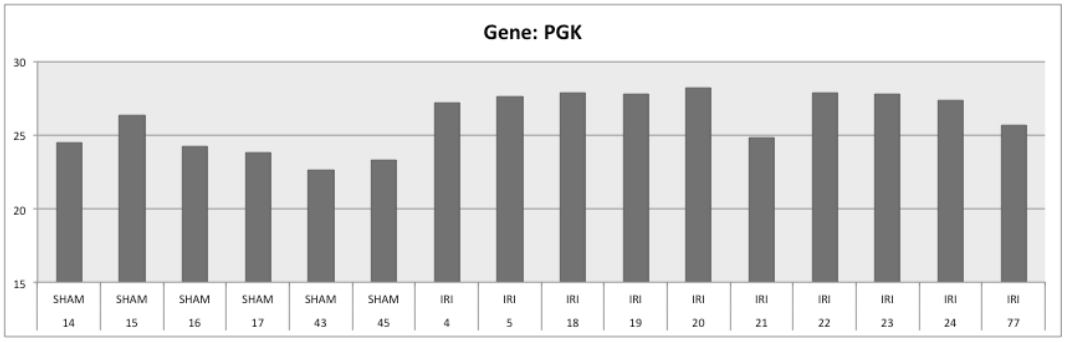
3.3.3.1. Normalising to a reference gene

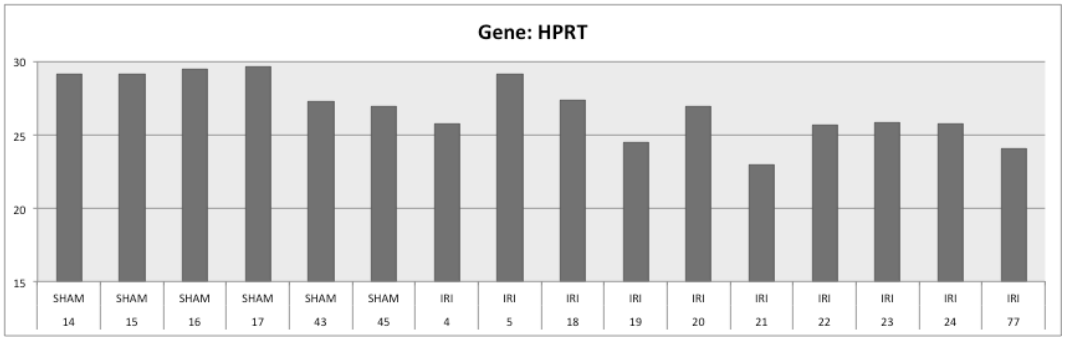
As disused in the methodology section, one of the techniques to assess changes in mRNA levels is to normalize RNA levels to an internal reference gene (367). The assumption is that reference gene is abundantly present in the tissue, and levels of expression don't vary

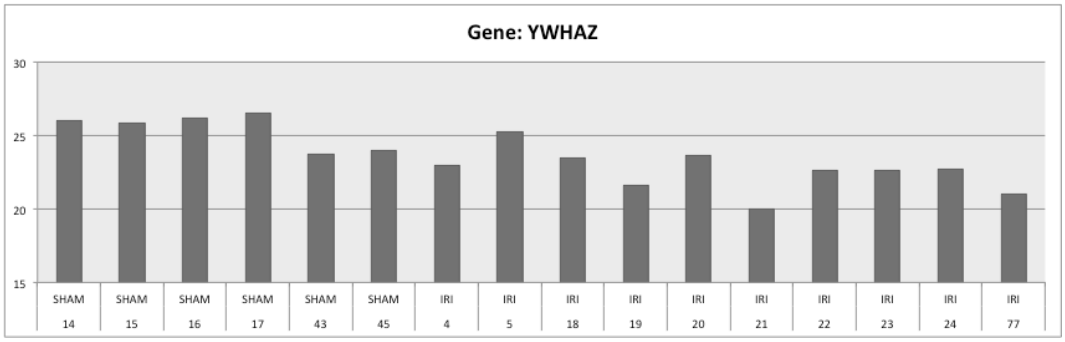
between the treated and untreated groups. In our study, we have also adopted this method as there are multiple groups with different treatments and differing RNA concentration, and normalising against reference gene would minimise variability. Hence, we have trialled seven genes that have been used in the literature in previous IRI studies and adopted with the lowest variation among the samples.

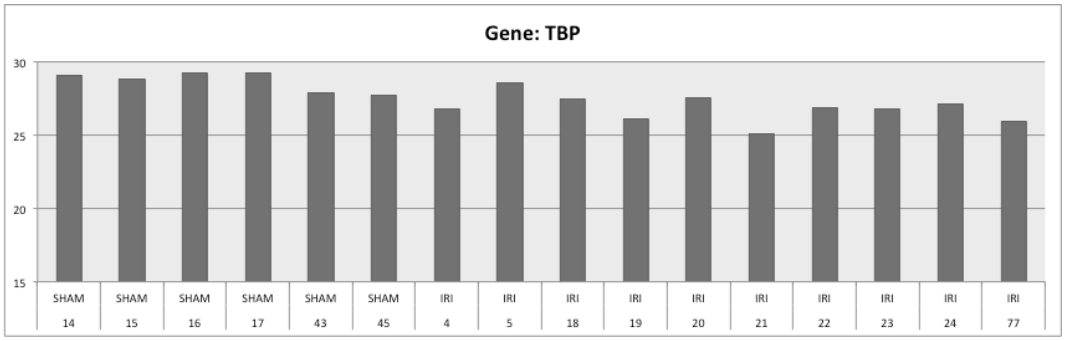
The CT values of the sham and the IR group of the seven genes are depicted in the graphs below.

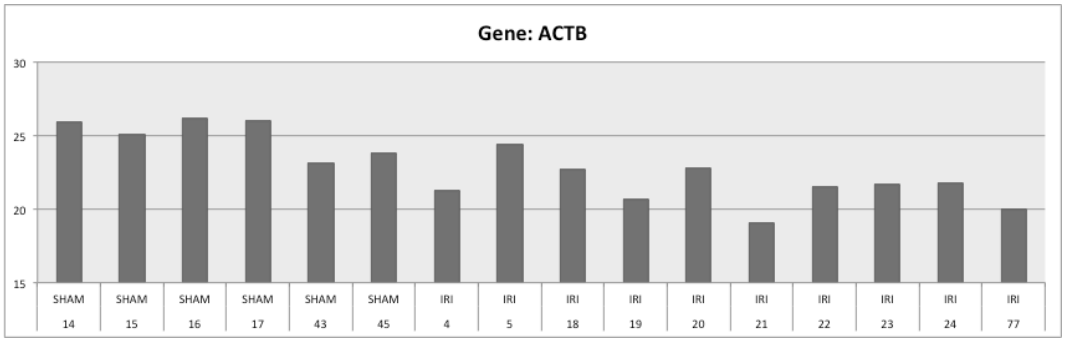
Figure 3.9: CT values of all individual genes across the individual rats

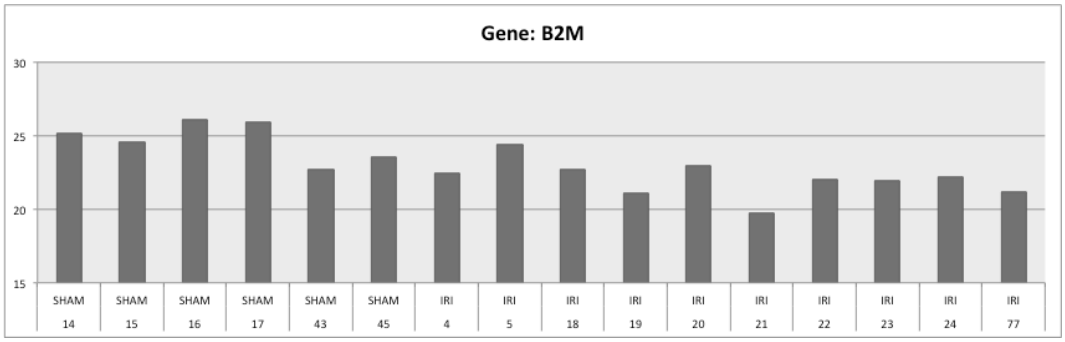





Gene: YWHAZ
30
25
20
15
SHAM 14
SHAM 15
SHAM 16
SHAM 17
SHAM 43
SHAM 45
IRI 4
IRI 5
IRI 18
IRI 19
IRI 20
IRI 21
IRI 22
IRI 23
IRI 24
IRI 77


Gene: TBP
30
25
20
15
SHAM 14
SHAM 15
SHAM 16
SHAM 17
SHAM 43
SHAM 45
IRI 4
IRI 5
IRI 18
IRI 19
IRI 20
IRI 21
IRI 22
IRI 23
IRI 24
IRI 77


Gene: ACTB
30
25
20
15
SHAM 14
SHAM 15
SHAM 16
SHAM 17
SHAM 43
SHAM 45
IRI 4
IRI 5
IRI 18
IRI 19
IRI 20
IRI 21
IRI 22
IRI 23
IRI 24
IRI 77


Gene: B2M
30
25
20
15
SHAM 14
SHAM 15
SHAM 16
SHAM 17
SHAM 43
SHAM 45
IRI 4
IRI 5
IRI 18
IRI 19
IRI 20
IRI 21
IRI 22
IRI 23
IRI 24
IRI 77

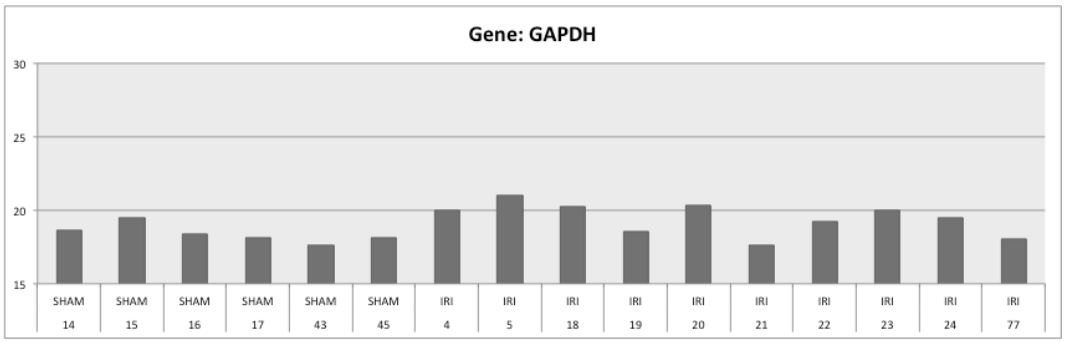


Figure 3.9: Ct (Cycle Threshold) values of seven genes that were evaluated by SYBR green fluorescence real-time reverse transcription PCR (RT-qPCR, using 7900 HT Fast Real-Time PCR System, Applied Biosystems. The x-axis depicts the Ct values while the Y-axis details the individual samples used. The individual Ct values of the sham (n=6) and the 40 minute-IR group (n=10) are depicted in the above figures.

Figure 3.10: Mean + Standard Deviation values of the genes

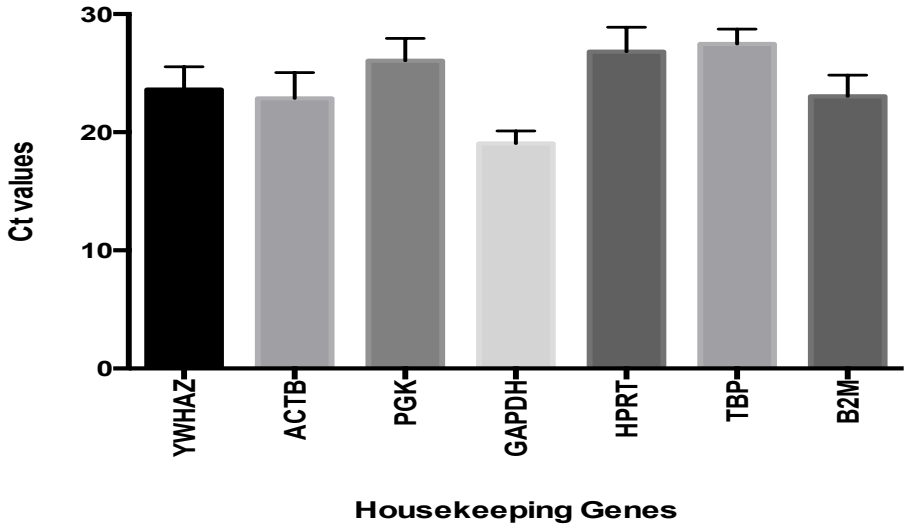


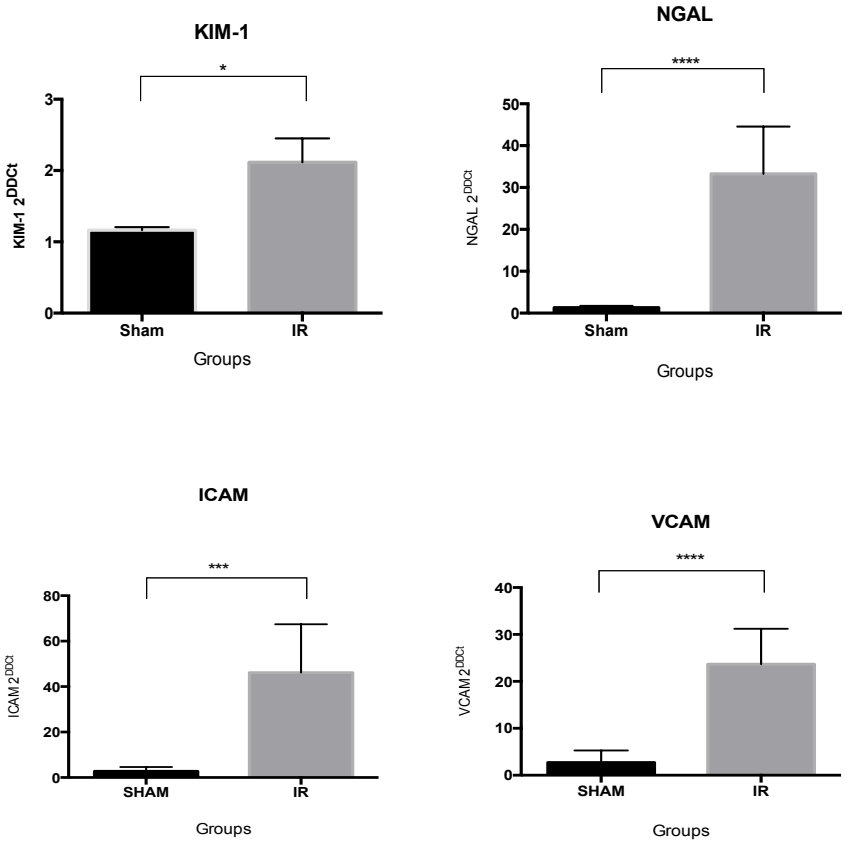
Figure 3.10: The seven reference (housekeeping) genes plotted as Mean + SD.

As evident by the graphs, the GAPDH demonstrated the narrowest SD (1.06) compared to YWAHZ (1.93), ACTB (2.19), PGK (1.90), HPRT (2.05), TBP (1.24) and B2M (1.78). Based on this, GAPDH was used as the reference gene for this study.

3.3.3.2. Genes of interest

Kidney injury markers (KIM-1 and NGAL), cellular adhesion molecules (ICAM-1, VCAM) and cytokine markers (TNF-alpha and IL18) were evaluated using RT-PCR. The results were analysed using Delta Delta Ct' (ΔΔCt) values as described by Livak et al(392) with GAPDH chosen as the reference gene for the relative quantification.

Figure 3.11: RT-qPCR analysis of six genes with expression normalized to GAPDH



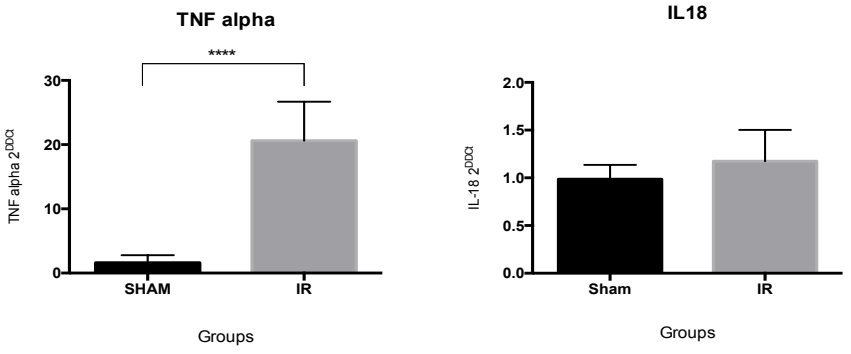


Figure 3.11: RT-qPCR analysis of six genes with expression normalized to GAPDH, and values are plotted as mean ± SEM. Unpaired –t tests used to test significance [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05 (*)]. Significant upregulation of both the kidney injury molecules- KIM-1 and NGAL are seen in the IR group compared to the sham. The adhesion molecules: ICAM-1 and VCAM-1 are also upregulated in the IR group compared to sham. Cytokines TNF alpha and IL-18 are also upregulated in the IR group compared to sham.

3.4. Discussion of Results

Histology:

We used a comprehensive 14-point scale to characterise injury encompassing tubular, glomerular, endothelial and tubulointerstitial damage. As shown in Figure 3.1, there was significant injury seen in the ischaemia group compared to the sham group and the baseline kidneys. Figure 3.2 clearly defines the changes seen in the IR group compared to the sham group with predominantly tubular, endothelial and tubule-interstitial injury contributing to the injury scoring compared to glomerular damage.

Immunohistochemistry:

Complement system:

Following activation of the complement system, activated **C3b** and C9 (marker for terminal membrane attack complex) act as effectors of the complement system in addition to C3a and C5a. As shown in figure 3.3 and 3.4, there was increase in C3b and C9 deposits in the renal interstitium and endothelium in the IR group compared to the controls; this is evidence of activation of the complement cascade. It was also interesting to note that we were able to stain for C3 in baseline (straight kill) kidneys indicating that there is residual level of C3 present that can be produced locally by epithelial and endothelial cells. However, there is still the need for an ischaemic insult to propagate the formation of C9 as there was no C9 seen in baseline kidneys and CD59 was still present. The increased deposits seen in the IR group may be a combination of local and systemic response to

ischaemia that eventually leads to formation of MAC and membrane injury. The phenomenon has been discussed in the literature as well (327, 328)

CD59 is a GPI-anchored membrane protein that acts as inhibitor acting at the terminal step, and prevents the assembly of the MAC by inhibiting the C5b-8 catalysed insertion of C9 into the lipid bilayer (217). CD55 and CD59 act synergistically to inhibit complement-mediated renal IRI, and its deficiency exaggerated renal IRI (393). So effectively, injury leads to disappearance of CD59 that was seen with IR. CD59 was seen in baseline kidneys as well, and the sham group also showed some reduction in deposits compared to the baseline kidneys, however this was not significant.

In summary, the complement system is activated with ischaemia leading to increased expression of C3 and C9, with disappearance of local regulator CD59. This phenomenon has been shown in literature (394), but most of the studies have shown it with CD59 as a target with reciprocal increase in c3 and c9, while we have shown that injury leads to loss of CD59 regulatory function with increase in MAC (C9) on cells.

