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Development of mouse models to investigate the effects of brain death on organ transplantation.

ZANE ZHANXIANG WANG

A thesis submitted in fulfillment of the requirements for the degree of Master of Philosophy

Submitted: 2nd September 2013
DECLARATION

This thesis is the result of full time study during the period from February 2011 to September 2013. The work, except where duly acknowledged, is my own, and has not been submitted for any other degree at this, or any other University.

Zane Zhanxiang Wang
BBiotech
September 2013
ACKNOWLEDGEMENTS

There are many people who I owe a great debt of gratitude to for their contribution to this project. First and foremost, I would like to express my deepest debt of gratitude to A/Prof Alexandra Sharland, my primary supervisor, for her continued support and interest as well as patience and generosity with her time. Second, I would like to thank Dr Chuanmin Wang for his guidance in the development of the brain death model as well as conducting all the renal transplants. At the same time, I would like to send my token of appreciation to my co-supervisor Dr Alexander Bishop for his knowledge and assistance. I would also like to express my gratitude to Moumita Paul for preparing the viruses and running the ELISAs.

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PUBLICATIONS ARISING FROM THIS THESIS


Chuanmin Wang, Zane Z Wang, Richard Allen, G Alex Bishop, Alexandra F Sharland. (2013), A modified method for heterotopic mouse heart transplantation, JoVE. (accepted)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BD</td>
<td>Brain Death</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced Glycation Endproduct</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Transaminase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>ATN</td>
<td>Acute Tubular Necrosis</td>
</tr>
<tr>
<td>B6</td>
<td>C56BL/6</td>
</tr>
<tr>
<td>BP</td>
<td>Blood Pressure</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C Chemokine Ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C Chemokine Receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cf</td>
<td>compared with</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C motif Chemokine</td>
</tr>
<tr>
<td>d1</td>
<td>day 1</td>
</tr>
<tr>
<td>d2</td>
<td>day 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>d4</td>
<td>day 4</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DN RAGE</td>
<td>Dominant Negative RAGE</td>
</tr>
<tr>
<td>eGFP</td>
<td>encoding Green Fluorescent Protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>esRAGE</td>
<td>endogenous secretory RAGE</td>
</tr>
<tr>
<td>F.IX</td>
<td>Factor IX</td>
</tr>
<tr>
<td>FIO2</td>
<td>Fraction of Inspired Oxygen</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Foxhead Box P3</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin staining</td>
</tr>
<tr>
<td>hAAT</td>
<td>Human α-1 Antitrypsin</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leokocyte Antigen</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-Mobility Group Protein 1</td>
</tr>
<tr>
<td>HPF</td>
<td>High Powered (40x) Field</td>
</tr>
<tr>
<td>HR</td>
<td>Heart Rate</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human Serum Albumin</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular Injection</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal Injection</td>
</tr>
<tr>
<td>IRI</td>
<td>Ischemia Reperfusion Injury</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous Injection</td>
</tr>
<tr>
<td>IVC</td>
<td>Inferior Veno Cava</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAP kinase</td>
<td>Mitogen-Activated Protein kinase</td>
</tr>
<tr>
<td>MCP 1</td>
<td>Monocyte Chemotactic Protein-1</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>MIP-2</td>
<td>Macrophage Inflammatory Protein 2</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>MyD88</td>
<td>Myeloid Differentiation factor 88</td>
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<td>NAD(P)H</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate-oxidase</td>
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<tr>
<td>NF</td>
<td>Nuclear Factor</td>
</tr>
<tr>
<td>NGAL</td>
<td>Neutrophil Gelatinase-Associated Lipocalin</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal Goat Serum</td>
</tr>
<tr>
<td>NHS</td>
<td>Normal Horse Serum</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural Killer Cells</td>
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</tbody>
</table>
NMS  Normal Mouse Serum
OCT  Optimum Cutting Temperature
PAMP  Pathogen-Associated Molecular Patterns
PBS  Phosphate Buffered Saline
PCNA  Proliferating Cell Nuclear Antigen
PCR  Polymerase Chain Reaction
PEEP  Positive End-Expiratory Pressure
PRR  Pattern Recognition Receptor
rAAV  recombinant Adeno-Associated Virus
RAGE  Receptor for Advanced Glycation Endproducts
RANTES  Regulated on Activation, Normal T cell Expressed and Secreted
ROS  Reactive Oxygen Species
RT  Room Temperature
SO2  Saturated Oxygen
sRAGE  soluble Receptor for Advanced Glycation Endproducts
ssDNA  single stranded DNA
TDW  Triple Distilled Water
TGF  Transforming Growth Factor
TIR  Toll-Interleukin-1 Receptor
TLR  Toll-like receptor
TNF  Tumor Necrosis Factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>Tregs</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid-Stimulating Hormone</td>
</tr>
<tr>
<td>Tx</td>
<td>Transplant</td>
</tr>
<tr>
<td>VG</td>
<td>Vector Genome</td>
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ABSTRACT

Organ transplantation is the most effective and at times the only method for treating patients with end stage organ failure. Studies have shown that kidney transplants from living donors are consistently more successful and longer lasting than those from brain dead donors, regardless of the degree of HLA matching. With more than 90% of transplantable organs coming from deceased donors, it is of vital importance to study the causes of the deterioration within these organs. The purpose of my study was to mimic the clinical setting of brain death (BD) in a mouse model and, to use this model to study the effects of brain death on donor organs as well as examining these organs post-transplantation. We showed that brain death in this model was accompanied by the expected physiological changes, and that as reported for other similar models, a systemic pro-inflammatory state ensues, with release of alarmins including S100B and HMGB1, and rapid elevation in circulating levels of cytokines and chemokines such as IL-6, TNF-α, IL-1β, MCP-1 and KC. Biochemical evidence of kidney damage was provided by increased serum concentrations of NGAL, an early biomarker of acute kidney injury.