Endothelium:

We used three endothelial markers in this study: Tissue factor (TF), Thrombomodulin (TM) and P-Selectin. Thrombomodulin is present normally in the endothelial cell possessing anti-inflammatory and anti-coagulant functions with the breakdown product in serum used to evaluate endothelial injury. Tissue factor on the other hand is released on injury from the sub-endothelial matrix, and promotes coagulation, thus accentuating the renal injury. Both these markers have been used to evaluate endothelial injury in isolation, but never in conjunction (262, 395). As evident in graphs 3.5 and 3.6, the IR group had increase in TF

deposits with contralateral decrease in TM deposits. P-Selectin is present, readily available within endothelial cells, in cytoplasmic deposits called Weibel-Palade bodies and is an adhesion molecule that promotes leukocyte recruitment and is increased in injury (84). Similarly, there was increased deposition of P-Selectin in the IR group compared to sham. In summary, we have evaluated the endothelium using three markers and have all shown consistent injury with ischaemia, and more importantly, all three being used and reported together is probably the first time in literature.

Leukocytes:

Cellular markers for lymphocytes (CD3, CD4 and CD8), macrophages (CD68) and neutrophils (CD 15) were used to assess inflammatory infiltrate in this study. All these cells were significantly increased in the ischaemia group indicating the inflammatory response that ensues following IR. As shown in graphs 3.8 and 3.8, there was also slight raise in CD68 and CD15 cells in the sham group compared to the baseline kidneys, indicating the laparotomy an recovery phase also triggers inflammatory pathways. The individual cells have been shown to be present in the post-ischaemic kidney in multiple studies (17, 112), and we have evaluated them together in this study.

RT- qPCR:

The most widely used and reliable technique to analyse variation in mRNA levels is to normalize RNA levels to an internal reference gene (370). The first step in this was to establish a reliable reference gene, and as evident by graphs 3.9 and 3.10, the GAPDH demonstrated the narrowest dispersion (1.06) compared to other reference genes. Hence, the genes of interest were normalised to GAPDH.

Kidney injury molecules KIM-1 and NGAL were upregulated in the IR group indicating injury, and correlating with the histology score and literature (396). Adhesion molecules: ICAM-1 and VCAM-1 were also upregulated keeping in trend with literature evidence, and reinforcing the immunohistochemistry findings of endothelial damage and activation (99). The cytokine TNF- alpha was also significantly raised in the IR group indicating the inflammatory response secondary to endothelial damage and leukocyte recruitment. IL-18 showed a trend to upregulation in the IR group, though this did not reach statistical significance.

Summary of Results:

In conclusion, the IR group compared to sham group has demonstrated:

- Significant endothelial injury
- Significant leukocyte infiltration
- Complement activation and MAC mediated cellular injury, associated with loss of local complement regulator protein CD59
- Increase in mRNA levels for kidney injury molecules, cytokines and adhesion molecules
- Low morbidity and mortality (discussed in the next chapter)

The results of this chapter will serve as the foundation for the next two chapters to assess changes with interventional agents.

CHAPTER IV:

RESULTS - ANTI-THYMOCYTE GLOBULIN AND RENAL ISCHAEMIA - REPERFUSION INJURY

4.1. Introduction

Anti-Thymocyte Globulin (ATG) is polyclonal antibody that binds primarily to peripheral blood lymphocytes, and displays differential specificity to antigens present on a variety of cells involved in immune responses, apoptosis, signal transduction, cell adhesion and trafficking (249, 296, 297). The predominant effect of ATG is through T-lymphocyte depletion, along with reduction in B-lymphocyte and Natural Killer cell counts (304).

With such a wide mode of action, ATG should potentially have effects on the cells mediating renal ischaemia-reperfusion injury. There have been only a handful studies looking into the effects of ATG on renal IRI, and they have had equivocal results. In a mouse model evaluating the effects of mouse anti-thymocyte globulin (mATG) on warm kidney IRI (models: 30 min bilateral, 60 min bilateral and 45 min unilateral), though there was consistent depletion of T-cells in blood, ATG did not confer functional or structural protection from the IR injury (311). In a rat transplant model, ATG given to recipient rats 2 hours before transplantation but not at the 'time of reperfusion' (syngenic kidneys) showed reduced tubular injury and better graft function (312). Similarly, ATG treated Wistar donor kidneys when transplanted into bilaterally nephrectomised recipient rats, the transplanted kidney analysed at 24 hours showed protection from renal IRI (313).

There has been considerable interest in therapeutic manipulation of leucocytes to improve outcome following renal IRI, and my results in Chapter 3 underscore the substantial leucocyte influx seen following renal IRI. However, the evidence from the previous intervention studies with ATG described above is equivocal.

Hence, the main aims of this chapter are to evaluate the overall effects of r-ATG on IRI with specific objective being

- Evaluate the effects of r-ATG on leukocyte infiltration
- Evaluate the effects of ATG on endothelial damage
- Evaluate the effects of ATG on mRNA levels of genes of interest used as sentinels for Acute Kidney Injury

4.2. Methods

ATG used in our study was a 'rabbit anti-rat thymocyte immonuglobulin (rATG)' produced analogously to commercial ATG (Thymoglobulin®), kindly provided by Genzyme Corporation (Cambridge, MA, USA). Briefly, rabbits were immunized with a mixture of thymocytes of four strains of rats (Sprague Dawley,F344 Fisher, Lewis and Long Evans). Thymocyte suspensions were prepared using thymi from several donor rats. Fifty New Zealand White rabbits were immunized twice, two weeks apart, and terminally bled two weeks after the last immunization. Total rabbit IgG obtained from the serum was pooled and purified similarly to Thymoglobulin® Genzyme, Cambridge, MA, USA). Control rabbit IgG was purified from whole normal rabbit serum (312). The dose used was recommended by the company, and has been used in other studies as well (313). The four groups for this study were:

Groups:

- *IR group* (n=10): 40 minutes of left unilateral renal ischaemia followed by 48 hours of reperfusion

- *ATG group* (n=8) received rat-specific ATG IV (10mg/kg BW) prior to the laparotomy followed by 40 minutes of left unilateral renal ischaemia, followed by 48 hours of reperfusion

- *ATG Isotype group* (n=8) received (10mg/kg BW) of a non-specific isotype immunoglobulin from the same laboratory prior to laparotomy, followed by 40 minutes of left unilateral renal ischaemia followed by 48 hours of reperfusion

- Sham group (n=6)

Analysis:

- Blood samples taken at T0 (prior to ATG administration) and at 48 hours (T1) analysed for CD3 blood count using flow cytometry to assess ATG efficacy
- Kidneys retrieved at 48 hours analysed for

  Histology

  Immunohistochemistry

  Rt-qPCR studies

4.3. Results

4.3.1. CD3 Count analysis

ATG effect can be measured by blood CD3 count analysis, and this method is used in clinical transplantation as well. Initially, we took blood samples at baseline, 24 hours and at 48 hours. As the CD3 count was depleted still at 48 hours, adhered to 48-hour blood collection and thus preventing the need to re-anaesthetise the rat at 24 hours for tail tip.

Figure 4.1: CD3 count analysis from ATG and Isotype groups

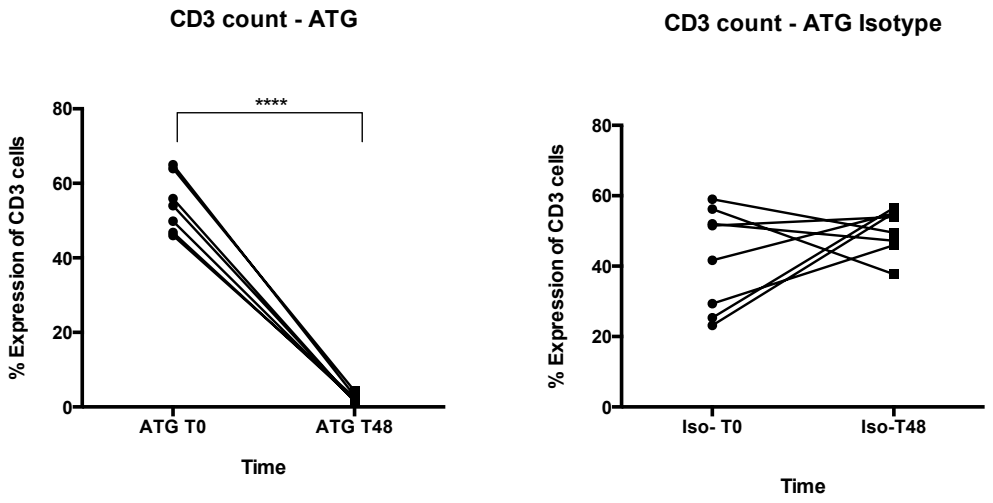


Figure 4.1: Graphs depicting the CD3 count analysis from the ATG group (n=7) and the isotype group (n=8). X-axis denotes the percentage expression of CD3 cells, and the Y-axis denotes the two time lines of blood sampling for CD3 count analysis: TO and t48 hours. Paired-t test shows significant reduction in the CD3 count in the ATG group, while no difference was seen in the Isoype group.

* *There are only seven ATG rat serum represented, as we had initial technical issues with the first sample; hence for the first rat we used correlations with IHC CD3 count, which proved reliable*

As evident by the graphs, the ATG group showed significant depletion of CD3 cells at 48 hours compared to the Isotype group implying that in this Lewis rat species, the ATG dose was effective, and effect lasted until 48 hours. The isotype group had a mixed response, but no depletion seen

4.3.2. Histology

A comprehensive histopathological scoring scale was used to evaluate renal injury.

Figure 4.2: ATG studies – Histology scoring of renal cortex sections

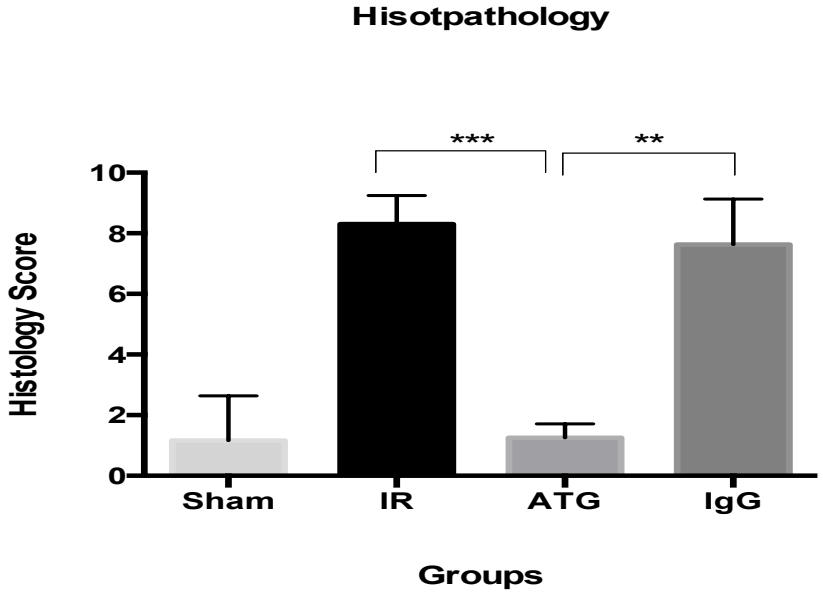


Figure 4.2: Scoring of renal cortex sections from sham (n=6), 40-minute unilateral IRI (n=10), ATG (n=8) and ATG isotype (IgG; n=8) in rats at 48 hours after reperfusion. Histology scores are plotted as Mean +/- SD, and Kruskal-Wallis tests used to test significance between the IR, ATG and IgG groups. If significance shown between groups, post hoc analysis (Dunn's test) was used to locate the significance. [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05 (*)]

Figure 4.3: ATG studies – Renal cortex H&E sections

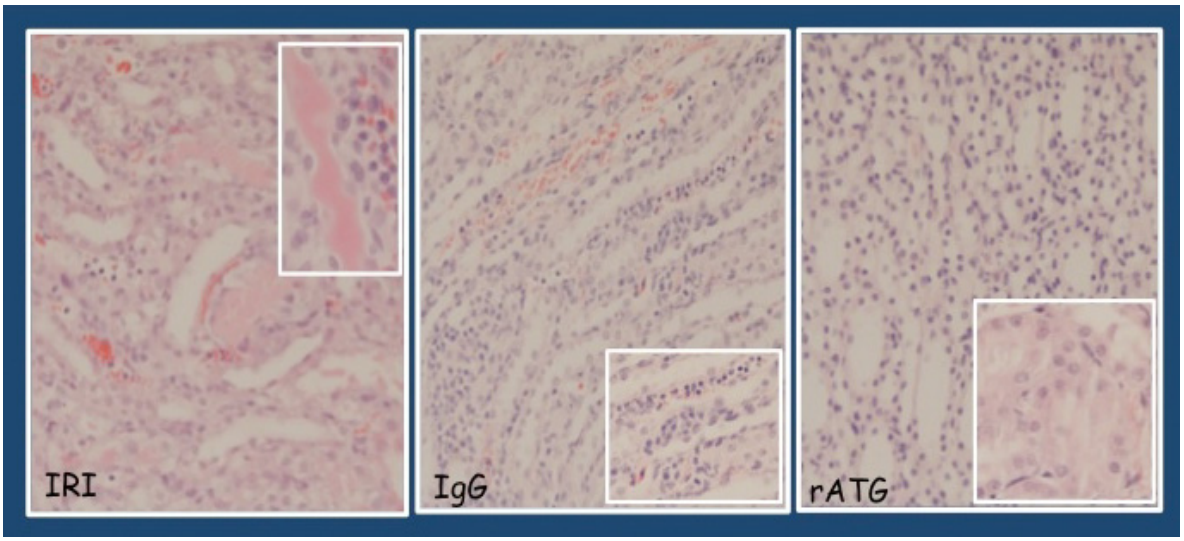


Figure 4.3: Rat Renal cortex H&E (x200) paraffin sections from IRI, IgG (ATG Isotype) and ATG, following 40 minutes of ischaemia and 48 hours after reperfusion. The slides were assessed for tubular, endothelial, glomerular and tubulo-interstitial cell damage. Magnifications as inserts.

IRI: *Untreated control animal showing venous thrombosis, multifocal necrosis and severe neutrophil infiltration (H&E). Insert: Severe neutrophil infiltration and partial defacement of vascular endothelium. Overall score = 9 (tubular =3, endothelial=2, glomerular=1 and tubulointerstitial=3)* ***IgG:*** *Rats pre-treated with isotype IgG (control) showing vascular congestion, haemorrhage and moderate neutrophil infiltration, more evident in the insert. Overall score=7 (tubular =2, endothelial=2, glomerular=1 and tubulointerstitial=2)*

rATG: *Rats pre-treated with ATG. The slide depicts preservation of anatomical integrity of the kidney with no endothelial damage and absence of neutrophil infiltration, which is obvious in the magnified insert. Overall score=2 (tubular =1, endothelial=0, glomerular=0 and tubulointerstitial=1).*

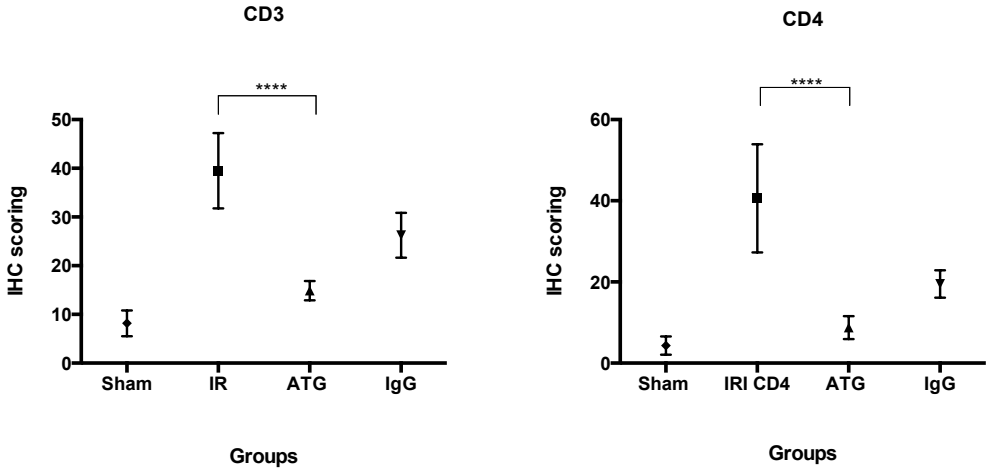
4.3.3. Immunohistochemistry

IHC was used to test cellular markers encompassing the leukocytes, endothelium and complement stainings. The results are shown in summative graphs, with representative IHC pictures showing the changes.

4.3.3.1. Leukocytes

Cellular markers for lymphocytes (CD3, CD4 and CD8), macrophages (CD68) and neutrophils (CD 15) were used to assess inflammatory infiltrates.

Figure 4.4: ATG studies – IHC scoring of cellular markers for inflammation mediators



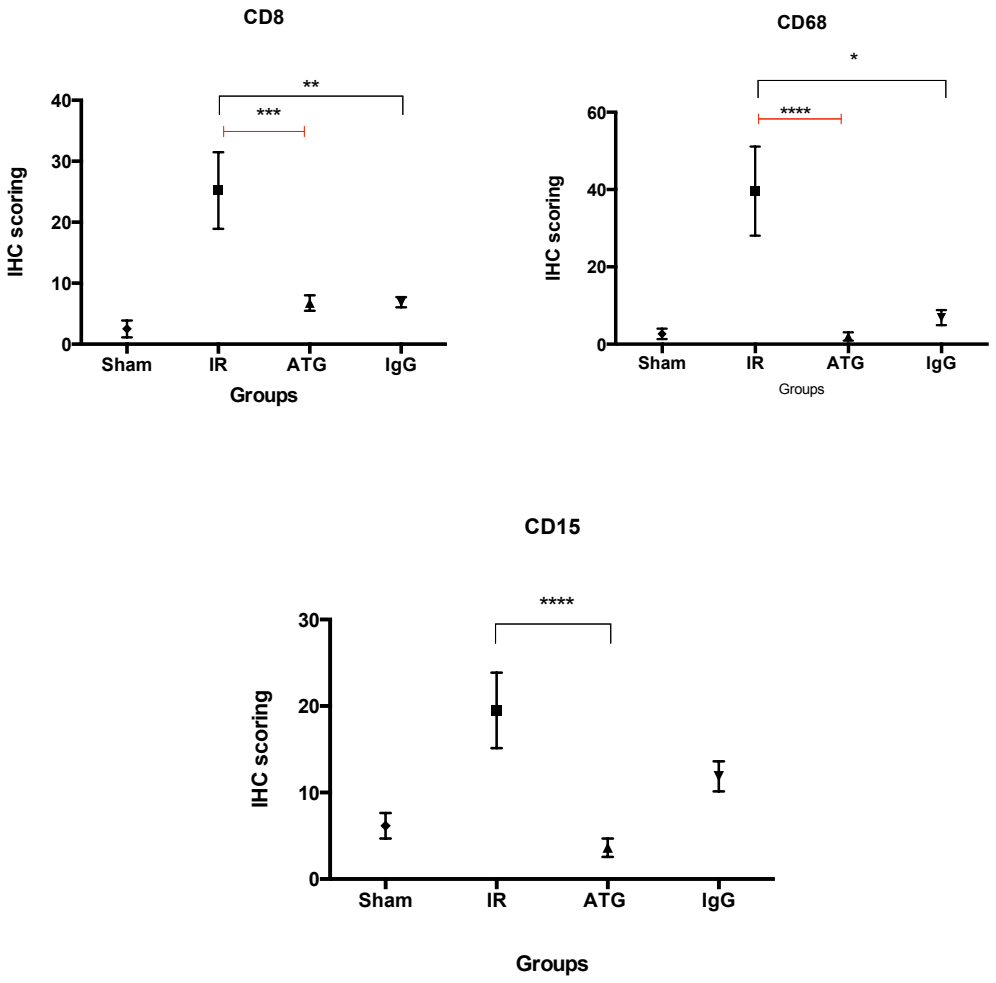


Figure 4.4: IHC scoring of renal cortex sections from sham (n=6), 40-minute unilateral IRI (n=10), ATG (n=8) and ATG isotype (IgG; n=8) in rats at 48 hours after reperfusion. Cellular markers for inflammation were stained as per protocol, and were counted: n/ avg 5x HPF (high power field). IHC scores are plotted as Mean +/- SD, and Kruskal-Wallis tests used to test significance between the IR, ATG and IgG groups. If significance shown between groups, post hoc analysis (Dunn's test) was used to locate the significance. [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05 (*)]