After determining the optimum method for ureteric reconstruction, we collected kidneys from BD donors, and performed syngeneic renal transplants to examine how donor brain death amplifies the ischaemia-reperfusion injury which inevitably accompanies any transplantation procedure. As anticipated, we found that donor brain death resulted in upregulation of a number of pro-inflammatory cytokines.
and chemokines in transplanted kidneys, and in substantially increased infiltration with neutrophils and macrophages.

It has been shown that esRAGE is capable of reducing inflammation by competitively binding to RAGE ligands. In order to study the effects of esRAGE upon IRI in brain-death and subsequent syngeneic renal transplantation, we generated an rAAV vector encoding esRAGE. Intraperitoneal injection of this vector resulted in strong, liver-specific expression of esRAGE. esRAGE significantly reduced the amount of HMGB1 in the serum of brain-dead mice. The most striking effect of esRAGE expression in mice undergoing brain death and syngeneic renal transplantation was a dramatic reduction in the numbers of infiltrating neutrophils and macrophages within the kidneys. Further, substantially increased numbers of proliferating cells were demonstrated in the kidneys of esRAGE-treated mice at d4 after syngeneic transplantation from BD donors, compared with those of uninjected mice and particularly with those of mice receiving the control vector. Given that the grafts from esRAGE–treated mice had reduced numbers of infiltrating leucocytes compared to those in the other groups, these are likely to be renal parenchymal cells. These effects are likely to be translated into improved graft function, and augmented ability of the graft to sustain life. RAGE signaling on T cells contributes to adaptive immune responses, including allore sponses. Testing of esRAGE at later timepoints when the transplanted kidneys are life-sustaining and in allotransplant models where its effects upon the adaptive immune response are predicted to be additive to those on non antigen-specific graft inflammation is the logical next step for this line of investigation, and will shortly be undertaken.
CHAPTER 1: INTRODUCTION

1.1 Deceased donor organ transplantation

Organ transplantation is the most effective and at times the only method for treating patients with end stage organ failure. In Australia, in the year 2011, a total of 1,192 organs were transplanted which consisted of 586 kidneys, 199 livers, 66 hearts, 306 lungs, 26 pancreas and 9 pancreatic islet grafts. Nonetheless, at the start of the year 2012, there were still 1518 patients on the organ transplantation waiting list [1]. This reflects not only the relative rarity of organ donation in Australia, compared with the incidence of end-stage organ failure, but also the need for re-transplantation following failure of the primary graft. Although transplantation is a well-established therapeutic modality, a number of challenges remain. Immunosuppressive drugs are used to dampen the host immune response and prolong graft survival. However, despite the introduction of increasingly powerful immunosuppressive regimes, long term graft survival has improved little over the past two decades [2-4]. The quality of the transplantable organ is emerging as an important factor in determining long term graft outcomes. A majority of the organs used for transplantation are donated from heart beating, brain dead donors. These organs are exposed to many insults, including brain death (BD) with catecholamine storm and vascular instability. Procurement, cold ischemia and transportation as well as warm ischemia and reperfusion in the recipient are also damaging to the transplantable organs. All these contribute to the ischemia reperfusion injury (IRI) which may potentiate graft rejection and oppose the
development of transplantation tolerance. Studies have shown that outcomes from organs of BD donors are significantly worse than those from living unrelated donors irrespective of the degree of HLA matching [5]. Understanding the mechanisms by which brain death damages donor organs will allow for the development of better therapeutic options for the management of deceased donors and ultimately will improve outcomes for transplant recipients.

1.2 Outcomes of deceased donor organ transplantation

Most organs for transplantation are procured from brain dead donors despite recent efforts to increase the rate of donation after circulatory death [6, 7]. BD in the donor is linked to impaired graft quality and is clinically associated with compromised patient and graft survival [8-12]. Despite advancements in immunosuppression, outcomes for patients receiving cadaveric organs are still consistently worse in both the short and long term than outcomes for those receiving organs from completely mismatched HLA living donors [5]. Similarly, patients with cadaveric organs experience more rejection episodes and are at a higher risk of primary graft failure [5, 8-10, 13-18]. According to the ANZDATA registry for the period 1991-2010, for kidney transplants performed on first time (primary transplant) recipients, the vast majority of the transplanted kidneys survived 1 year after transplantation with the survival rate from living donor kidneys being 96.8%, and that of deceased donor kidneys being 92.1%. From the same database, the 5-year survival rate for primary transplants is reported as 87.7% for living donor transplants and 80.9% for deceased donor transplants. Graft
survival rates progressively drop off 10, 15 and 20 years after transplantation. Nevertheless, primary transplants from a living donor on average result in longer transplant survival than deceased donor transplants [19]. This survival benefit extends to HLA mismatched living donors. These results are widely reflected by many animal studies [8, 16, 20-22].