Figure 4.5: ATG studies – Renal cortex IHC sections of cellular markers for inflammation mediators

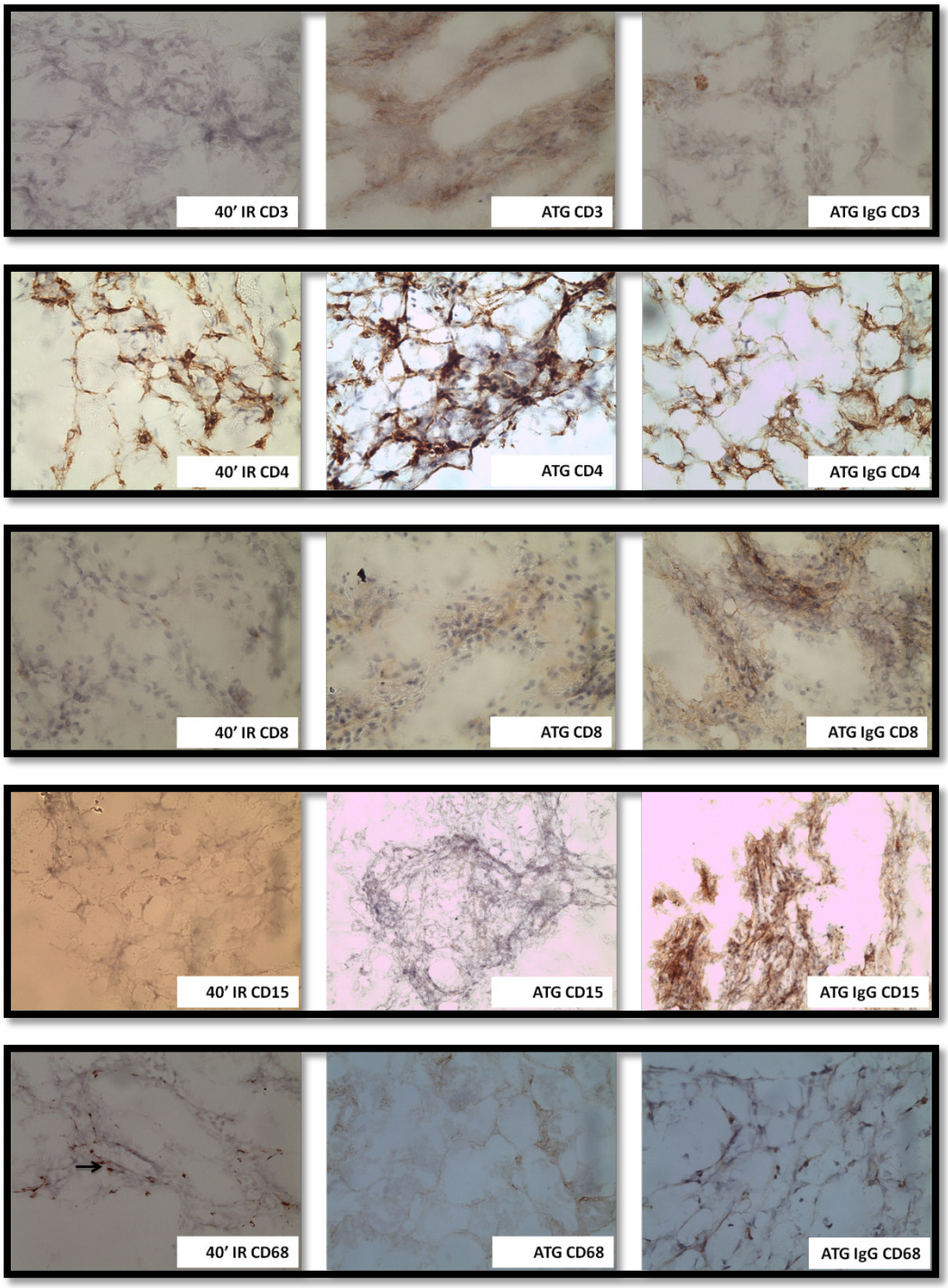


Figure 4.5: Representative rat renal cortex (x200) IHC sections from 40-minute unilateral IRI, ATG and ATG isotype (IgG) groups at 48 hours after reperfusion. Cellular markers for inflammation were stained as per protocol, and were counted: n/ avg 5x HPF (high power field). Row 1 and Row 2 depict CD3 and CD4 staining in all the three groups, with decrease in the ATG group compared to IR and isotype group. Row 3 and 4 show decreased CD8 and CD68 deposits in both the ATG and Isotype group compared to IR group. Row 5 show decreased neutrophil (CD15) infiltration in the ATG group compared to both the Isotype and IR group.

4.3.3.2. Endothelium

Thrombomodulin and Tissue Factor were used as markers to assess endothelial damage, and these markers have not been used in ATG studies before. Results depicted as summative graphs and representative slides.

Figure 4.6: ATG studies – Summative IHC scoring of endothelial markers

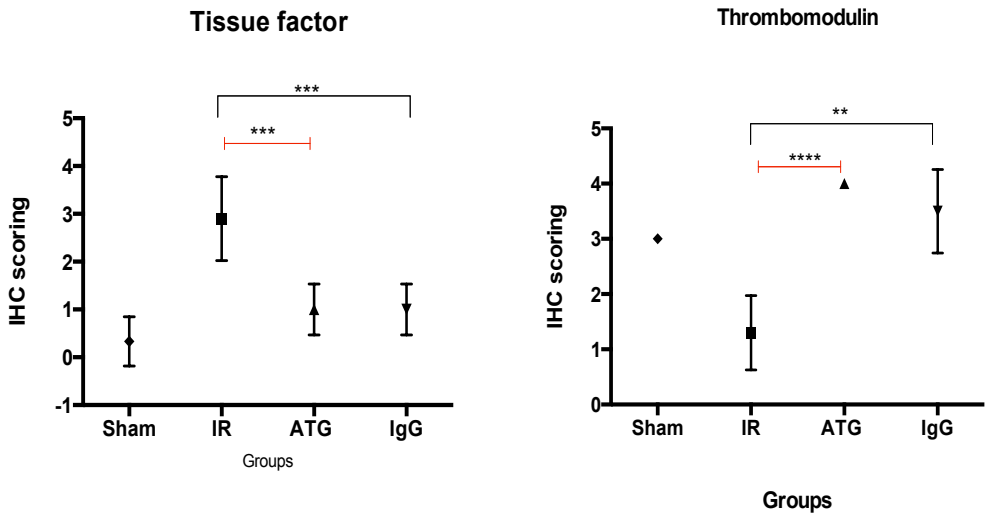


Figure 4.6: IHC scoring of renal cortex sections from 40-minute unilateral IRI (n=10), ATG (n=8) and ATG isotype (IgG; n=8) in rats at 48 hours after reperfusion. Endothelial markers Tissue factor and Thrombomodulin were stained as per protocol and scored 0-4 (vascular and stromal staining). IHC scores are plotted as Mean +/- SD, and Kruskal-Wallis tests used to test significance between the IR, ATG and IgG groups. If significance shown between groups, post hoc analysis (Dunn's test) was used to locate the significance.

Figure 4.7: ATG studies – Renal cortex IHC sections of endothelial markers

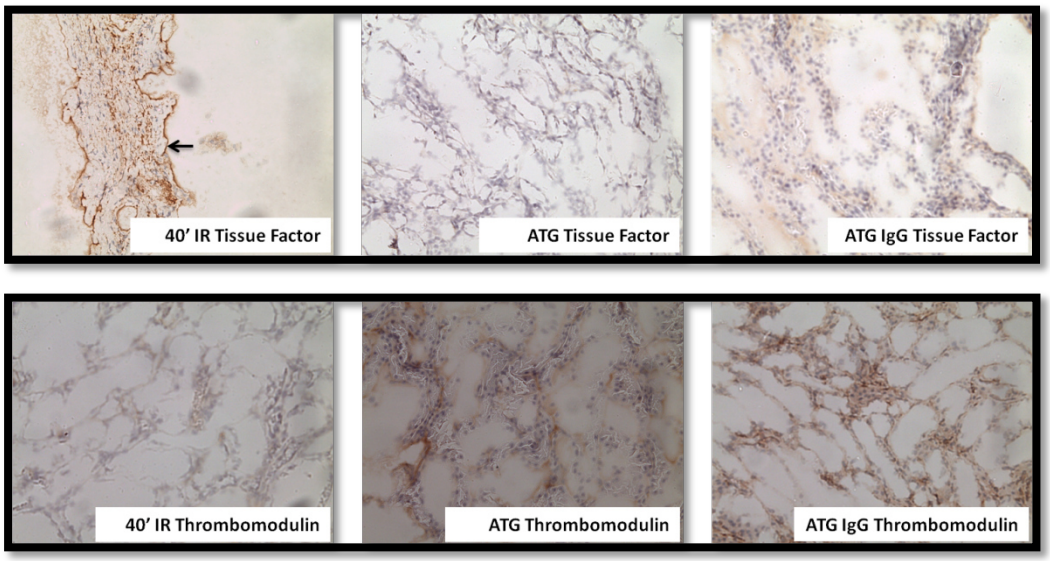


Figure 4.7: Representative rat renal cortex (x200) IHC sections from 40-minute unilateral IRI, ATG and ATG isotype (IgG) groups at 48 hours after reperfusion. Endothelial markers: Tissue factor and Thrombomodulin were stained as per protocol and scored 0-4 (vascular and stromal staining). Row1 shows staining for tissue factor, which is increased in IR group (compared to sham, slide not shown) with decreased deposits in both ATG and IgG groups. Row 2 shows thrombomodulin staining that is present normally (sham), but is decreased with injury (IR) and protected in both ATG and IgG groups.

4.3.3.3. Complement C3

As discussed in the first chapter, Complement C3 deposits are increased following injury. We evaluated activated C3b deposits in this study on the hypothesis that decreased inflammatory infiltrate effect may have effect on C3 deposits.

Figure 4.8: ATG studies – Summative IHC scoring of complement markers

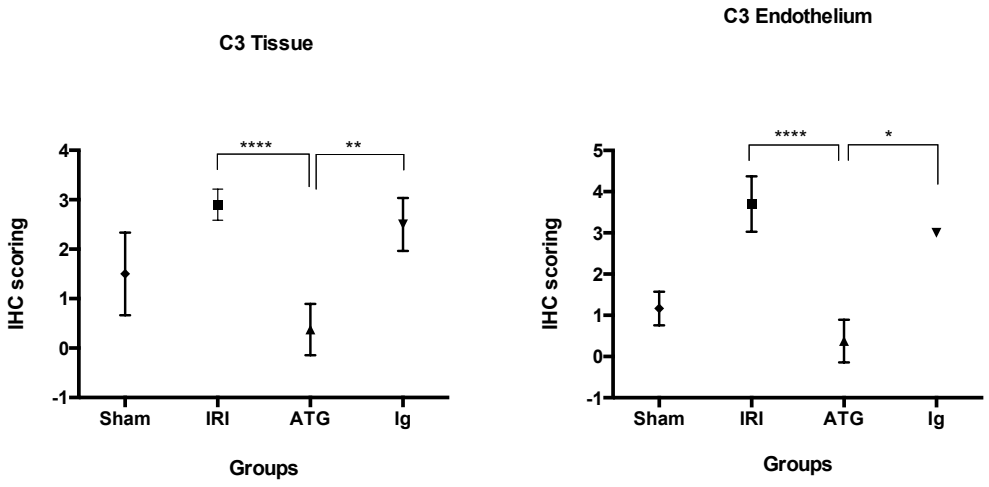


Figure 4.8: IHC scoring of renal cortex sections from 40-minute unilateral IRI (n=10), ATG (n=8) and ATG isotype (IgG; n=8) in rats at 48 hours after reperfusion. Complement marker C3 was stained as per protocol and scored 0-4 (vascular and stromal staining). The graph depicts the IHC scores plotted as Mean +/- SD,and Kruskal-Wallis tests used to test significance between the IR, ATG and IgG groups. If significance shown between groups, post hoc analysis (Dunn's test) was used to locate the significance [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05 (*)]

Figure 4.9: ATG studies - Renal cortex IHC sections of complement markers

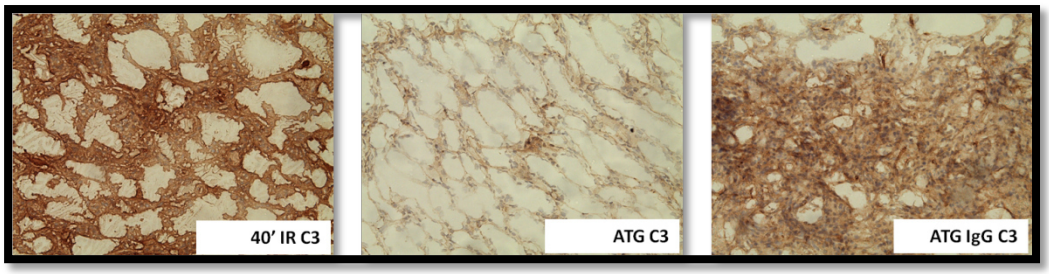


Figure 4.9: Representative rat renal cortex (x200) IHC sections 40-minute unilateral IRI, ATG and ATG isotype (IgG) groups. Complement marker C3 was stained as per protocol and scored 0-4 (vascular and stromal staining) based on intensity of staining. The slides show increased deposits of C3 in renal interstitium in the IR and the IgG group compared to ATG treated kidneys.

4.3.4. RT-qPCR

Kidney injury markers (KIM-1 and NGAL), cellular adhesion molecules (ICAM-1 and VCAM) and cytokine markers (TNF-alpha and IL18) were evaluated using RT-PCR. The results were analysed using Delta Delta Ct' (ΔΔCt) method) with GAPDH chosen as the reference gene for the relative quantification.

Figure 4.10: ATG studies – RT-qPCR analysis of six genes expression normalized to GAPDH

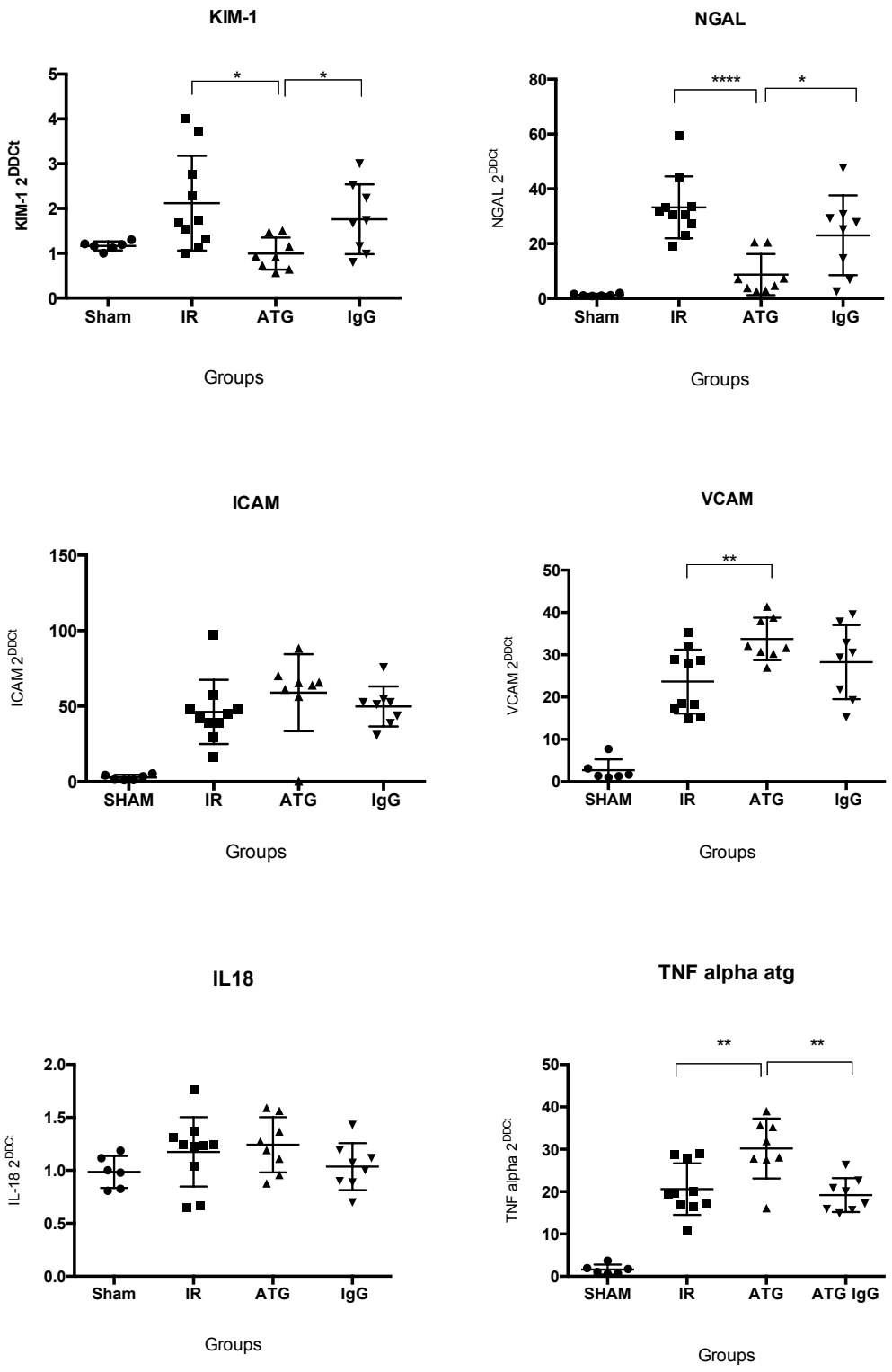


Figure 4.9: RT-qPCR analysis of six genes with expression normalized to GAPDH (reference gene), and values are plotted as mean ± SEM. One way-Anova used to test significance between the IR, ATG and IgG groups, and if significance seen, post hoc (Newman-Keuls test) analysis carried out to locate the significance. [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05

4.3.5. Discussion of Results

CD3 blood count:

As evident in Graph 4.1, ATG had a profound effect on decreasing the CD3 count at 48 hours compared to the baseline. The ATG Isotype had a mixed response with majority showing increase in CD3 count and some showing decrease; however it did not have any significant effect on the CD3 count. CD3 count is used to measure the effect of ATG in clinical studies (358), and our methodology correlated well with established literature. ATG has a long-half life on pharmacokinetics assays in human study, and this may explain the prolonged effect of ATG seen (397).

Histology:

ATG offered significant structural protection compared to the IR and the Isotype group. The H&E staining demonstrated endothelial protection and reduced neutrophil infiltration. When analysing the histopathological scores, it was evident that tubular and endothelial protection was prominent in the ATG treated group compared to the Isotype and the IR group.