1.3 Physiological changes of brain death

The Cushing reflex is a well-recognised sign of brain death on the onset of raised intracranial pressure with a rise in systolic blood pressure. Brain death is accompanied by an immediate short period of hypertension and bradycardia followed by a massive release of catecholamines leading to vasoconstriction, fluctuations in blood pressure, endothelial injury, hypothermia, electrolyte abnormalities, tachyarrhythmias and coagulopathies [12, 23]. This phenomenon has been termed the ‘catecholamine storm’. Following this outpour of catecholamines, hypotension and reduced peripheral vascular resistance may result from a combination of the body failing to maintain circulating catecholamines and a loss of sympathetic tone [24-26]. Reduced oxygen delivery no longer meets myocardial oxygen demand causing global myocardial ischemia. This in turn further compromises the perfusion of peripheral organs, leading to endothelial activation and ischemia-reperfusion injury [27-31]. All of these processes contribute to a pro-inflammatory environment in the donor, which has been demonstrated in various animal studies [22, 32, 33].
Hormone depletion occurs as a result of pituitary infarction caused by brain death. The pituitary is a small gland connected to the hypothalamus and produces many of the hormones that control essential body processes. These include thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH) and vasopressin, also known as anti-diuretic hormone. Deficiency of anti-diuretic hormone is manifested clinically as diabetes insipidus, where production of large volumes of dilute urine further destabilises fluid balance and end-organ perfusion [34].

1.4 Innate immunity in Brain Death and Ischemia Reperfusion Injury

Allograft injury negatively affects both short and long term graft function, and amplifies graft rejection [35]. The causes of allograft injury can be linked to brain death, warm and cold ischemia and reperfusion [8-10, 13, 36, 37]. Peri-mortem events in BD donors lead to haemodynamic, hormonal, metabolic and inflammatory changes, all impacting the quality of transplantable organs and increasing graft immunogenicity. Many facets of the host immune system have been identified as contributors to the poor function of allografts. Initially, it was thought that the adaptive immune system was responsible for these effects but more recent findings have pointed towards the innate immune system playing an important role in both the acute and chronic rejection of allografts. The mechanisms by which donor organs are damaged include the up-regulation and activation of toll-like receptors and other pattern recognition receptors, release of cytokines and chemokines, upregulation of adhesion molecules and class II MHC on allograft endothelium and,
complement activation and thrombin deposition with depletion of tissue plasminogen activator [11, 12, 38-45].

1.5 Components of the innate immune response

1.5.1 Pattern-recognition receptors

Pattern-recognition receptors involved in innate immune responses include C-type lectin receptors, NOD-like receptors, RIG-like receptors, Toll-like receptors and RAGE [46]. Of these, the best characterised in terms of their role in ischaemia-reperfusion injury of the various transplantable organs are the Toll-like receptors and RAGE.

1.5.1a Toll-like receptors

Toll like receptors (TLRs) are a set of germline-encoded receptors that play an important role in the innate immune response. They are expressed on a variety of cell types including macrophages, dendritic cells, neutrophils, B cells, NK cells, eosinophils, mast cells, basophils as well as on the parenchymal cells of some organs including tubular epithelial and mesangial cells within the kidney [47-49]. TLRs recognise conserved molecular patterns known as pathogen-associated molecular patterns (PAMPs) which are shared by large groups of microorganisms. They are also able to recognise endogenous ligands released by damaged tissues which are known as alarmins [50, 51]. Various alarmins have been implicated in TLR engagement. Some of these include high-mobility group box 1 (HMGB1), hyaluronan, fibrinogen, fibronectin, heparan sulphate, surfactant protein A and β-
defensin 2 lymphoma antigen [43, 50, 52-57]. When TLRs are activated, adaptor molecules are recruited to the receptors through their respective Toll/Interleukin-1 receptors (TIR) domain, which interacts with the TIR domains of TLRs. Myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) are the major adaptor molecules for TLRs, and TLR-activated signalling pathways can be classified into MyD88-dependent and –independent pathways, depending upon utilisation of these adaptors [58].

MyD88 recruitment predominates, and activates a downstream family of kinases, IL-1 receptor-associated kinases (IRAKs) 1, 2 and 4. Signalling events further downstream the release of NF-κB from inhibitor of NF-κB (IκB). The released NF-κB then moves to the nucleus and promotes an inflammatory response through increasing proinflammatory cytokine gene expression [58, 59]. TLRs can contribute to both innate and adaptive immune responses depending upon the cell types receiving signals. For instance, TLR4 engagement on renal tubular cells results in chemokine production and neutrophil recruitment, as well as the expression of activating ligands for natural killer cells, thus mainly influencing the innate response, illustrated in Figure 1 and 2 below. In contrast, TLR4 binding on dendritic cells potentiates adaptive immunity by upregulating MHC class II and co-stimulatory molecules which are important for antigen presentation to naïve T cells, and by modulating the expression of chemokine receptors CCR6 and CCR7, thereby directing the DC to the T cell regions of peripheral lymph nodes where antigen presentation can occur [60-62], Figure 3.
Figure 1 Downstream effects of TLR4 signalling on parenchymal cells. Engagement of TLR4 on renal tubular cells activates the MyD88-dependent pro-inflammatory pathway, resulting in the production of chemokines such as IL8, MCP-1 and RANTES and recruitment of neutrophils. At the same time, this engagement also results in the expression of activating ligands such as NKG2D, for natural killer (NK) cells, thus, mainly influencing the innate response.
Figure 2 Activation of the endothelium via TLRs and cytokines. An inflammatory response can occur through the engagement of TLRs and/or the binding of cytokines and chemokines, such as those released from TLR4 signalling on parenchymal cells (Figure 1), to the endothelium. Bound chemokines attract leucocytes and this, in turn, increases local levels of cytokines, such as TNF-α. The release of cytokines then up-regulates E-selectin and intercellular adhesion molecule-1 (ICAM-1) and this facilitates leucocyte migration into tissues.
Figure 3 Process of monocytes maturing into myeloid dendritic cells (DC) in response to TLR signalling. TLR binding on dendritic cells potentiates adaptive immunity by upregulating MHC class II and co-stimulatory molecules which are important for antigen presentation to naïve T cells, and by modulating the expression of chemokine receptors (decrease in CCR6 and increase in CCR7), thereby directing the DC to the T cell regions of peripheral lymph nodes where antigen presentation can occur.
TLRs are recognised as one of the most potent contributors to pathogen- and injury-induced inflammation. During IRI, both endogenous and exogenous ligands are released by damaged tissues which can bind to TLRs and in turn, activate the innate immune system [63].