Immunohistochemistry:

Leukocytes:

Effect of ATG on leukocytes is predominantly via lymphocyte depletion and this was evident on our study where there was significant depletion of CD3, CD4 and CD8 lymphocyte cells compared to the IR group. ATG also had significant depletion of CD68 (macrophages) and CD15 (neutrophils) cells, and this finding contributes to the finding that ATG has an effect on wide array of leukocytes (313). ATG effects on leukocytes are well defined in clinical practice especially in transplantation and haematological malignancies (249). Previous studies have demonstrated lymphocyte depletion, with one study showing decrease in macrophage count, but we have shown a wider array of the leukocytes being affected by ATG in renal IRI. The Isotype also had some effect on the CD8 and CD68 cells (decreased count), but this did not lead to any effect on the histological scoring. The effect of isotype has been discussed under the discussion chapter.

Endothelium:

There have been no previous studies looking into the effect of ATG on the endothelium. A study looking at the effects of ATG and other immunosuppressants on microvascular thrombus formation in normal and post ischemic tissue in mice, reported a thrombogenic effect of ATG with increase in serum concentrations of P-Selectin and VCAM (308). However, the aims of this study were different, and the model was different. Most of the studies have evaluated the adhesion molecules (306) but we are the first to investigate effect of ATG and its Isotype on Tissue factor and Thrombomodulin. ATG treated rats showed reduced TF expression and preserved TM expression compared to the IR group.

Interestingly, even the Isotype control showed endothelial protection. These findings are new in literature. From this we can hypothesise, endothelial protection alone would not be sufficient for attenuating IRI, and augments our hypothesis that IRI is a complex interplay between multiple mediators and affecting just one mediator may not offer desired results. The ATG also had significant effect of leukocyte infiltrates and this may partly explain the effects seen on injury scores by ATG.

Complement C3:

We evaluated C3 deposits in the endothelium and interstitium. We hypothesised that considering ATG has a profound effect on leukocyte infiltration and on endothelium, there may be decreased effect on complement activation leading to reduced C3 production locally, and systematically. As evident by figure 4.4, there were decreased complement deposits in both the interstitium and the endothelium in the ATG treated rats compared to the Isotype or IR group.

RT-qPCR:

ATG significantly down regulated both the kidney injury molecules: KIM-1 and NGAL compared to the IR and Isotype group, indicating renal protection. This correlates with the histological findings. There was no differences seen in the ICAM expression between the groups, and other authors have reported similar effect (313). The VCAM expression was increased in the ATG group, and this finding was also observed in a mice skin model where ATG was used as an interventional agent (308), while there was no effect of ATG on VCAM on human umbilical endothelial cells (309). TNF-alpha expression was significantly increased in the ATG group compared to the isotype or the IR group. This

phenomenon has been seen in humans receiving ATG treatment where increase in serum TNF-alpha levels were observed (398). Other groups have shown increased TNF-alpha levels in kidney with ATG treatment in brain dead rat model at 2 hours (313). Decreased leukocyte count should probably lead to decreased TNF-alpha count as seen with ATG on other IRI models (364, 399), however it's also been shown that TNF- alpha activation can happen through many mechanisms. ATG is known to cause profound cytokine release following administration, and this may have contributed to the effect seen in our study. No differences were seen in IL-18 expression between the groups, and this may be due to the degraded RNA samples, or frankly, no effect of ATG on IL-18 levels.

Summary of Results:

Our study has clearly demonstrated that ATG attenuates IRI contradicting the previous report from Jang et.al who showed no structural protection from renal IRI (models: 30 min bilateral, 60 min bilateral and 45 min unilateral) (311). Their model was similar to ours with differing grades of severity, and in spite of lymphocyte depletion, the kidneys were not protected. The main difference between their study and ours was the administration of ATG. In out study, ATG was given IV just prior to the laparotomy, while they gave ATG intraperitoneally either 2-3 days before, or just after reperfusion. Timing of ATG may also be crucial, as we have adopted a model close to clinical practice. In a transplant model, ATG given 2 hours pre-transplant to recipients showed protection of the kidney, but no protection was observed when ATG was given just prior to reperfusion (312).

However, we have to be careful in interpreting results from different species and different models. Most of the ATG effects on leukocytes and adhesion molecules were seen in

primate models. The effect of ATG on rat renal transplant models are also difficult to gauge as there has been only one article (313) who administered ATG to donor rats, and transplanted their kidneys into recipients with a 24-hour observation. The effects on leukocytes and lymphocytes seem to be consistent across the board, however none of these studies evaluated the endothelial response, which we have incorporated.

In conclusion, we can summarise the following findings from our study

- ATG offers significant protection from renal IRI in this warm model of unilateral IRI with ATG administered just prior to laparotomy
- ATG causes significant decrease in leukocyte infiltrates
- ATG and its Isotype showed significant endothelial protection which is new in literature, however the Isotype effect was not translated into renal protection
- ATG decreases inflammatory response and reduces Complement C3 deposits
- ATG down regulated expression of kidney injury molecules

CHAPTER V:

COMPLEMENT SYSTEM MODULATION: sCR1 AND ANTI-MASP2

This chapter is discussed in two sections:

- sCR1 and its effects on renal IRI
- Anit-MASP2 antibody and its effects on renal IRI

5.1. Soluble Complement Receptor 1(sCR1)

5.1.1. Introduction

CD35/Complement Receptor-1 (CR1) is a transmembrane glycoprotein that facilitates the decay of C3/C5 convertase in both the classical and alternate pathways, and acts as a co-factor for factor I in the degradation of C3b and C4b(214). sCR1 is a complement regulator that has been shown to inhibit the activation of classical and alternative pathways in vitro and suppressed complement activation in vivo (257). sCR1 has been shown to attenuate IR injuries in myocardium, liver and intestinal models, however, reports of its effect on renal IRI are sparse with some beneficial effects seen in a renal transplant model with a longer period of observation and repeated doses of sCR1 (327).

The aims of the this chapter were to evaluate the overall effects of sCR1 on renal IRI, with specific objectives being:

- Evaluate the effects of sCR1 on serum complement activity, and on the activated products of the complement system: C3b and CD59
- Evaluate the effects of sCR1 on leukocytes
- Evaluate the effects of sCR1 on endothelium

5.1.2. Methods

The sCR1 utilized in this study was a kind gift of SmithKline Beecham, Pharmaceuticals, Harlow, Essex, UK and has been used by previous investigators (320, 327). The dose used was similar as previous publications on sCR1 (5.3mg/ml): 25 mg/kg body weight.

The three groups for this study are

- *IR group* (n=10): 40 minutes of left unilateral ischaemia followed by 48 hours of reperfusion
- *sCR1 group* (n=8) received sCR1(25mg/kg BW) administered intravenously 20-minutes prior to the laparotomy, followed by 40 minutes of left unilateral ischaemia and 48 hours of reperfusion
- Sham group (n=6)

Analysis:

- CH50 Complement assay: Blood samples: TO (prior to laparotomy), T1 (prior to reperfusion), T2 (24 hours) and T3 (48 hours)
- Kidneys harvested at 48 hours were analysed for histological changes. Immunohistochemistry (IHC) and real-time PCR were used to assess complement deposits and cellular infiltration

5.1.3. Results

5.1.3.1. Complement activity and CH50 analysis

Complement haemolytic activity (CH50) is a functional test of the complement cascade, and is a standard test used to determine the effect of sCR1 on complement activity in serum (320, 327). CH50 is based on the lysis of sensitised sheep erythrocytes in the presence of Ca++ and Mg++, and the methods have been discussed earlier.

Figure 5.1: SCR1 Studies – Complement activity in rats at 1/20 dilution

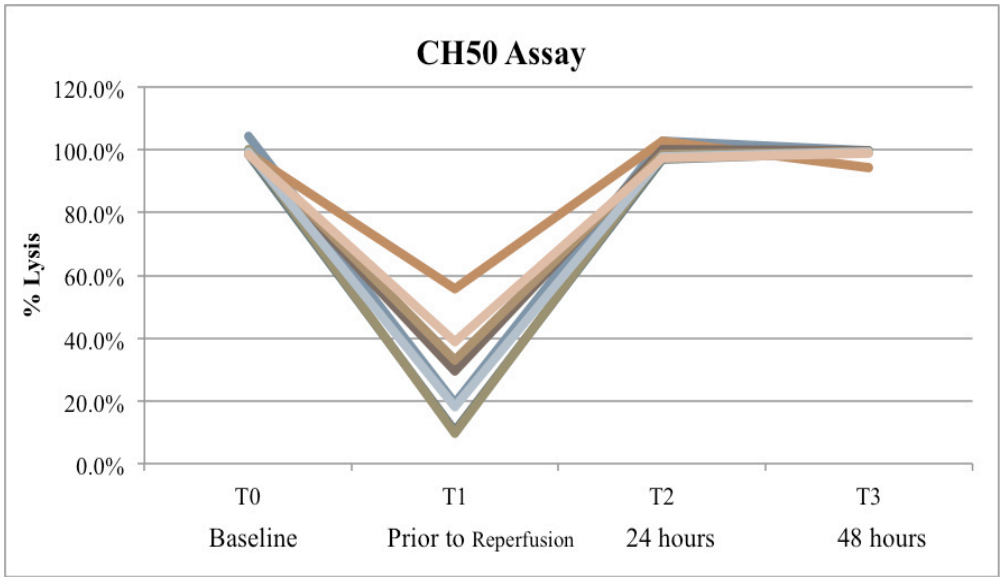


Figure 5.1: Graph depicting the complement activity of (n=8) rats at 1/20 dilution. The Y-axis represents the time scales of blood being taken over a 48-hour period. The X-axis denotes the percentage lysis of sheep erythrocytes read on the optical reader.

The graph clearly shows that at T0 (prior to laparotomy), the complement system is competent, leading to 100% lysis of sheep erythrocytes. Following sCR1, the T1 sample shows ablation of the complement system with decreased haemolysis of the sheep erythrocytes. By 24 hours, the complement system is completely back to baseline activity,

implying that sCR1 is efficacious in ablating the complement system for a short period of time.

5.1.3.2. Histology

A comprehensive histopathological scoring scale was used to evaluate renal injury. The results are shown as summative graphs for the groups, with representative H&E pictures showing the pathological changes.

Figure 5.2: SCR1 studies – Histology scoring of renal cortex sections

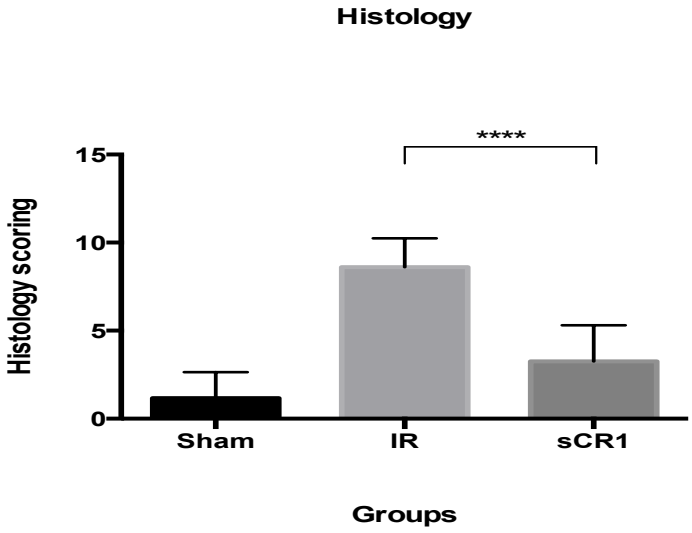


Figure 5.2: Scoring of renal cortex sections from sham (n=6), 40-minute unilateral IR (n=10) and sCR1 treated (n=8) rats at 48 hours after reperfusion, stained with H&E and assessed using a comprehensive scoring system comprising of tubular, endothelial, glomerular and tubulo-interstitial cell damage. Histology scores are plotted as Mean +/- SD, and Mann Whitney tests used to test significance [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05 (*)]

Figure 5.3: SCR1 studies – Renal cortex H&E sections

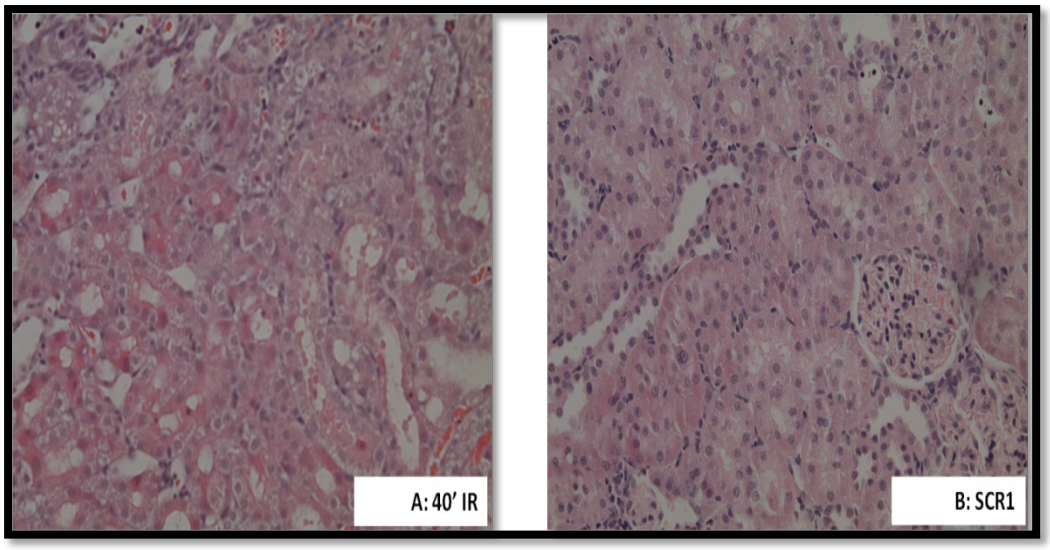


Figure 5.3: Representative Rat Renal cortex H&E (x200) paraffin sections from IR and sCR1group following 40 minutes of ischaemia and 48 hours after reperfusion. The slides were assessed for tubular, endothelial, glomerular and tubulo-interstitial cell damage.

IRI: Untreated control animal showing venous thrombosis, multifocal necrosis and severe neutrophil infiltration (H&E). Overall score = 9 (tubular =3, endothelial=2, glomerular=1 and tubulointerstitial=3)

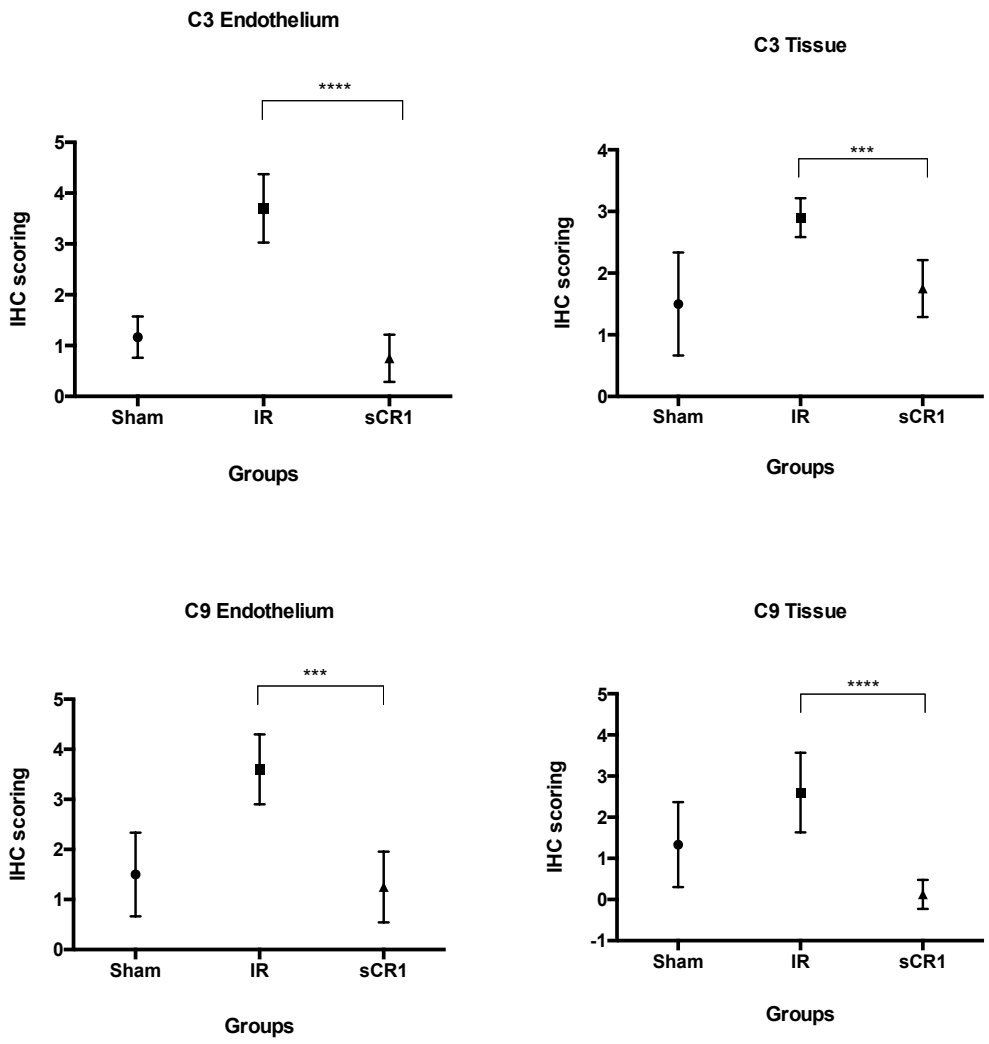
sCR1: Rats pre-treated with sCR1. The slide depicts decreased neutrophil with no endothelial damage. Overall score=2 (tubular =0, endothelial=0, glomerular=1 and tubulointerstitial=1).

5.1.3.3. Immunohistochemistry

IHC was used to test markers encompassing the complement system, endothelium and leukocytes. The results are shown in summative graphs, with representative IHC pictures showing the changes.

5.1.3.3.1. Complement markers

Figure 5.4: SCR1 studies – Summative IHC scoring of complement markers



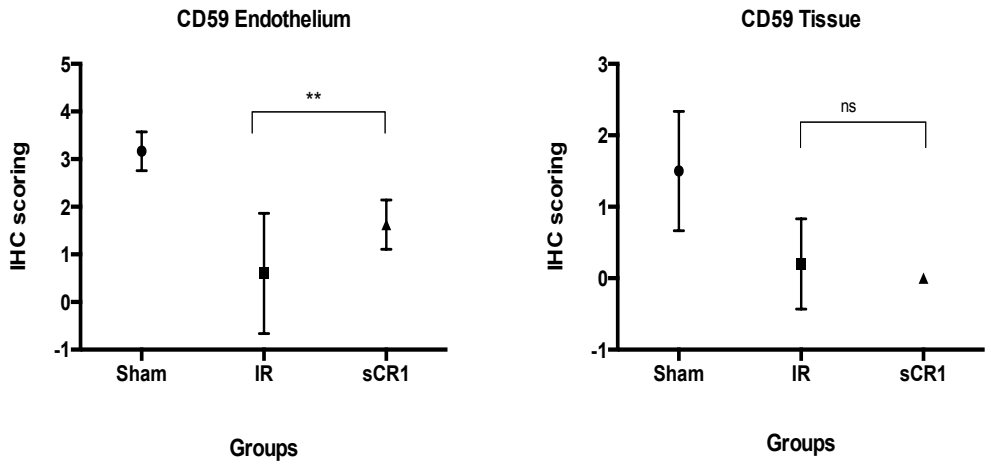
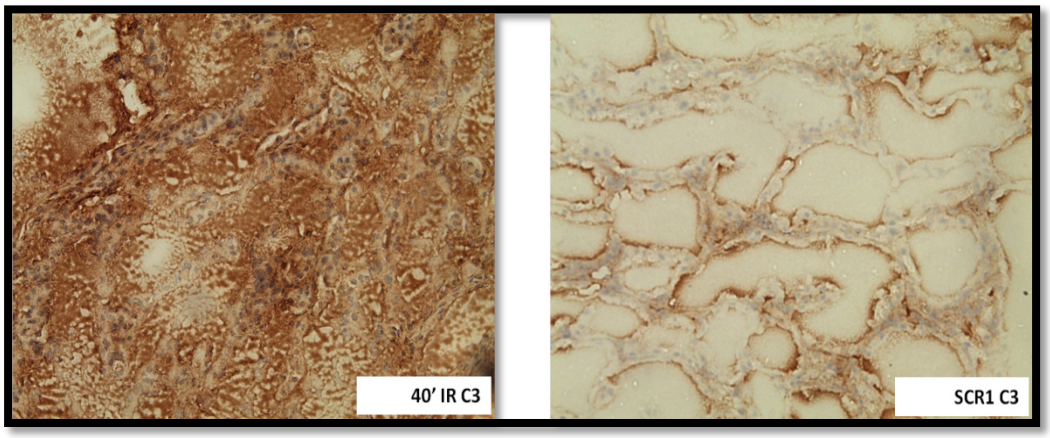


Figure 5.4: IHC scoring of renal cortex sections from sham (n=6), 40-minute unilateral IR(n=10) and sCR1 treated (n=8) in rats at 48 hours after reperfusion. Complement markers C3, C9 and CD59 stained as per protocol and scored 0-4 (vascular and stromal staining). IHC scores are plotted as Mean +/- SD, and Mann Whitney tests used to test significance [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05 (*)].