The expression and roles of TLR 2 and 4 have been widely studied in many organs following IRI. Studies have shown that TLR 4-dependent signals following IRI promote injury of the heart, lungs, liver and kidneys [24, 43, 44, 64, 65]. TLR engagement during IRI results in the release of proinflammatory cytokines and chemokines, with recruitment of neutrophils macrophages and T cells to the ischaemic tissues. In a positive feedback loop, cytokines such as IL-1β and TNF-α have been shown to further increase TLR 2 and TLR 4 mRNA levels in hepatocytes [66, 67]. Similarly, in kidneys, levels of TLR 2 and TLR 4 mRNA in the renal tubular epithelial cells are significantly increased by ischemia [68, 69]. Whereas TLR4 deficiency was universally protective in renal, liver, lung or myocardial IRI, there are differences between organs in the relative importance of TLR4 engagement on different cell types, the contributions of the various adaptor molecules and the involvement of additional TLRs such as TLR2 in the pathogenesis of IRI. Ischaemic insults to the liver trigger innate immunity by activating TLRs in both parenchymal and nonparenchymal cells [43, 66, 67, 70], whereas in the kidney, signalling via TLRs on cells of the parenchymal compartment plays the predominant role in IRI propogation [42, 44]. Equivalent protection against renal IRI was afforded by either TLR4 or MyD88 deficiency [44], whilst Shigeoka et al showed that TLR 2 deficient
mice were better protected against IRI than MyD88 deficient mice, suggesting that the pathways contributing to TLR 2-mediated kidney damage following ischaemia involve other adaptors in addition to MyD88 [71]. Further studies attempting to determine the relative contributions of TLR 2 and 4 to renal IRI showed that IRI was attenuated in both single TLR 2 and TLR 4 deficient mice. Surprisingly, in mice with both TLR 2 and 4 deficiencies, no increased protection was observed when compared to the single TLR 2 or 4 deficient mice [72].

TLR 4-deficient mice exposed to warm liver ischaemia developed significantly less inflammation and hepatocellular damage than wild-type controls. However, in contrast to renal IRI, the liver injury was not reduced in MyD88 deficient mice [73], implying that TLR 4 induced inflammation in liver following ischaemia is mainly mediated via the MyD88 independent pathway. Similar to TLR 4, TLR 2 is an important player in the initiation of inflammation following renal ischaemia [42, 71]. Abrogation of TLR2 signalling by either genetic deficiency of TLR2 or antisense RNA treatment resulted in less tubular apoptosis, reduced cellular infiltrate, less renal damage and preservation of renal function [42]. Conversely no protective effect of TLR 2 deficiency could be demonstrated in a mouse model of liver IRI [74].

1.5.1b RAGE

The receptor for advanced glycation endproducts (RAGE) is a member of the immunoglobulin superfamily, encoded in the Class III region of the major histocompatibility complex. The extracellular components of RAGE comprise an Ig variable domain (the principal site of ligand binding), VC1 and C2 domains, a
transmembrane region and a short intracellular tail. RAGE forms oligomers at the cell membrane, and dimerisation of RAGE via interaction of the V regions is essential for signal transduction through the cytoplasmic tail. The domain structure of RAGE is shown in Figure 4 below.

![Domain structure of RAGE](image)

Figure 4 The domain structure of RAGE.

RAGE is a multiligand receptor which plays a crucial role in innate immunity against pathogens, but also interacts with endogenous ligands, resulting in chronic inflammation [75, 76]. RAGE is expressed in many tissues and cell types usually at low levels under steady-state conditions. RAGE is expressed in all transplantable organs, but is particularly abundant in lung where it is produced by type I alveolar epithelial cells [76-78]. Of note, RAGE becomes upregulated at sites where ligands accumulate [76-78]. RAGE recognises tertiary structures rather than amino acid
sequences, thus, giving it the ability to engage classes of molecules rather than individual ligands [76, 78], a key feature of pattern-recognition receptors. RAGE binds inflammatory mediators of the S100 family and, in common with TLRs 2, 4 and 9 is a receptor for HMGB1. Binding of ligands such as S100/calgranulins and HMGB1 to RAGE activates a range of signalling pathways depending on the cell types involved. Intermediates in these pathways include erk1/2 (p44/p42) MAP kinases, p38 and SAPK/JNK MAP kinases, rho GTPases, phosphoinositol-3 kinase, the JAK/STAT pathway and the downstream activation of NF-κB [78-83] leading to the production of pro-inflammatory cytokines, chemokines and adhesion molecules [75]. In addition to the upregulation of adhesion molecules which, in turn, promotes inflammatory cell recruitment, RAGE is directly involved in the recruitment of inflammatory cells as a counter-receptor for leukocyte integrins, interacting with β2-integrins [84]. RAGE suppression reduces the migration and proliferation of macrophages as well as the vascular expression of inflammatory molecules [85].