Figure 5.5: SCR1 studies – Renal cortex IHC sections of complement markers



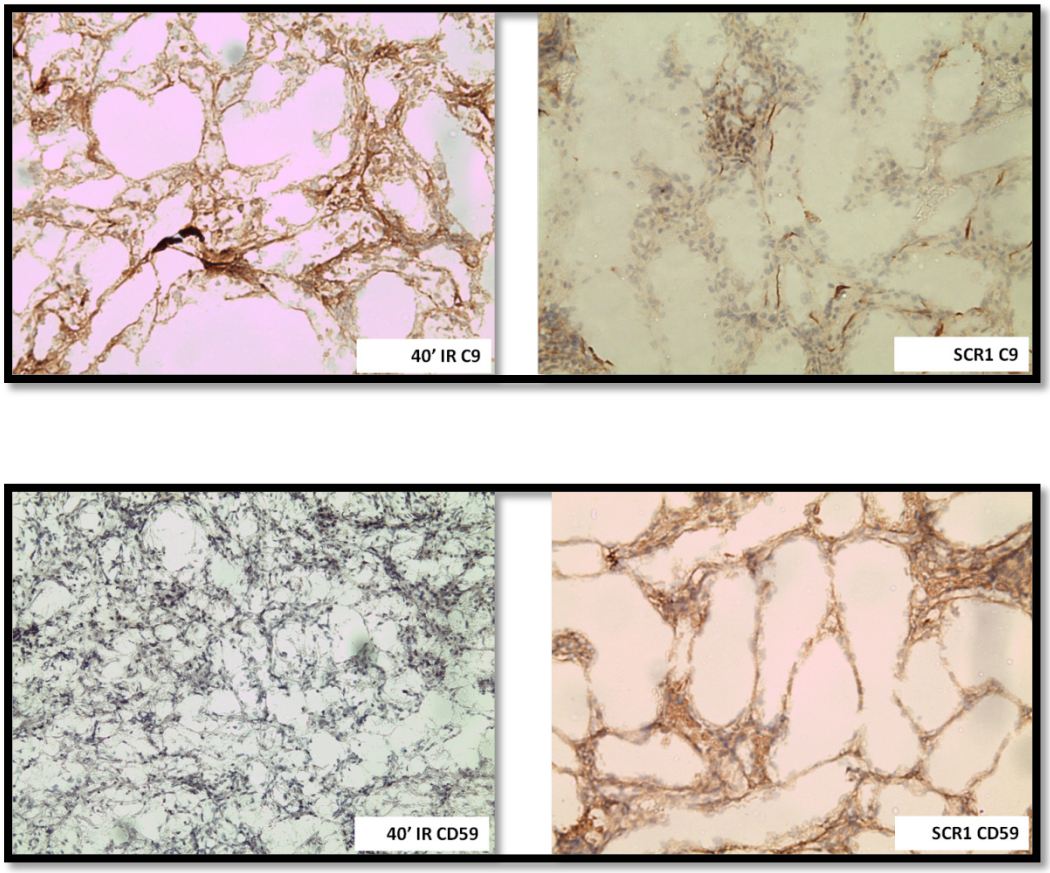


Figure 5.5: Rat Renal cortex IHC sections (200x) from IR and sCR1 groups, following 40 minutes of ischaemia and 48 hours after reperfusion. Complement markers C3, C9 and CD59 stained as per protocol and scored 0-4 (vascular and stromal staining). The 40' IR slides are on the left depict increased deposits of C3 and C9 compared to the sCR1 group, which have decreased deposits. There is also decreased CD59 staining in the IR group compared to relative protection in the sCR1 group.

5.1.3.3.2. Endothelium

Figure 5.6: SCR1 studies – Summative IHC scoring of endothelial markers

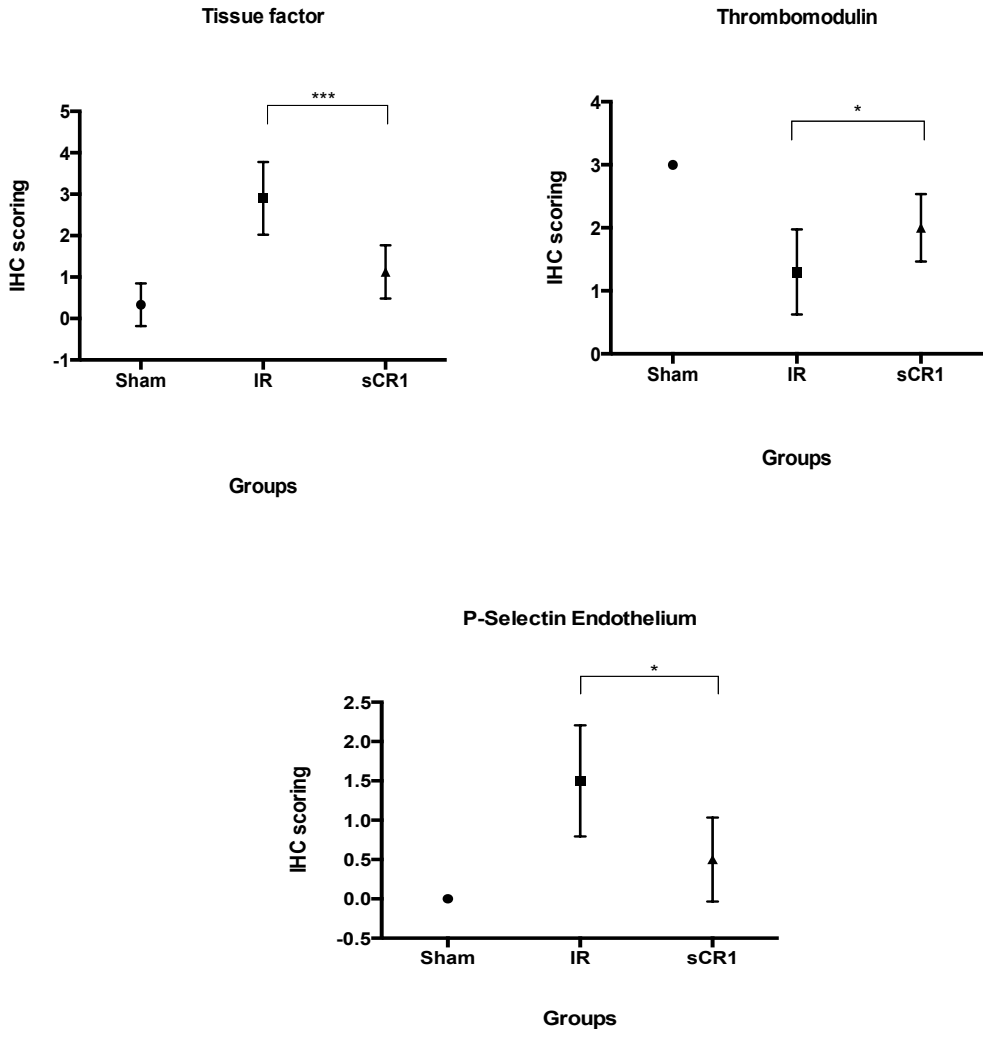


Figure 5.6: IHC scoring of renal cortex sections from sham (n=6), 40-minute unilateral IR (n=10) and sCR1 treated (n=8) in rats at 48 hours after reperfusion. Endothelial markers Tissue factor, Thrombomodulin, and P-Selectin were stained as per protocol and scored 0-4 (vascular and stromal staining). IHC scores are plotted as Mean +/- SD, and Mann Whitney tests used to test significance [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05 (*)].

Figure 5.7: SCR1 studies – Renal cortex IHC sections of endothelial markers

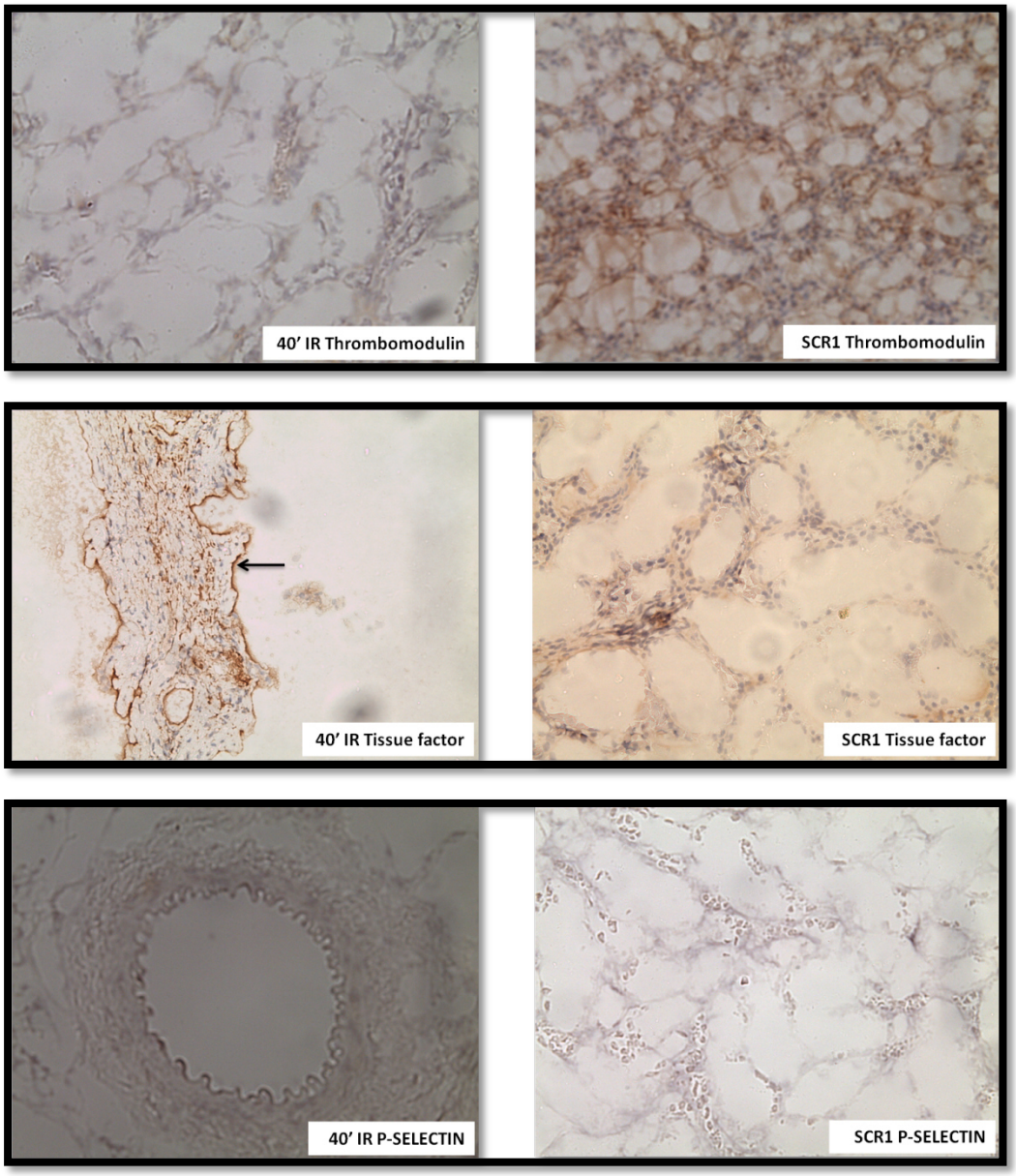


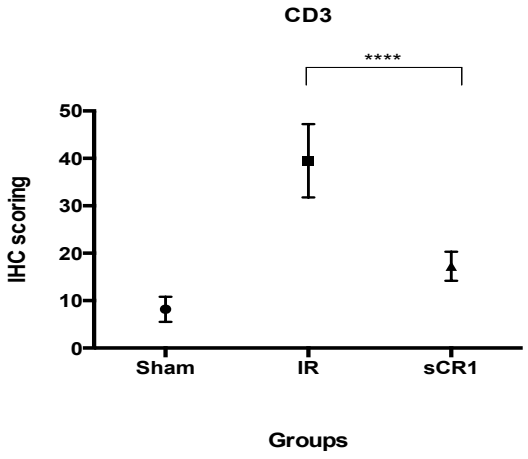
Figure 5.7: Rat Renal cortex IHC (x200) sections from IR and sCR1 group, following 40 minutes of ischaemia and 48 hours after reperfusion. Endothelial markers: Tissue factor and Thrombomodulin, and P-selectin were stained as per protocol and scored 0-4

(vascular and stromal staining. There are decreased deposits of Tissue factor and P-Selectin in the sCR1 treated rats (right column) compared to the IR group (left column). There is also presence of thormbomodulin in the sCR1 group compared to the IR group. The quantitative figures are provided in figure 3..

5.1.3.3.3. Inflammatory Cells

Cellular markers for lymphocytes (CD3, CD4 and CD8), macrophages (CD68) and neutrophils (CD 15) were used to assess inflammatory infiltrates.

Figure 5.8: SCR1 studies – Summative IHC scoring of cellular markers for inflammation mediators



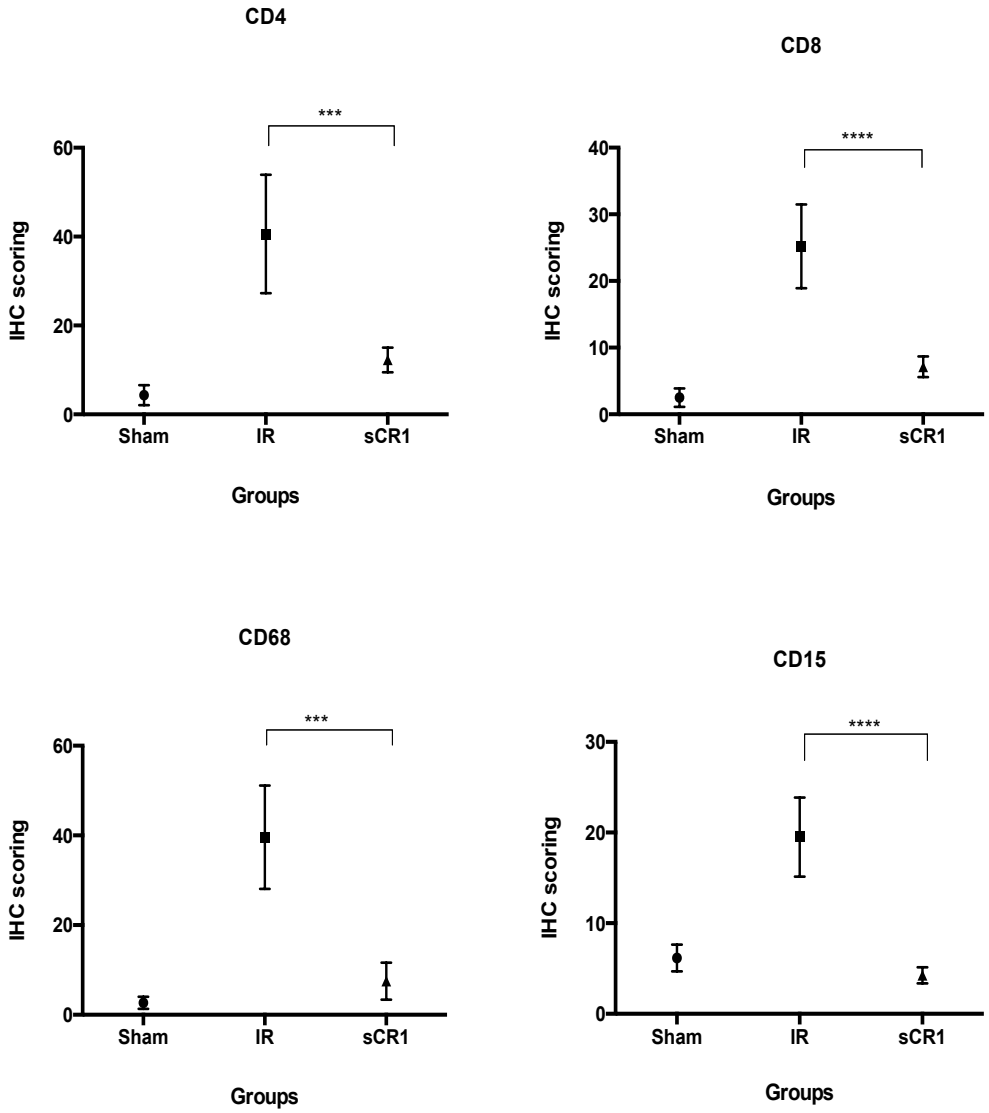
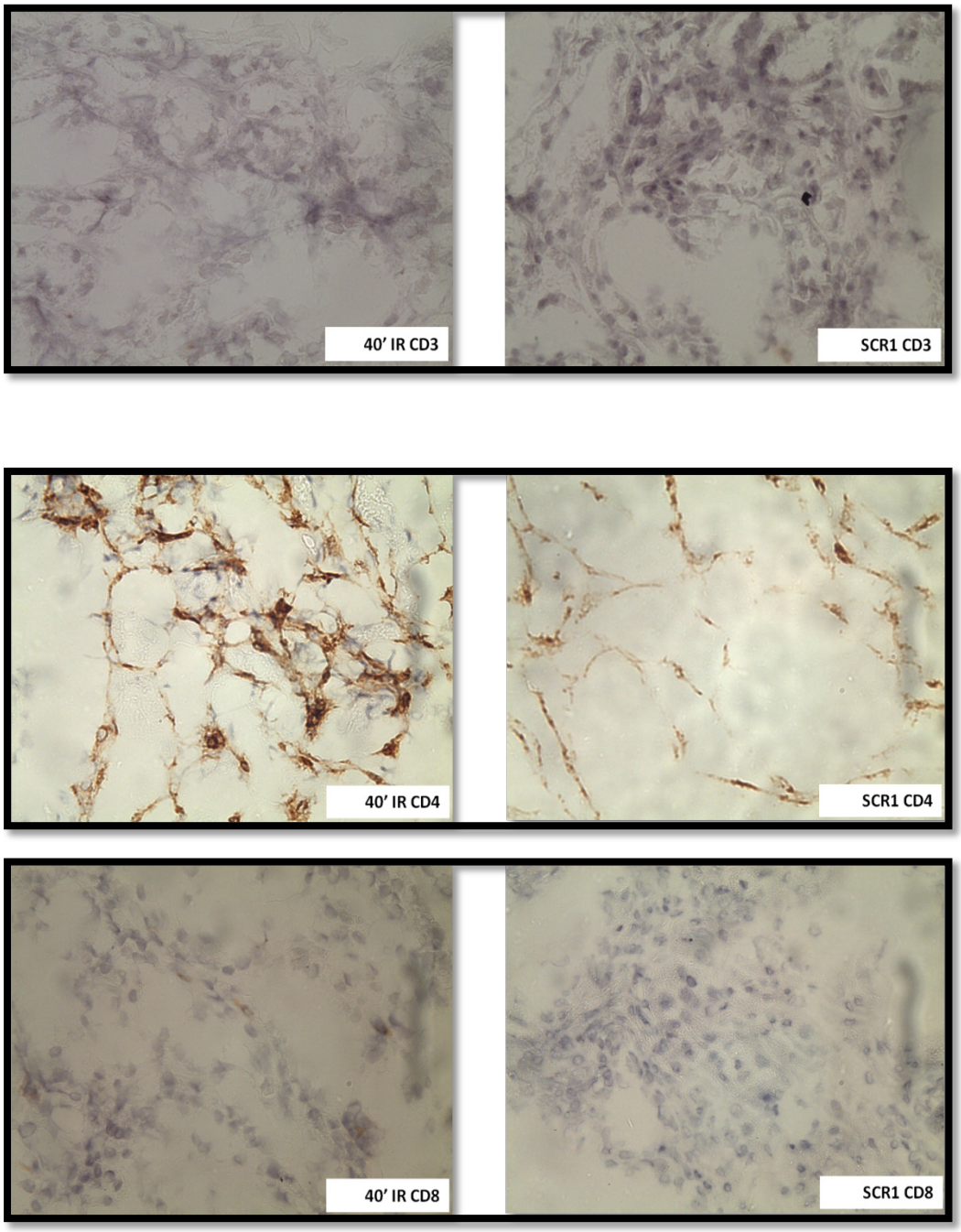


Figure 5.8: IHC scoring of renal cortex sections from sham (n=6), 40-minute unilateral IR (n=10) and sCR1 treated (n=8) in rats at 48 hours after reperfusion. Cellular markers for inflammation mediators were stained as per protocol, and were counted: n/ avg 5x HPF (high power field). IHC scores are plotted as Mean +/- SD, and Mann Whitney tests used to test significance [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05 (*)].