RAGE exists in a number of isoforms. esRAGE is the major splice variant of RAGE and lacks the c-terminus of the full-length form, but isoforms lacking the N-terminus and therefore without ligand binding activity exist, as does a dominant negative (DN) form with the transmembrane region but lacking the intracellular tail [86, 87].

The relationship between transmembrane and esRAGE is depicted below in Figure 5 below.
Many of the isoforms of RAGE have regulatory effects upon RAGE signalling. DN RAGE binds ligands but cannot transduce a signal [86, 87]. esRAGE has been postulated to inhibit RAGE signaling via several distinct mechanisms. esRAGE is a naturally-occurring decoy receptor which can bind to HMGB1, S100B, AGEs and other RAGE ligands. In healthy human subjects, plasma levels of HMGB1 are inversely related to those of esRAGE, suggesting that esRAGE mediates the clearance of HMGB1 from the circulation [88]. Soluble RAGE V domain binds to the V domain of TM RAGE, preventing dimerisation and thus blocking signal transduction [89]. Expression of RAGE is positively regulated by ligand binding, so that chronic administration of soluble RAGE in mouse models of diabetic
vasculopathy leads to clearance of RAGE ligands from the vessel wall, and to decreased RAGE expression [76].

During IRI, expression of RAGE and its ligands is significantly increased. Suppression of RAGE binding via the injection of soluble RAGE (sRAGE) decreases the infiltration of inflammatory effector cells into tissues along with decreased expression of proinflammatory cytokines [90, 91]. Administration of soluble RAGE promotes survival after partial warm liver IRI and resection of the non-ischaemic fragment. In a seeming paradox, levels of some inflammatory cytokines such as IL-6 and TNF-alpha was increased by sRAGE treatment. Proliferation of hepatocytes was also increased [92].

1.5.2 Danger signals or “alarmins”

Alarmins are endogenous mediators that can simultaneously induce the chemotactic migration and activation of antigen-presenting cells (APCs), and consequently promote the induction of immune responses [93, 94]. They are present in leukocytes and various epithelial cells as either granular products or nuclear proteins which are produced rapidly by cells of the host innate immune system in response to infections and/or tissue injuries. Alarmins include defensins, cathelicidin, eosinophil-derived neurotoxin (EDN) and high-mobility group box 1 (HMGB1) protein. They function not only as potent effectors of innate defence but also act to alarm the immune system by promoting the recruitment and activation of host leukocytes through interaction with distinct receptors.
1.5.2a High mobility group box 1

High mobility group box 1 (HMGB1) is a nuclear protein that is released upon cell stress or necrosis, and when extracellular, can exert proinflammatory activities [43, 71, 79, 90, 95, 96]. Apoptotic cells do not release HMGB1 upon death as they modify their chromatin to bind to HMGB1 tightly [65]. HMGB1 can form specific complexes with various molecules such as ssDNA, LPS, IL-1β and nucleosomes which interact with TLR 9, TLR 4, IL 1R and TLR 2 respectively [48, 54, 97]. These complexes elicit a much greater inflammatory response than those due to the uncomplexed danger signal. As an example, as little as 0.5 ng/ml LPS associated with 0.5 µg/ml HMGB1 induces levels of IL-6 release comparable to those produced by 100 ng/ml of LPS alone [55]. HMGB1 can also bind to RAGE to induce inflammatory responses. Amplification of inflammatory responses to CpG DNA in the presence of HMGB1 involves physical association and co-operation between HMGB1 and RAGE [98]. It has not been determined whether similar mechanisms account for the synergistic interactions between HMGB1 and the ligands for TLRs 2 and 4.

HMGB1 is upregulated in ischaemic mouse kidneys, both at the mRNA and at the protein level [44]. During IRI, HMGB1 induces DC cell maturation as well as promotes the secretion of proinflammatory cytokines [68, 70, 99]. Adhesion and migration of macrophages and neutrophils is increased upon stimulation with HMGB1 [69, 100, 101]. In part, this is due to the interaction of MAC-1 and RAGE, thus, activating the adhesive and migratory function of these cells [102].
Furthermore, HMGB1 with the help of neutrophils stimulates production of reactive oxygen species through a TLR 4 dependent activation of NAD(P)H oxidase as well as increases activation of NF-κB, resulting in production and release of cytokines [67, 103].

Many studies using HMGB1 neutralizing antibodies in mice have shown lessening of damage and inflammation following IRI. Wu et al showed that treating mice with neutralizing anti-HMGB1 antibody protected them against kidney IRI, mediated by HMGB1/TLR 4 signalling through the MyD88 dependent pathway, reflected in lower serum creatinine level, less tubular damage, less neutrophil and macrophage infiltrates and significantly reduced apoptosis of tubular epithelial cells than untreated mice [44, 104]. In liver, HMGB1 acts as an early mediator of inflammation and organ damage as early as 1 hour after reperfusion and up to 24 hours. When mice were treated with HMGB1 neutralizing antibodies, significantly less damage was observed after liver IRI, whereas administration of recombinant HMGB1 worsened IRI [77, 97]. This worsening may, in part, be caused by HMGB1 interacting with functional TLR 4 on dendritic cells (DCs) [105]. However, in a seeming paradox, preconditioning with HMGB1 conferred protection against hepatic IRI. This protection was associated with higher expression of IL-1R-associated kinase M which is a negative regulator of TLR 4 [97].

In a porcine model of brain death, HMGB1 expression is upregulated in the liver and kidney within hours of after brain death induction [38]. Moreover, immunostaining demonstrates that early HMGB1 migration out of the nucleus and into the
cytoplasm, an appearance which correlates with its release and function as an alarmin [43]. TLR4 but not TLR2 was strongly upregulated in both liver and kidney early after brain death. Brain death differs from warm ischaemia of isolated individual organs in that the erratic peripheral perfusion which accompanies brain death can compromise gut integrity, resulting in translocation of bacterial products and elevated endotoxin levels in the systemic circulation [106, 107]. The kidneys normally express high levels of TIR8, an inhibitory receptor which dampens TLR signalling by sequestering signalling intermediates such as IRAK-1 and TRAF-6 [108]. Endotoxin downregulates TIR-8 expression, thus, releasing TLR signalling from inhibition, as well as acting as an agonist for TLR4 [108]. The convergence of HMGB1 release and systemic endotoxaemia which occur during brain death imply that HMGB1-LPS complexes should be potent stimulators of TLR4 signalling, and that interruption of this pathway will attenuate inflammation in donor kidneys following brain death.

1.5.2b S100B

S100B is a protein belonging to the family of S100/calgranulins and is also a ligand of RAGE. It is particularly abundant within astroglia of the CNS, but also found in many other tissues and cells including dendritic cells, langerhans cells, and mononuclear phagocytes, myocardium, renal epithelium, adipocytes, melanocytes and chondrocytes [109]. S100B is released into the systemic circulation during acute brain injury, is detectable in peripheral blood samples following brain death and has been proposed as a potential biomarker for the severity of CNS injury [110].
S100B is also released from other organs upon damage and is mainly eliminated in the kidney [109, 111]. Intracellular S100B is responsible for the regulation of cytoskeletal structure and cell proliferation but high levels have deleterious effects [112, 113]. At high extracellular levels, S100B stimulates endothelial, vascular smooth muscle cells, monocytes, macrophages and T cells via RAGE which results in the generation of cytokines, chemokines and proinflammatory adhesion molecules [84, 90, 91, 114], recruits inflammatory cells to sites of tissue damage and induces apoptosis [115]. Apoptotic death also results from RAGE signalling with downstream elevation of reactive oxygen species, cytochrome C release and activation of the caspase cascade [113]. During brain death, S100b is primarily released by cells in the CNS but can also be readily released from other tissues undergoing ischaemic injury subsequent to the initial CNS damage.

1.5.3 Effector cells of the innate immune response

Many cells play part in the innate immune response. During IRI, neutrophils, macrophages, natural killer (NK) cells and also T cells, in particular CD4\(^+\) and CD8\(^+\), which are derived from pluripotent stem cells in bone marrow, are rapidly recruited to the site of injury [116].

1.5.3a Neutrophils

Neutrophils form the first and most prominent line of cellular defense against invading microorganisms and injury. Active recruitment of neutrophils to the site of injury is important to the innate immune system. Neutrophils are mobilized from
circulation and bone marrow reserves in response to host-derived chemotactic factors. Neutrophils roll along the walls of postcapillary venules, surveying connective tissue, mucosal membranes, skeletal muscle, and lymphatic organs for signs of tissue distress and the presence of chemoattractants [117]. Some of the well-studied neutrophil chemoattractants are IL8, INF-γ, C5a and leukotriene [118-121]. Once at the site of injury, neutrophils can be primed for enhanced adhesion, phagocytosis, production of reactive oxygen species (ROS), cytokine secretion, leukotriene synthesis, degranulation and bactericidal activity [122-124].

Neutrophils can function at the site of injury by binding and ingesting the invading cells by the process of phagocytosis. Neutrophils are able to directly recognize surface-bound or freely secreted molecules produced by bacteria, including peptidoglycan, lipoproteins, lipoteichoic acid, lipopolysaccharide (LPS), CpG-containing DNA and flagellin. These molecules produced by bacteria are known as PAMPs and can interact directly with TLRs expressed on neutrophils [125, 126]. Ligation of neutrophil TLRs, in particular TLRs 2 and 4, activates transduction pathways that ultimately prolong cell survival, facilitate adhesion and phagocytosis, and enhance release of cytokines and chemokines [125, 127, 128].

Alternatively, under the condition of IRI, neutrophils can be activated through the release of oxygen free radicals, cytokines and proinflammatory mediators [129, 130]. The activation of neutrophils brings about an inflammatory-like response and plays a central role in releasing oxidants and proteases that damage or kill tissues, and release inflammatory products, such as TNF-α and IL6, that amplify the
recruitment and activation of greater numbers of neutrophils into the effected tissue, thereby extending the severity of tissue damage [131, 132].