Figure 5.9: SCR1 studies – Renal cortex IHC sections of cellular markers for inflammation mediators



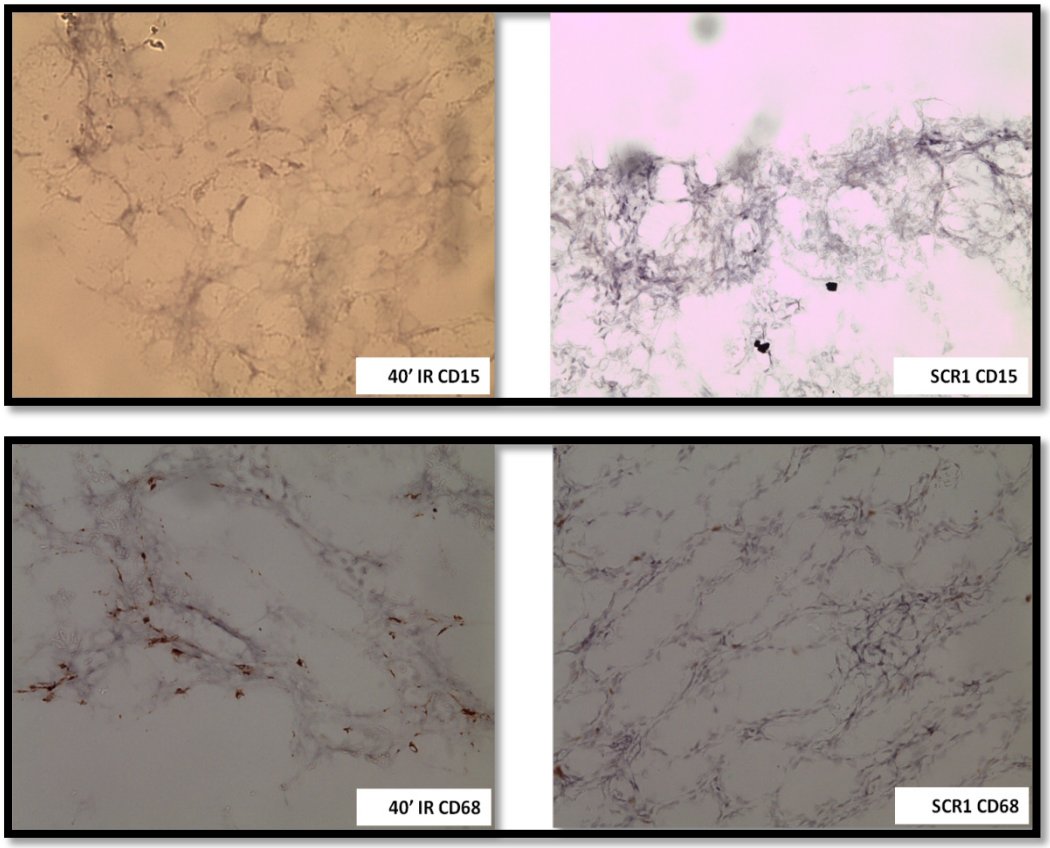
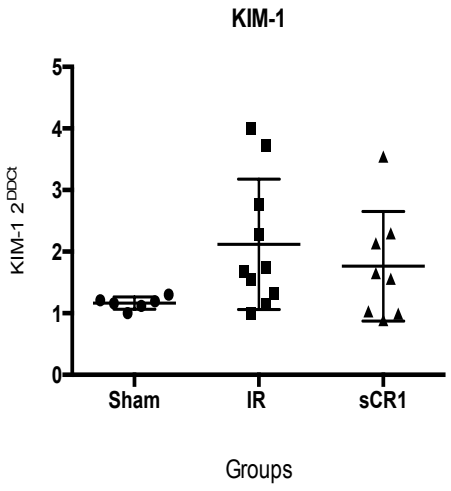


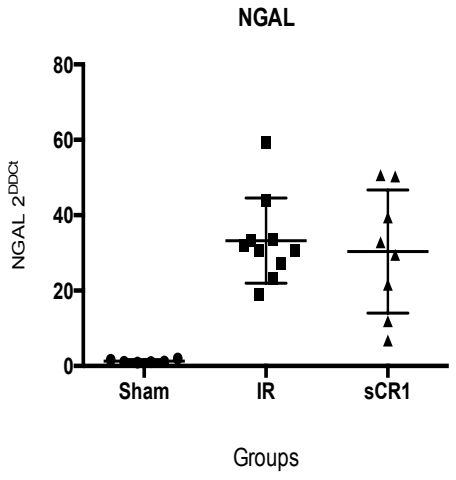
Figure 5.9: Rat Renal cortex IHC sections (200x) from IR and sCR1 groups, following 40 minutes of ischaemia and 48 hours after reperfusion Cellular markers for inflammation mediators were stained as per protocol, and were counted: n/ avg 5x HPF (high power field). There is decreased infiltrate of CD3, CD4, CD8, CD15 and CD68 cells in the sCR1 treated group compared to the IR group.

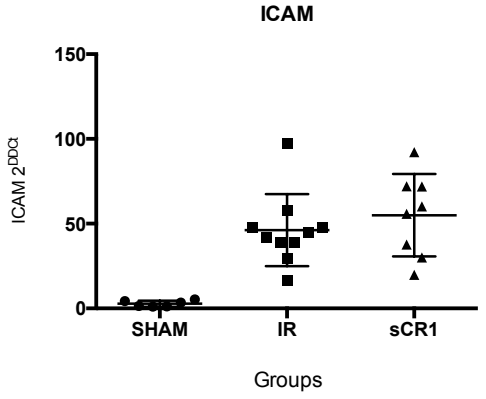
5.1.3.4. RT-qPCR

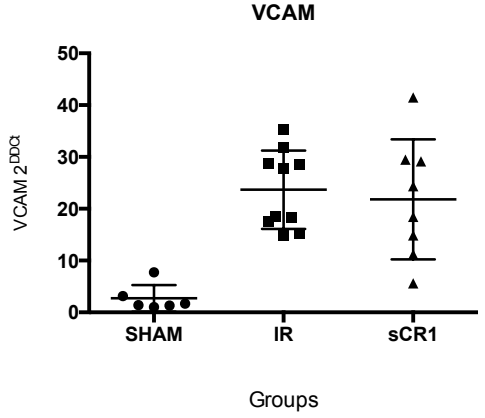
Kidney injury markers (KIM-1 and NGAL), cellular adhesion molecules (ICAM-1 and VCAM) and cytokine markers (TNF-alpha and IL18) were evaluated using RT-PCR. The results were analysed using Delta Delta Ct' (ΔΔCt) values with GAPDH chosen as the reference gene for the relative quantification.

Figure 5.10: SCR1 studies – RT-qPCR analysis of six genes expression normalized to GAPDH









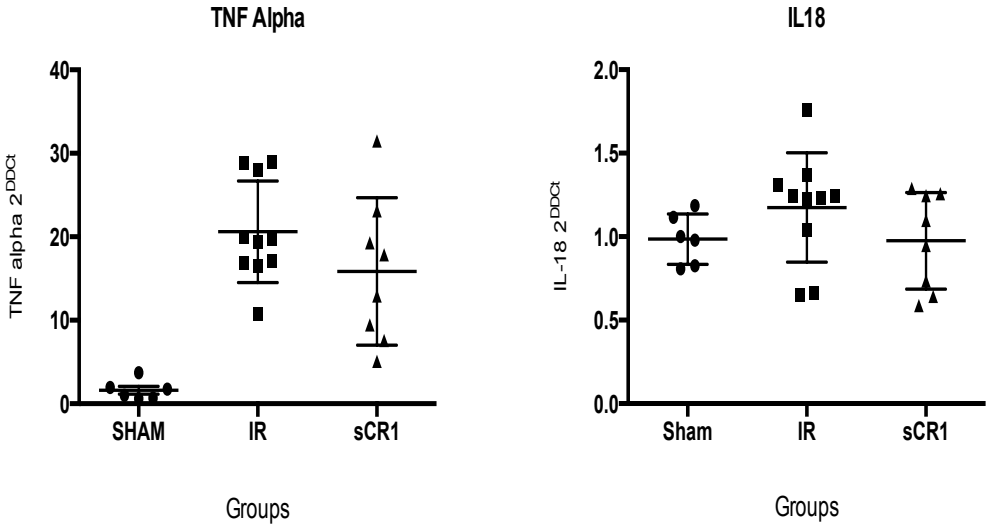


Figure 5.10: RT-qPCR analysis of six genes with expression normalized to GAPDH. Values are plotted as mean ± SEM. *Unpaired –t tests used to test significance [p value < 0.0001 (****), p= 0.0001 to 0.001 (***), p = 0.001 to 0.01(**), p = 0.01 to 0.05 (*)].*

There was no statistical difference seen in any of the genes of interest between the IR and the sCR1 treated group.

5.1.4. Discussion of Results

CH50 Analysis:

As evident by the graph 5.1, the 1/20 dilution of sCR1 rat serum showed ablation of the complement activity just prior to reperfusion, and the complement system was back to near normal by 24 hours. In our model, the sCR1 was injected 20 minutes prior to laparotomy followed by ischaemia time of 40 minutes. Pharmacokinetics of sCR1 revealed that the sCR1 levels (25mg/kg IV) was reduced to 50% within 30 minutes and declined to trace levels by 24 hours (327). This may explain the reconstitution of the complement pathway at 24 hours, and is directly proportional to the pharmacokinetics of the sCR1.

Histology:

sCR1 showed significant structural protection compared to the IR group. Figure 5.3 shows the key differences between the sCR1 and IR group on H &E staining, predominantly being tubular and endothelial protection with decreased neutrophil infiltrate.

Immunohistochemistry:

Complement

The complement cascade leads to formation of C3b, anaphylotoxins and C5B-9 as end product. We evaluated C3b and C9 deposition in both the endothelium and the renal interstitium. sCR1 significantly reduced C3 deposits indicating suppression of the complement system cascade. This led to decreased assembly of MAC (C9) in the cells in the sCR1 treated rats. We also evaluated the local regulatory protein CD59, which in

normal circumstances acts as local complement regulator. Loss of CD59 with injury in the IR group, was protected in sCR1 treated kidneys. The effect of reduced complement staining's were predominantly seen at the endothelium level compared to the tissues, highlighting that endothelial protection offered by complement system ablation may be a key mechanism for attenuating IR injury

The phenomenon of sCR1 induced decreases in C3 and MAC has been shown in renal transplant models(327), and other IRI models (320). However, there is no warm renal IRI model used before for comparison. The renal transplant model is a step-up compared to our model, but our methods and drug dosing were different to the transplant model.

Endothelium

To further investigate the effect of sCR1 on endothelium, we investigated markers of endothelial activation/injury: Tissue factor, Thrombomodulin and P-Selectin. As depicted in figures 5.6 and 5.7, there were decreased deposits of Tissue factor and P-Selectin in sCR1 treated rats compared to IR group indicating endothelial protection. Similarly, Thrombomodulin was present in sCR1 treated animals indicating endothelial structural integrity.

There is very limited evidence of the effect of sCR1 on vascular endothelium in literature. Pratt et.al demonstrated histological protection of endothelium in a renal transplant model (327). In a hepatic IRI model, sCR1 was shown to reduce leukocyte sticking to endothelium, but they did not evaluate endothelial injury or used markers to quantify it (321). In our study, we have evaluated three specific markers for endothelial injury and shown endothelial protection on immunohistochemistry and histology.

Leukocytes

sCR1 treated rats showed marked decrease in all the evaluated leukocyte markers: CD3, CD4, CD8, CD15 and CD68 cells compared to the IR group. This effect may be partly attributed to endothelial protection, and also suppression of the complement system. C3a and C5a (anaphylotoxins) are key players in recruitment of leukocytes to site of injury, and the suppression of the complement cascade at C3 convertase level prevents the formation of these anaphylotoxins, and subsequent leukocyte recruitment. Similar effects of sCR1 on leukocytes have been seen on other IRI models involving liver and myocardium in literature (318, 328).

RT-qPCR:

The kidney injury molecules: KIM-1 and NGAL both showed down-regulation compared to the IR group, however it did not reach statistical significance. There was also similar down-regulation of cytokines TNF-alpha and IL-18, but did not reach statistical significance. There were no differences in the expression of VCAM and ICAM.

Of the few studies using sCR1/TP-10 in renal ischaemia, there has been no RT-qPCR done on these renal samples. So, it is difficult to compare against literature evidence. The ATG group had a significant effect on the injury scoring compared to sCR1 (mean histology scores: IR (8.70) vs. ATG (1.25) vs. sCR1 (3.25), and the probability exists that the down regulation of kidney injury molecules may be directly proportional to the 'degree' of protection offered. The other explanation may be due to the degradation of the mRNAs seen in the scR1 samples that precluded the Ct values to reach significance.

Summary of Results:

In conclusion, we can summarise the following findings from this part of our study

- Single dose sCR1 offered protection from renal ischemia reperfusion injury through reduced complement activation
- sCR1 treated rats showed reduced leukocyte infiltrates which may be due to reduced anaphylotoxins
- sCR1 offered significant endothelial protection
- The complement system was ablated at the time of reperfusion and was reconstituted by 24 hours, thus indicating that suppression of complement system during the phase of ischemia and reperfusion may provide an avenue for mitigating IRI.

5.2. Anti-MASP2 antibody and Lectin pathway

5.2.1. Introduction

Lectin pathway is activated by the binding of multimolecular lectin complex with a carbohydrate moiety on pathogens, with the three MBL-associated serine proteases MASP-1, MASP-2 and MASP-3 able to bind to the carbohydrate recognition subcomponents and activate the lectin pathway (329). MASP-2 cleaves C4 and C2 to form C3 convertase, and this cleaving effect was shown by recombinant MASP-2 suggesting that MBL was able to activate the lectin pathway via MASP-2 (331). Murine studies have shown sMAP (small MBL-associated protein (sMAP) deficient mice showing decreased expression of MASP-2, and injection of recombinant sMAP and MASP-2 reconstituted the MBL-MASP-sMAP complex in deficient serum, suggesting that MASP-2 was essential for the activation of C4 in the activation of the lectin pathway (332).

A recent study employing MASP-2 deficient mice showed protection from myocardial and intestinal IR injuries, and this was further supported by the use of an inhibitory monoclonal antibody (selectively binds to MASP-2) pre-ischemia that reduced intestinal tissue IRI (329). Furthermore, this study also showed that lectin pathway could activate complement C3 in the absence of C4 via a unique MASP-2–Dependent C4-Bypass, and lectin pathway-mediated activation of C3 in the absence of C4 was demonstrated in vitro and shown to require MASP-2, C2, and MASP-1/3. In a later study, in a murine study of renal transplantation, WT grafts transplanted into MASP-2-deficient recipients showed structural and functional protection of transplanted kidney. In the absence of donor or recipient complement C4, the WT to WT phenotype was preserved, indicating that the MASP-2-

mediated damage could occur through MASP-2-dependent activation events independent of C4 (400).

The effect on anti-MASP-2 antibody (329) in renal IRI has not been explored, and we trialled this antibody with the aims of evaluating the effects of Anti-MASP 2 antibody on renal IRI in a rat model.

5.2.2: Methods

The anti-MASP-2 antibody was provided by Omeros Inc., Seattle, WA and has been used to evaluate its efficacy in intestinal IR model (329). Its preparation is as follows: recombinant antibodies against MASP-2 (AbDH3 and AbD04211) were isolated from Human Combinatorial Antibody Libraries (Serotec; AbD) using recombinant human and rat MASP-2A as antigens. An anti-rat Fab2 fragment that potently inhibited lectin pathway-mediated activation of C4 and C3 in mouse plasma was converted to full-length IgG2a antibody. Polyclonal anti-murine MASP-2A antiserum was raised in rats.

The three groups for this study are

- *IR group* (n=10): 40 minutes of left unilateral ischaemia followed by 48 hours of reperfusion
- *Anti-MASP2 group* (n=6): antibody (1 mg/kg body weight, manufacturer recommended) administered intravenously just prior to the laparotomy followed by 40 minutes of left unilateral ischaemia and 48 hours of reperfusion

- *Anti-MASP2 Isotype* (n=6) received the isotype (1 mg/kg body weight, manufacturer recommended) administered intravenously just prior to the laparotomy followed by 40 minutes of left unilateral ischaemia and 48 hours of reperfusion

- Sham group (n=6)

Analysis:

- Lectin Pathway Inhibition: Blood samples: TO (prior to laparotomy), T1 (prior to reperfusion), T2 (24 hours) and T3 (48 hours)

- Kidneys harvested at 48 hours were analysed for histological damage and RT-qPCR studies

5.2.3. Results

5.2.3.1. Lectin Pathway Ablation

Omeros Corporation (USA) provided guidance for methods to check the ablation of lectin pathway activity in serum, which was adapted in our lab under the guidance of Dr Brad Spiller. Functional activity of the lectin pathway in rat serum was assessed by detection of C4b deposition on mannan-coated plates. The methodology is discussed in the methods section.