1.5.3b Macrophages

Monocytes circulate in the blood stream. In response to infection or an inflammatory stimulus, monocytes migrate to the site of the immune-inflammatory activity where they differentiate into macrophages [133]. They play an important role in host defense against microorganisms and in the removal of senescent erythrocytes and leukocytes [134, 135]. Their primary modes of action are phagocytosis and subsequent digestion, antigen presentation and cytokine production. Macrophages can perform phagocytosis of opsonized microbes or via binding of PRRs to microbe-associated molecular patterns (MAMPs). Microbial fragments that remain after digestion are served as antigen. The fragments are incorporated into major histocompatibility complex (MHC) molecules and then transported to the cell surface of the phagocyte. This process leads to the activation of T lymphocytes, which then mount a specific immune response against the source of the antigen. Microbial products can directly activate macrophages leading to the production of pro-inflammatory cytokines. Typical cytokines produced by macrophages include TNF, IL1, IL6 and IL12 [136-138].

1.5.3c Natural Killer (NK) cells

NK cells have an important role in the innate immune system because they are able to recognize infected cells, cancer cells and stressed cells, and kill them. In addition, they are able to produce a variety of cytokines, including proinflammatory
cytokines, chemokines, colony-stimulating factors, and other cytokines that function as regulators of body defenses. Through cytokine production, NK cells are also able to suppress and/or activate macrophages, suppress and/or activate the antigen-presenting capabilities of dendritic cells, and suppress and/or activate T-lymphocyte responses [139, 140].

NK cells use a dual receptor system in determining whether to kill or not kill a cell.

When cells are either under stress, are undergoing malignant transformation, or are infected, various stress-induced molecules such as MHC class I polypeptide-related sequence A (MICA) and MHC class I polypeptide-related sequence B (MICB) are produced and expressed on the surface of those cells [141, 142]. The first type of receptor on NK cells, called the killer-activating receptors (KAR), can bind to these stress-induced molecules, and this sends a positive signal that enables the NK cell to kill the cell to which it has bound unless the second receptor cancels that signal. The second receptor type, called the killer-inhibitory receptors (KIR), are able to recognize MHC-I molecules [143]. This helps NK cells distinguish between self and non-self. If the self MHC-I molecules have peptides from the body's own proteins bound to them, NK cells will recognize those cells as self, and the receptor triggers a negative signal that overrides the original kill signal and prevents the NK cell from killing the target cell to which it has bound. On the other hand, if the MHC-I molecules have peptides from viral, bacterial, or mutant proteins bound to them, NK cells may fail to recognize that cell as self and kill it [143].
NK cells can destroy target cells in two ways. The first is via the release of pore-forming proteins called perforins, proteolytic enzymes called granzymes, and chemokines. Granzymes pass through the pores and activate the enzymes that lead to apoptosis of the infected cell by means of destruction of its structural cytoskeleton proteins and by chromosomal degradation [144]. As a result, the cell breaks into fragments that are subsequently removed by phagocytes. The second is by binding of the Fas ligand (FasL) of NK cells to Fas of the target cell. This binding causes a conformational change in Fas, making it bind to the death domain, and sending downstream signals to induce apoptosis by a caspase-dependent process [145].

Figure 6 NK cell interacting with a normal body cell [146].
Figure 7 Two mechanisms NK cells utilize to destroy target cells [147]. The first mechanism in which NK cells are capable of destroying target cells is by the release of perforins and granzymes. Granzymes pass through the pores, created by perforins, and activate the enzymes that lead to apoptosis of the target cell. The second mechanism is binding of NK cell FasL to target cell Fas. This process sends downstream caspase-dependent signaling in the target cell leading to the induction of apoptosis.
1.5.3d T cells

Although T cells are principally thought of as components of the adaptive immune system, both CD4⁺ and CD8⁺ T cells also contribute to the innate immune system. CD4⁺ T cells are capable of producing proinflammatory cytokines and cellular immune responses whilst CD8⁺ T cells, also known as cytotoxic T cells (CTL), are very capable as effectors of target cell destruction, and function like NK cells (Figure 7 above). The cytokines released by CD4⁺ T cells promote the activation and proliferation of cytotoxic T cells and NK cells as well as the recruitment and activation of macrophages, described in Figure 8 below.

Figure 8 Pathways utilized by CD4⁺ T cells to destroy target cell. CD4⁺ T cells are capable of releasing Th1 cytokines used to activate CD8⁺ T cells and, at the same time, assist with the recruitment and activation of macrophages. They are also able to release Th2 cytokines which mature B cells into antibody-producing plasma cells, contributing to antibody-dependent cellular cytotoxicity.
The role of T cells in innate immunity has been shown in a number of animal studies. T cells are mediators of IRI in mice. Studies using T cell-deficient mice showed better outcomes for both liver and renal IRI [148, 149]. Further study into the roles of the T cell subsets, CD4$^+$ and CD8$^+$, showed that organs from mice deficient in CD4$^+$ T cells, but not mice deficient in CD8$^+$ T cells, were significantly protected against IRI [148]. These results were confirmed by the reconstitution of CD4-deficient mice with wild-type CD4$^+$ T cells where the cellular inoculum restored renal injury [148]. The mechanism by which CD4-deficient mice are protects against renal IRI is dependent on CD28 and IFN-γ. CD4-deficient mice adoptively transferred with enriched CD4$^+$ T cell lacking in either CD28 or IFN-γ retained protective effects against renal IRI in both cases [148]. However, this finding was not replicated in liver IRI. Blocking of CD4-derived IFN-γ signaling in liver IRI was not seen to have any protective effects [150]. Rather, the mechanism by which CD4-deficient mice are protected against liver IRI is dependent on CD154 and CD28 [150]. Shen et al., showed that, in liver IRI, CD4$^+$ T cells functions are dependent on CD154-CD40 signaling. They also showed that CD4$^+$ T cells can function in liver IRI without the requirement of de novo Ag-specific activation [150].