In our study using the current methodology and dosing, we were unable to show LP pathway ablation at T1 (prior to reperfusion) and T2 (24 hours) as evident by residual C4b

deposits. We were unable to pinpoint if this effect was due to residual Classical pathway activation or a bypass mechanism of activating C4 that the antibody was not able to suppress. This aspect is further analysed later in this chapter.

5.2.3.2. Histology

A comprehensive histopathological scoring scale was used to evaluate renal injury.

Figure 5.11: MASP studies – Histology scoring of renal cortex sections

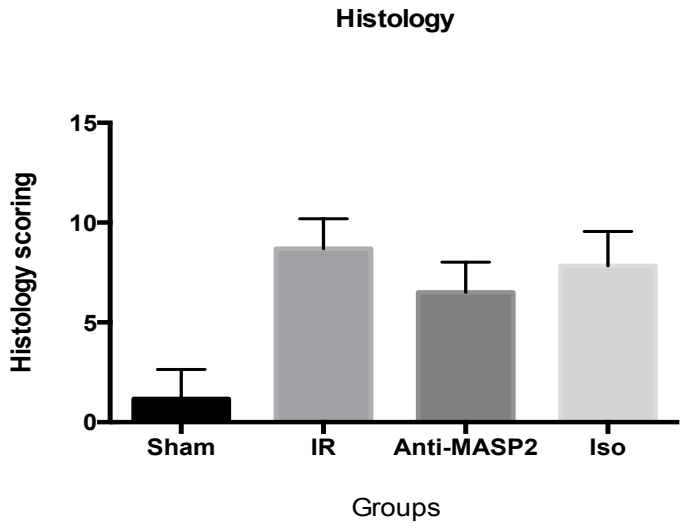


Figure 5.11: Scoring of renal cortex sections from sham (n=6), 40-minute unilateral IR (n=10), Anti-MASP2 (n=6) and Anti-MASP2 Isotype (Iso, n=6) at 48 hours after reperfusion, stained with H&E and assessed using a comprehensive scoring system comprising of tubular, endothelial, glomerular and tubule-interstitial cell changes. Histology scores are plotted as Mean +/- SD, and Kruskal-Wallis test used to test significance between the IR and the treatment groups.

Figure 5.12: MASP studies – Renal cortex H&E sections

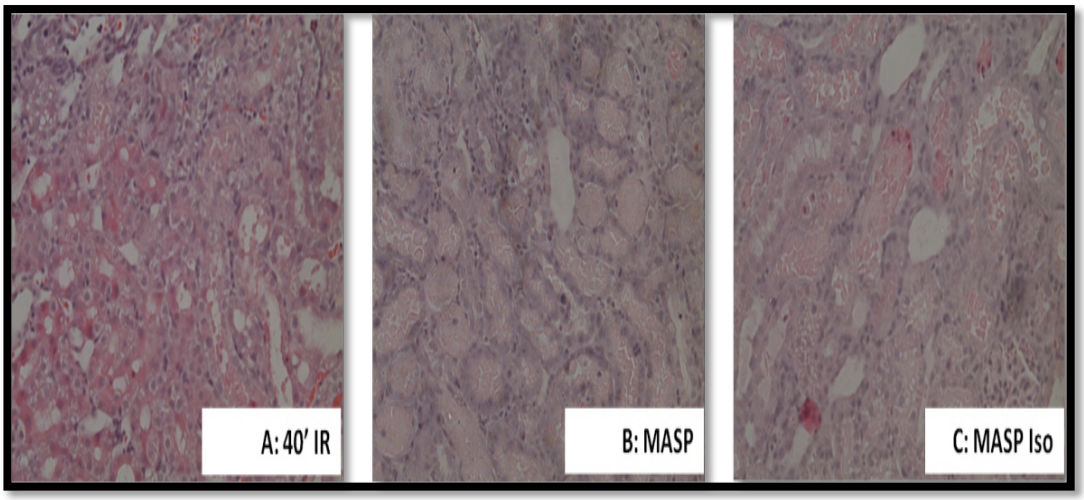


Figure 5.12: Rat Renal cortex H&E (x200) paraffin sections from sham (n=6), 40-minute unilateral IR (n=10), Anti-MASP2 (n=6) and Anti-MASP2 Isotype (Iso, n=6) at 48 hours after reperfusion, stained with H&E. The slides were assessed for tubular, endothelial, glomerular and tubulo-interstitial cell damage.

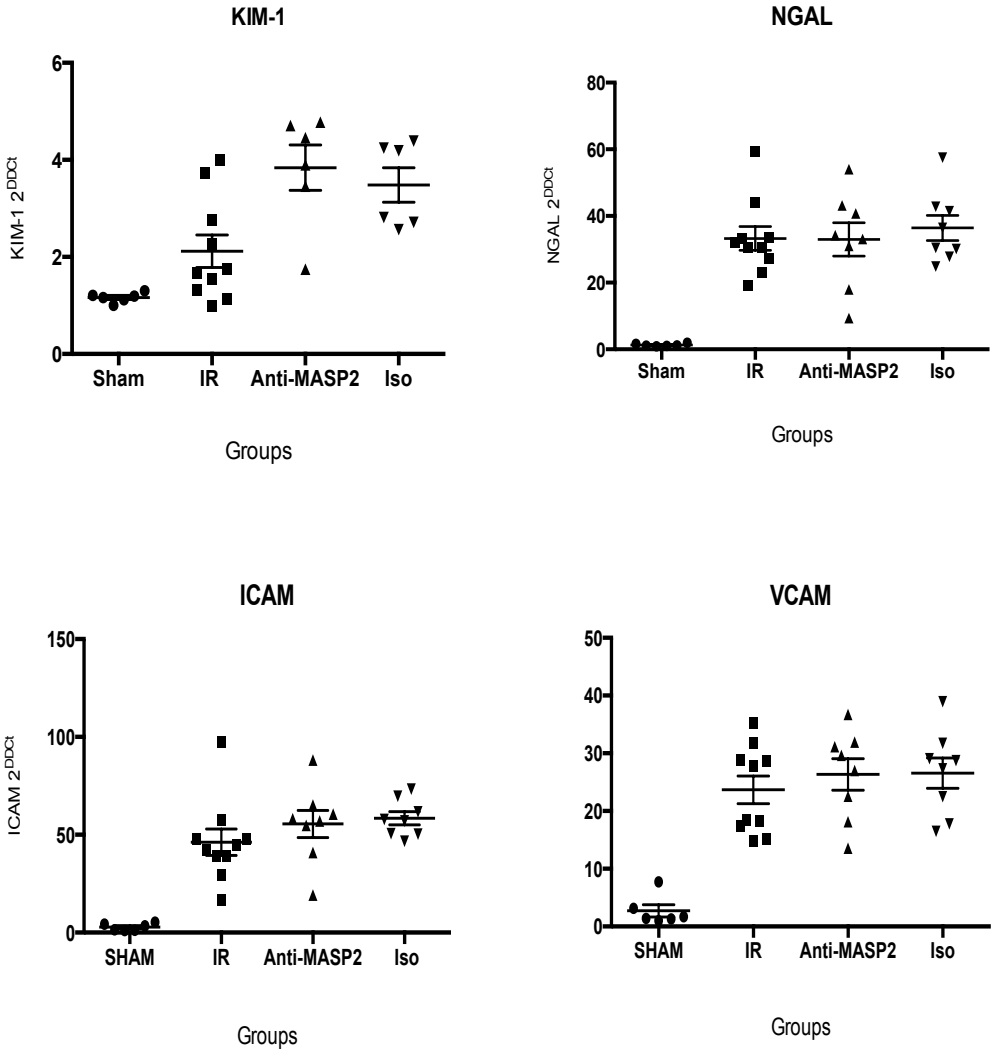
***IRI**: Untreated control animal showing venous thrombosis, multifocal necrosis and severe neutrophil infiltration (H&E). Overall score = 10 (tubular =3, endothelial=2, glomerular=2 and tubulointerstitial=3). **Anti-MASP2**: Anti-MASP2 antibody shows neutrophil infiltrate along with tubular injury and cast formation. There is endothelial swelling along with glomeruli tuft retraction. Overall score=8 (tubular=3, endothelial=2, glomerular=2 tubulointerstitial=1).*

***Anti-MASP2 Isotype**: The slide shows neutrophil infiltrate along with tubular injury and cast formation. There is endothelial swelling along with glomeruli tuft retraction. Tubulointerstitial damage seen with necrosis in parts. Overall score=9 (tubular=3, endothelial=2, glomerular=2 tubulointerstitial=2).*

5.2.3.3. RT-qPCR

Kidney injury markers (KIM-1 and NGAL), cellular adhesion molecules (ICAM-1 and VCAM) and cytokine markers (TNF-alpha and IL18) were evaluated using RT-PCR. The results were analysed using Delta Delta Ct' (ΔΔCt) values with GAPDH chosen as the reference gene for the relative quantification.

Figure 5.13: MASP studies – RT-qPCR analysis of six genes with expression normalized to GAPDH



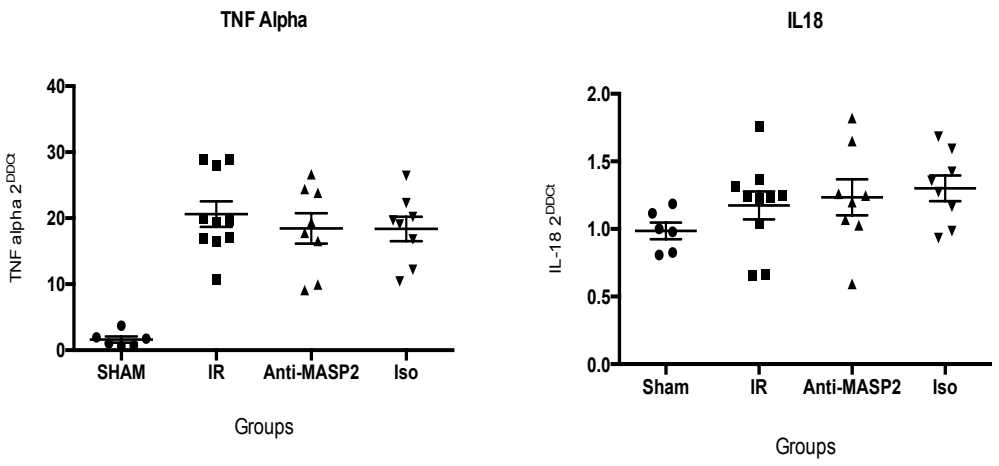


Figure 5.13: RT-qPCR analysis of six genes with expression normalized to GAPDH. Values are plotted as mean ± SEM. One-way-Anova used to test significance between the IR, Anti-MASP2 and Anti-MASP2 Isotype groups. There were no differences seen in the genes of interest between the IR and the intervention groups.

5.2.4. Discussion of results

This is the first time we have trialled this antibody, and had limited stock available. The dosing schedule was as advised by the manufacturer.

Histology:

As evident by the graphs 5.11 and 5.12, there was no protection offered by anti-MASP-2 antibody on the IR injury

RT-qPCR:

There was no difference seen in the mRNA levels of kidney injury molecules (KIM-1, NGAL), adhesion molecules (ICAM-1, VCAM) and cytokine levels (TNF-alpha, IL-18).

Lectin pathway (LP) ablation:

We were unable to block the lectin pathway completely and there were still C4 deposits seen in the plate for T1 and T2 samples. The reasons I see that may have contributed to the effect not seen:

- The dosing was as advised by Omeros, Inc at 1mg/kg BW. The dose they used in the original study was 0.6mg/kg BW and we used 1mg/kg BW as advised. In the original study, they were able to show LP ablation at 6 hours and lasting effect for 48 hours in a mouse model. Our 24-hour samples did not show ablation as evident by C4 deposits. Though pharmacokinetics was not part of the methodology, on checking for the probable dilution required, nearly 20% concentration was required which would amount to nearly 3-4x the does currently used.
- The antibody was shown to be effective on a mice myocardial and intestinal reperfusion model and the dose was effective on that particular model. No studies have been carried out on the rat renal model in literature, and whether MASP-2 blockade would work in renal IRI still needs to be established.
- Probability that there is always a residual level of complement activation in the assays that is resistant to anti MASP-2 antibody. Also, it is difficult to interpret whether there was any contribution of the classical or alternate pathways to the effect seen.

Summary of Results:

Ideally, I would have liked to develop this model further, however due to paucity of time, I am reporting the outcomes seen in the preliminary study and if given an opportunity to develop this model, this would be the methodology I would employ

- Determine baseline LP activity in IR and sham groups to determine any involvement of the LP, ideally in a mouse model so that knock-out mice could be used to classify and quantify changes
- Determine involvement of Classical and Alternate pathway involvement by using knock-out mice model
- Determine assays to measure MASP-2 in IR and sham models
- Determine pharmacokinetics of the Anti-MASP2 antibody to determine the appropriate dosing, timing of delivery (compared to IR event), mean half life and effect on LP pathway
- Immunohistochemistry to determine complement deposits and probably in a model with varying time periods of reperfusion (24-hours, 48 hours and 5 days)

CHAPTER VI:

DISCUSSION AND CONCLUSIONS

6.1. Discussion of methodology

6.1.1. Choice of Model

Animal studies are a key resource for investigating renal ischemia-reperfusion injury, and potential novel therapies. Important considerations in selecting a model include species (eg. rat vs. mice vs. swine), the methodology of injury and the timing of analysis (eg. 24 hours vs. 48 hours vs. days). To test therapeutic manipulation of leucocytes and complement, I have adopted a unilateral model of renal ischaemia, in the Lewis rat, and a 40' minute ischemia period. This was selected based on prior demonstration in the literature of reproducible, significant injury, that may be ameliorated by therapy (348, 349).

6.1.2. Alternative model approaches

It is important to acknowledge the limitations of this model, and potential advantages/disadvantages of the model adopted for this work.

The major disadvantage of rat when compared to mouse models is the relative difficulty of genetic manipulation in the rat. However, there were additional considerations for my planned testing of therapies. For example, frequent blood sampling and circulatory volume are issues in the mouse –frequent blood sampling in studies would potentially complicate the models. An important future direction may be adaption of the model to use of mice, allowing additional genetic approaches to pathway manipulation to be used in conjunction with testing of therapeutic approaches in the rat. A second alternative is a large animal species, for example a porcine model. This would have additional advantages in terms of

histopathological similarity and increased similarity of pathological and therapeutic responses, but brings major additional resource and infrastructure considerations.

The unilateral model would also limit measurement of physiological functional measurements like serum creatinine and other serum markers for kidney damage. Having established a safety profile with our animal work, a bilateral renal IRI model would be the next step forward, and we have adopted this for the next project. The right kidney also served as internal controls and has been discussed in the methodology chapter.

The 40' minute ischaemia time showed moderate degree of injury in our study. It can be speculated that having a more increased time of ischaemia (60 minutes/120 minutes) could have shown a higher degree of injury especially if interventions are used to attenuate the injury. On the contrary, more severe injury may also have carried increased morbidity to the rat, but having produced a model that was safe, reproducible and efficient, future work would evolve into exploring more severe models of injury.

6.1.3. Mortality and Morbidity

Morbidity included wound infection/dehiscence, failure to thrive and any other features that would have needed the animal to require additional treatment, or early sacrifice. In this study, we had two mortalities and no morbidities. The two mortalities were unexpected and happened very early in the project, and have been discussed below.

- Rat 1 (IR group): the subject experienced laboured breathing 30 minutes into ischaemic time and showed some recovery following titration of anaesthesia. At the time of closure, the rat appeared pale and was recovered in a warm chamber

to negate any hypothermic insult. However, the rat passed away in the camber itself, and a systematic post mortem was carried out to identify any iatrogenic causes. Laparotomy and thoracotomy revealed no bleeding or other explainable causes

- Rat 2 (sham group): the subject was well until the end of 40 minute observation period, and was recovering in the heat chamber. However, within 5 minutes it passed away, and a systematic post-mortem carried out did not show any iatrogenic causes

Overall, we had a relatively safe and efficient model that adhered to the Animals (Scientific Procedures) Act 1986 (ASPA).

6.2. Discussion of Results

Renal ischaemia-reperfusion is an important clinical problem affecting patient's health, and has wide ranging effects on the health service and health economy. My research has focussed on three important components of the IRI mechanism, and my results have shown that modulating these can attenuate renal IRI. In the next few sections, I will evaluate the results under these three components with a perspective on clinical application, and future avenues.

6.2.1. Leukocytes

Ischaemia-Reperfusion injury is an inflammatory process characterised by leukocyte infiltrates, a finding seen in this study and correlates with literature. As discussed in the introduction chapter, there is a family of leukocytes (neutrophils, lymphocytes, macrophages, NK cells, Tregs etc.) which all have a role in the mediation and propagation of IRI. The inflammatory state of the IR kidney may lead to fibrosis and chronic kidney disease in the long term. Leukocyte infiltration also coincides with platelet accumulation and activation of coagulation cascade, which further accentuate renal injury. The complexity and diversity in leukocyte recruitment at the site of injury, from endothelial stimulus to anaphylotoxins, makes it challenging to address. Though the individual cells and their antagonists have shown promise in individual studies, they still haven't translated into clinical practice.

Previous studies using Activated protein C (physiological anticoagulant with anti-inflammatory and anti-apoptotic activities), vinblastine and nitrogen mustard have shown leukopenia-induced benefits in other hepatic and spinal cord IRI (293, 294). Recently,

thrombomodulin-dependent APC was shown to ameliorate renal IRI in a murine model (401). Neuronal guidance cues: Slit and Roundabout (Robo) have shown to prevent the migration of multiple leukocyte subsets towards inflammatory chemoattractants, and possess anti-platelet functions in vitro and in vivo (402). The last two articles had potential benefit on the platelet and coagulation cascade in addition to the leukocyte influence, indicating that a multi-system mediator is needed to address the complex mechanisms of IRI.

ATG and Isotype:

Rat specific ATG in our study ameliorated IRI via significant reduction in leukocyte infiltrate and endothelial protection. It can be hypothesized that the leukocyte depletion using ATG may be a combination of direct effect on the leukocytes themselves, or secondary to the endothelial protection (TM and TF). The recent evidence of TM-linked APC and its benefits in renal IRI can be linked to our study where ATG showed TM protection. We haven't measure APC in our study, and this may be an avenue to be explored. We also showed decreased C3 deposits in the renal tissue and endothelium with ATG treatment. This is probably due to decreased activation of the complement system from the endothelium and along with the dampened inflammatory response.

One of the interesting findings was the Isotype Ig effect on the endothelial cells and inflammatory cells (CD8 and CD68 cells). The ATG isotype is a rabbit IgG purified from whole normal rabbit serum. IVIg (Intravenous Immunoglobulin products) has been used for the treatment of primary immunodeficiency disorders, inflammatory diseases (Kawasaki disease, Guillain-Barre syndrome, myasthenia gravis etc.), in reduction of anti-HLA

antibodies in human leukocyte antigen (HLA) sensitized patients and in treatment of antibody-mediated rejection (AMR) (403). AMR,also known as B-cell-mediated/ humoral rejection, is treated with plasmapheresis and IVIg (404). As the isotype is also an immunoglobulin, we feel that some of the effects seen may be partly due to its 'antibody' nature, however it did not confer protection against IRI. This alludes to the possibility that just endothelial protection in itself may not confer overall structural protection.

6.2.2. Complement System

The complement system has had a renaissance in the last few years, and is being increasingly recognised as being a key mediator of renal ischaemia-reperfusion injuries. In one of the earliest sentinel papers, role of complement in renal IRI was questioned as C3 knock-out mice did not show protection compared to WT (405). However, in this model, the warm ischaemia time was only 20-30 minutes, and many subsequent articles questioned the validity of such a short ischaemic time in inducing complement activation, or renal injury. In the same decade, Zhou et.al showed that C3-, C5-, and C6-deficient mice were protected from renal IR injury, whereas C4-deficient mice were not protected, thus implying a role for alternative pathway (19). In this study, the renal ischaemia time was 40-58 minutes with predominant injury seen in the tubular epithelium with relative sparing of the renal endothelium. This implies that in any model of IRI, the ischaemic time could have a bearing on the aims of the study, and has to sufficient enough to activate/upregulate the cells in question. Recent review articles have suggested that among all the pathways implicated in mediating renal IRI, alternative and lectin pathways probably have a more definitive role than the classical pathway (406, 407).

Complement-targeted therapeutics:

The complement system in involved a host of clinical pathologies, from autoimmune disorders to ischaemia-reperfusion injuries in almost every organ. Its involvement is more easily characterised due to the simplicity of the cascade and a definitive end product (MAC) that can be measured.

Complement-targeted therapeutics currently target five main avenues (408):

- *Serine proteases* that are integral to the complement cascade (C1r, C1s, C2a, MASP-1, MASP-2, factor D, factor B, factor I) with only C1-inhibitor (C1-INH) currently used in clinical practice for treatment of hereditary angioneurotic edema
- *Complement antibodies* like monoclonal antibodies to C5, and this has translated into clinical practice in the form of Eculizumab which is used in the treatment of Paroxysmal nocturnal hemoglobinuria, atypical HUS and refractory humoral allograft rejection (409)
- *Complement component inhibitors* which target specific complement components like drug Compstatin (prevents the cleavage of C3 to its active fragments C3a and C3b) and drug Pegaptanib (short aptamer molecule directed against C5) (410)
- *Anaphylatoxin receptor antagonists* that selectively inhibit the binding of C5a to its receptors (C5a receptor and C5L2), like PMX-53 which is still in animal trials (411)
- *Soluble Complement Regulators* like sCR1/TP-10, TP20 (sCR1 with sialyl LewisX –glycosylation), Mirococept/APT070 (a truncated sCR1 with a membrane-tethering motif) and soluble forms of MCP, DAF and CD59 which are still in early development phase (408)

Though complement system research has been on going for the last many years, very few studies have translated into clinical practice. sCR1/TP-10 has been applied into clinical practice especially in human coronary artery bypass studies, however results were inconclusive. Single dose sCR1 immediately before surgery (like in our model) was found to inhibit complement activation for up to 3 days postoperatively with significant improvement of the clinical end-points in males, but no benefits in female patients (412).

sCR1 and renal IRI:

As discussed earlier in the results section, there is a paucity of research in the field of complement and renal IRI. sCR1 showed promise in a transplant model, however it did not translate into further studies. In our study, we have clearly demonstrated the role of complement cascade in the IRI process, and the effect of sCR1 in attenuating the renal IRI. sCR1 also showed decreases leukocyte infiltrate which is most likely related to the prevention of formation of the anaphylotoxins. The endothelial protection especially using three markers is new to literature, and may offer further avenues to explore in future. We also demonstrated the loss of local complement regulator CD59 with injury, and protection with sCR1. We also showed that ablation of complement system in the ischaemia-reperfusion phase offers a 'time slot' where complement intervention could be initiated.

The results should be borne in mind with the model used in our study. We had a moderate injury scale, and a 48-hour observation period. Whether the effects will vary with increased severity of injury (60-120 mins of ischaemia) or longer observation period needs to be explored. The ideal model would be a transplant model with treatment started in donor and

carried all the way in to the recipient. We hope to establish such a model in the future based on the findings from this study.

6.2.3. Endothelium

Endothelium is the main target, activator and propagator of renal IRI. Endothelial protection using sphingosine 1-phospate (that maintains endothelial cell integrity and inhibits lymphocyte extravasations) or prostacyclin analogue iloprost have conferred protection against IRI (12, 337). Coating of renal endothelial cells with vaccinia virus complement control protein (VCP) via fusogenic lipid vesicle (FLVs) delivery system prior to renal IR in a rat model showed structural and functional protection from IRI (338). Endothelium also expresses various adhesion molecules that facilitate leukocyte adhesion and rolling. Blocking the action of these adhesion molecules also has shown protection from IRI, and has been reviewed in the introduction chapter. Manipulating endothelial dysfunction offers an attractive proposition, but the complex and intricate relations that endothelium shares with a variety of mediators may make it hard for it to be an 'isolated treatment target'.

The early stages of IR lead to endothelial damage, with the ensuing hypoxia shown to activate angiogenic factors like vascular endothelial growth factor (VEGF), and mediators like Hypoxia Inducible Factor-1α (HIF-1α) and mTOR, that facilitate the angiogenic response (413). Targeting these mediators early in the IR phase may also offer an avenue to improve the vascular response to IR. Endothelial activation also leads to aggregation of platelets, and activation of the coagulation cascade. In a swine transplant model, addition of antithrombin to the collection and preservation protocols showed protection of the

transplanted grafts with reduced inflammatory infiltrate and endothelial protection (413). The activated coagulation cascade leads to formation of fibrin plugs with activated platelets leading to hemostasis, but now evidence is emerging showing the ability of the coagulation system to orchestrate long term inflammatory and fibroproliferative responses (414), and future studies should evaluate this with both short-term (days) and long-term models (weeks).

Endothelium and IRI:

In our study, histological scoring, immunohistochemical stainings and RT-qPCR evaluated the endothelial response to IRI. Compared to the sham group, the IR group demonstrated significant endothelial injury. Both ATG and sCR1 offered significant endothelial protection. This may be secondary to the dampened inflammatory response or the ablated complement cascade. Measuring coagulation cascade products should be explored in future studies especially with increasing evidence that angiogenic factors orchestrate fibroproliferative response. Studies should also be considered in clinical practice to evaluate transplant renal biopsies post ATG induction for endothelial response. Complement modulation is being introduced clinical practice at organ storage level, and using biomarkers for endothelial injury will add another dimension to these studies.

6.3. Conclusions

To conclude, I set out on this project with the hypothesis that there are 'three' key determinants of outcomes following IRI in the context of kidney transplantation: The responses of leukocytes, endothelium and the complement system. I have performed experimental work to advance understanding of these processes. My main objectives prior to the study (italics) and my conclusions from this study are:

1. To determine the nature of ischaemia-reperfusion injury and classifying the leukocyte infiltrates, evaluate endothelial damage and discuss the role of the complement system.

I have comprehensively shown that IRI is an inflammatory process with increased leukocyte infiltrates and upregulated cytokine response following injury. I have quantified endothelial damage on a wide scale using histology, immunohistochemistry and RT-qPCR, and have used markers that have not been used before to quantify endothelial damage. I have also evaluated activated complement system products (C3, C9), and also demonstrated the loss of local complementary regulator (CD59) in this model. In the IRI process, all the three key determinants were involved and contributed to the injury.

2. Using ATG as a 'leukocyte modulator' and determining whether it attenuates IR injury in rats as the reports are inconclusive. We will also ascertain the effect of ATG on the leukocytes and the endothelium.

Contrary to previous reports, I have shown that ATG confers protection from renal IRI in this model. The leukocyte response is well documented in the literature, and I have shown again in this study, albeit be using more markers (CD3, CD4, CD8, CD15, CD68). I have also shown new insights into the effect of ATG on the endothelium, with the protection

offered contributing to reduction in the injury scores. I have also shown decreased complement C3 deposits indicating a decreased activation of the complement system, which is also new in literature and this is probably due to reduced injury, but further studies would be needed to see if any specific pathway of complement activation is targeted by polyclonal ATG.

3. Using SCR-1 and anti-MASP-2 antibodies, we aim to modulate the effects of the complement system on renal ischaemia reperfusion, along with evaluating the effects on leukocytes and the endothelium

With regards to sCR1, we demonstrated that a single dose of SCR1 was able attenuate IRI, which coincidentally links with TP-10 studies in cardiac bypass, and is new in literature especially the model we have used. I have also shown that attenuation of complement system is the IR phase offers an avenue to mitigate IRI, and these interesting finding needs to be explored in future models. I have also shown endothelial protection using sCR1 on a comprehensive scale, and demonstrated reduced leukocyte infiltrates. Unfortunately, I was unable to fully evaluate the anti-MASP-2 antibody in this current period of research, but have set out plans in the methodology section that can pave way for future research project.

Clinical perspective:

Clinical transplantation involves the donor, the organ and the recipient, and all these three elements contribute to IRI. ATG currently is being used in clinical practice and has some benefits in reducing the DGF/IRI in clinical practice, but the results are equivocal. My research study and other animal studies have shown that ATG can attenuate IRI in the native kidney and in transplant models (donor ATG treatment). Currently, due to ethical

issues, donor treatment prior to donation is still in infancy. We are aware that ATG preservation solutions are being trialled and we await the outcomes from that. With regards to recipient treatment, ATG has shown to affect leuckocyte count and is used in induction and rejection episodes following transplantation, mainly as an immunological agent. Further clinical studies may demonstrate the effect of ATG on endothelium, and needs to be explored. There are multiple variables in the IRI process, and the polyclonal antibody nature of ATG makes it an ideal IRI attenuating drug and further studies, both animal (with increased severity and transplantation models) and clinical may help define the role of ATG as an IRI attenuating agent.

Complement inhibition also offers an attractive proposition with human studies confirming the role of complement system. However, the review of complement-targeted therapeutics revealed that very few drugs have made it to the clinical shelf. The reasons are multifactorial, but the overriding reason seems to be the complexity of targeting one system that has multiple interactions with other systems. There is reason for optimism, but needs to carefully explored in models having severe injury /transplant models. Currently, Cardiff is part of a multicentre randomised controlled trial in renal transplantation, EMPIRIKAL, where mirococept (similar to sCR1) will be given as a treatment to the donor kidney prior to transplantation. The outcomes from this study will also define the role of preservation solutions with additives in attenuating IRI.

Overall, I have shown that IRI is mediated by three key elements that interact in complex mechanisms, and having a drug that targets all these systems may offer any avenue to attenuate IRI in the transplantation setting.

APPENDICES

Appendix A: Protocol

ATG protocol

Operative part:

- General Anaesthesia
- Time zero blood sample from tail tip (T0) 0.00 minutes
- Inject ATG at the desired dose in the penile vein
- Laparotomy + Left renal pedicle IRI for 40.00 minutes
- Recovery

48 hours later:

- Blood sample (T1) and retrieval of kidneys : 48 hours

Blood sampling:

- 24 blood sample was collected in EDTA lined tubes/syringe (by Dr. Brad Spiller)
- 48 hour sample collected in EDTA eppendorfs from the inferior vena cava
- Protocol for CD3 analysis explained later in this chapter

sCR1 PROTOCOL

sCR1 dose: 5.3 mg/ ml Dosage: 25mg/kg body weight

Operative part:

- General Anaesthesia
- Time zero blood sample from tail tip (T0) **: 0.00 minutes**
- Inject sCR1 at the desired dose in the penile vein
- Laparotomy + Left renal pedicle IRI : **20 minutes**
- 1 hour post blood sample prior to reperfusion (T1) : **60 minutes**
- Recovery

24 hours later:

- GA and blood sample (T2) : **24 hours**

48 hours later:

- Big blood sample (T3) and retrieval of kidneys : **48 hours**

Blood sampling:

- Tail tip into 500ul tubes and place it on ice
- Spin at 1300 G for 15 minutes at 5 degree centigrade
- Transfer amber plasma cells into fresh tubes
- Store in -70 deg centigrade

Anti-MASP2 ab protocol

Anti- MASP2 antibody dose: 1 mg/kg body weight

Each vial: 250 ug – mixed with cold saline and appropriate volume injected according to the body weight

Operative part:

- General Anaesthesia
- Time zero blood sample from tail tip (T0)- 0.00 minutes
- Inject MASP2 at the desired dose in the penile vein
- Laparotomy + Left renal pedicle IRI at 20.00 minutes
- 1 hour post blood sample prior to reperfusion (T1) at 60 minutes
- Recovery

24 hours later:

- GA and blood sample (T2) : 24 hours

48 hours later:

- Big blood sample (T3) and retrieval of kidneys : 48 hours

Blood sampling:

- Tail tip (0.1 to 0.2 mls) into 500ul tubes , and place it on ice
- Spin at 1300 G for 15 minutes at 5 degree centigrade
- Transfer amber plasma cells into fresh tubes
- Store in -70 deg centigrade

Appendix B: Reagents and Materials

Reagents:

1. Hydrogen Peroxide solution, 30 wt. % in H_2O, ACS reagent, ACS REAGENT (Cat :21,676-3, Lot – S28647-265, Sigma- Aldrich)
2. Phosphate Buffered SALINE (10X solution, BP 399-20, Fischer Scientific limited, Lot – 102355)
3. Amboceptor (2 ml, Catalog Number: 9002; Institut Virion\Serion GmbH
4. Sulfuric acid solution 5M (10N) volumetric analysis (J/8400/24, Fischer Scientific Ltd)
5. Tween 20 (BP337-500, Fischer Scientific Ltd)
6. OPD EASY-tablets for ELISA, 2mg/tablet(Code : 327972000 ; Acros Organics)
7. Code Number: 711-035-152
8. Peroxidase AffiniPure Donkey Anti-Rabbit IgG (Code Number: 711-035-152 , Jackson Immuno Research Lab)
9. Complement Fixation Tablets (Product No. BR0016, Oxoid Limited, UK)
10. Mannan from *Saccharomyces cerevisiae* (Product Number: M7504, Sigma-Aldrich)
11. Veronal Buffered Saline (NC9460913; Fischer Scientific limited
12. 96 well high affinity plate (Immuno 96 MicroWell™ Solid Plates, . MaxiSorp. Thermo Scientific Nunc MicroWell ELISA,), maxisorb-442404,

qPCR Related :

1. MicroAmp Fast optical 96-well reaction plate with Barcode, 0.1 mL (Cat: 4346906, Applied Biosystems, Invitrogen)
2. MicroAmp Optical Adhesive Film PCR Compatible,(Cat :4311971, Applied Biosystems, Invitrogen)
3. QPCR Analyses– 7900 HT Fast Real-Time PCR System, Applied Biosystems
4. RNAse free Water, Millipore Milli-Q Synthesis
5. Power SYBR® Green PCR Master Mix and Power SYBR® Green RT-PCR Reagents Kit (Catalog Number 4368577, 4367659, 4367660, 4368706, 4368702, 4368708 (Master Mix) and 4368711 (RT-PCR Reagents Kit), Applied Biosystems, Life Technologies).
6. NanoDrop-1000 spectrophotometer (Thermo Fisher, Pittsburgh, PA)
7. Bioanalyzer 2100 (Agilent Technologies, USA)
8. TRIzol® Reagent (Cat: 15596-026, Invitrogen)
9. Chloroform (Cat: C2432, Sigma-Aldrich)
10. 2-Propanol (Cat: I9516, Sigma-Aldrich)
11. Ethanol Absolute (Cat: E/0650DF/17, Fischer Scietific)
12. High Capacity cDNA Reverse Transcription Kit (Cat: 4368814, Invitrogen)
13. Ethanol absolute (Cat: 1.08543.0250, Merck KGaA)
14. FlameStrip Strips for QPCR (Cat: 4ti-0785/B, 4titude Ltd)

Immunohistochemistry:

1. DAKO EnVision™ Detection Systems Peroxidase/DAB, Rabbit/Mouse (Cat no: Code K5007, DAKO)

Appendix C: Nano drop and RIN values

	Sample ID	weight (mgs)	ng/ul	A260	A280	260/280	260/230	RIN VALUES
40 min IRI	1	305	285.41	7.135	3.725	1.92	2.19	3.9
	2	168	272.05	6.801	3.524	1.93	2.23	2.4
	3	250	242.59	6.065	3.136	1.93	2	2.7
	4	250	336.51	8.413	4.357	1.93	1.99	3.8
	5	302	299.28	7.482	3.904	1.92	2.11	2.3
	6	296	260.79	6.52	3.332	1.96	2.16	4.6
	7	220	326.3	8.158	4.138	1.97	2.05	3.9
	8	253	242.04	6.051	3.125	1.94	2.25	2.7
	9	152	250.22	6.255	3.264	1.92	2.27	3.3
	10	217	344.16	8.604	4.431	1.94	2.25	3.3
SHAM	1	190	718.61	17.965	9.11	1.97	2.28	2.2
	2	200	493.4	12.335	6.246	1.97	2.31	2.2
	3	187	446.55	11.164	5.783	1.93	2.2	2.4
	4	190	426.19	10.655	5.537	1.92	2.25	2.4
	5	125	324.14	8.104	4.173	1.94	2.26	2.5
	6	162	522.61	13.065	6.535	2	2.25	2.5
ATG	1	155	498.43	12.461	6.434	1.94	2.27	3.2
	2	146	578.32	14.458	7.403	1.95	2.29	2.8
	3	230	538.02	13.451	6.733	2	2.27	5.4
	4	204	816.66	20.416	10.128	2.02	2.3	3.2
	5	214	1032.1	25.803	12.757	2.02	2.27	3.9
	6	160	620.94	15.524	7.832	1.98	2.29	4.6
	7	205	559.28	13.982	7.002	2	2.26	3.6
	8	197	543.59	13.59	6.795	2	2.3	3
ATG Isotype	1	314	601.27	15.032	7.504	2	2.29	5.8
	2	317	411.42	10.285	5.205	1.98	1.68	3.1
	3	201	374.04	9.351	4.808	1.94	2.28	2.7
	4	199	406.04	10.151	5.254	1.93	2.26	3
	5	302	605.86	15.147	7.653	1.98	2.28	6.1
	6	253	1010.47	25.262	12.483	2.02	2.29	2.6
	7	240	1126.18	28.155	13.806	2.04	2.26	6.7
	8	201	574.27	14.357	7.118	2.02	2.28	6.1

	Sample ID	weight (mgs)	ng/ul	A260	A280	260/280	260/230	RIN VALUES
MASP	1	340	508.93	12.723	6.326	2.01	2.19	3.5
	2	215	301.6	7.54	3.9	1.93	2.21	4.3
	3	236	615.11	15.378	7.753	1.98	2.21	5.4
	4	240	426.43	10.661	5.439	1.96	2.21	7.2
	5	238	684.67	17.117	8.489	2.02	2.21	4.2
	6	225	701.94	17.548	8.749	2.01	2.2	4.2
MASP Isotype	1	263	321.33	8.033	4.108	1.96	2.22	6.3
	2	204	631.81	15.795	7.956	1.99	2.25	4.6
	3	238	681.55	17.039	8.412	2.03	2.23	4.9
	4	234	599.33	14.983	7.519	1.99	2.16	5.9
	5	302	441.75	11.044	5.64	1.96	2.18	4.4
	6	225	632.43	15.811	7.877	2.01	2.21	7.8
SCR1	1	240	443.71	11.093	5.613	1.98	2.24	3.1
	2	300	357.33	8.933	4.513	1.98	2.17	4.3
	3	191	674.47	16.862	8.358	2.02	2.3	2.6
	4	212	617.51	15.438	7.628	2.02	2.25	3.1
	5	200	473.62	11.84	6.052	1.96	2.19	3.6
	6	240	243	6.075	3.09	1.97	2.22	3.4
	7	240	147.11	3.678	1.9	1.94	2.25	5.1
	8	272	614.79	15.37	7.596	2.02	2.28	4.1

rin test on 7/12/12 on new solutions from stock created (1/20 dilutions: 57 ul of H20 + 3 ul of stock solution ; 5 ul of this solution sent for RIN testing)

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