1.6 Pathological features of renal and liver warm IRI.

IRI is the tissue damage that ensues following reperfusion of ischaemic tissues. Whilst prolonged anoxia results in cell death, tissues experiencing sub-critical ischaemic periods may still receive a lethal injury after restoration of blood supply to them. This is the ischaemia-reperfusion injury, a phenomenon that appears to be
mediated by inflammatory processes. A proposed cascade of IRI sees accumulation
of free radicals and anaerobic metabolites during the ischaemic period. Upon
restoration of blood supply, oxidative cell damage occurs, resulting in cytokine and
adhesion molecule synthesis and activation. This in turn attracts leucocytes to the
reperfused tissues, and thus ensues cellular damage and necrosis. The mechanisms
of this leucocyte-mediated cell damage are multiple and include microvascular
occlusion, oxygen free radical damage, cytotoxic enzyme release, increased
cytokine release and increased vascular permeability [151-155]. Despite the
necessity of restoration of blood supply for tissue survival, the event of reperfusion
can, paradoxically, exacerbate the cellular injury [156].

1.7 Innate immunity opposing tolerance

TLR-mediated signalling has been shown in a number of studies to have an
inhibitory effect upon the induction of transplantation tolerance [157, 158]. Studies
using small animal models have shown that administration of Pam3CSK4,
lipopolysaccharide (LPS) and CpG-ODN, agonists to TLR 2, 4 and 9 respectively,
promotes allograft rejection. In the absence of TLR signalling, anti-CD154-treated
animals sustain long term cardiac graft survival assisted by the recruitment of Tregs
to heart allografts. Nonetheless, engagement of a single TLR (TLR9) by CpG-ODN
was all it took to abrogate this anti-CD154-mediated survival prolongation, via
mechanisms including reduced recruitment of CD4+/FoxP3+ Tregs [159, 160]. In
subsequent studies, Chen et al were able to demonstrate that CpG functions by
targeting recipient hemopoietic cells and promotes acute cardiac allograft rejection
in a manner that depends on IL6 and IL17, and that its pro-rejection effects correlate both with prevention of anti-CD154-mediated conversion of conventional CD4⁺ T cells into induced regulatory T cells and with the expression of IFN-γ and IL-17 by intragraft CD4⁺ T cells [161]. Further studies in the same model demonstrated that the elimination of IL6 and IL17 signalling suppressed the ability of CpG to promote cardiac allograft rejection [161].

Donor brain death results in upregulation within and release from the transplantable organs of TLR ligands such as HMGB1 [38]. The erratic peripheral perfusion that occurs with BD can compromise gut integrity resulting in translocation of LPS and other bacterial products into the systemic circulation. Thus it seems highly likely that the inflammatory processes triggered by donor brain death could impede the development of transplantation tolerance.

1.8 rAAV vectors for gene transfer to the liver

AAV possesses many qualities ideally suited for gene transfer. It is non-pathogenic and naturally replication-deficient, with a low risk of insertional mutagenesis. Vector safety is further enhanced by removal of all virus coding sequences leaving only the 145-nucleotide ITRs required for genome packaging [162]. Liver-directed transgene delivery in mouse models has been shown to result in tolerance to a number of expressed proteins, both secreted and cytosolic [162]. Studies using mice transgenic for the D011.10 T cell receptor which specifically recognizes chicken ovalbumin (OVA) demonstrated antigen-specific CD4⁺ T cell anergy and deletion following AAV-mediated liver-specific expression of OVA. The proportion
of thymocytes expressing the D011.10 receptor declined over time, suggesting that central, as well as peripheral, deletion was occurring. Moreover, these mice failed to produce anti-OVA antibodies after a subsequent challenge with OVA, implying that the absence of T cell help resulted in operational B cell tolerance. Similarly, wild-type mice did not produce antibodies upon challenge with Factor IX (F.IX) following AAV-mediated expression of this antigen in the liver [163]. Cytotoxic T cell responses to transgene products are also abrogated in mice by liver-specific gene expression [164-167].

In contrast to skeletal muscle expression of these genes, challenge with a highly-immunogenic adenoviral vector following earlier AAV-mediated liver-specific expression of the same transgene does not result in the destruction of transduced hepatocytes [164]. Tolerance induction after hepatic gene transfer is promoted by higher levels of gene expression. For each transgene, a threshold level of expression exists, below which tolerance cannot be achieved [162]. The combination of AAV8 capsid with the efficient liver-specific ApoE/hAAT promoter results in substantially higher levels of transgene expression at equivalent vector doses, when compared with AAV2-ApoE2/hAAT [167]. Correspondingly, AAV8-ApoE2/hAAT-driven gene transfer was superior to that mediated by AAV2-ApoE2/hAAT in inducing tolerance to Human F.IX across a range of F.IX-deficient mouse strains, including C3H/HeJ F9⁻/⁻ mice which had previously been resistant to tolerance induction [167]. An AAV2 vector genome pseudo-serotyped with the type 8 capsid (rAAV2/8), and incorporating the human α-1 antitrypsin (hAAT) promoter has been used
extensively in the sponsoring laboratory. In our hands, this vector provides high-level, long-lasting liver-specific transgene expression, with no inflammation in rodent livers expressing a range of proteins including GFP, indoleamine dioxygenase [168], esRAGE, human albumin, or allogeneic mouse MHC [169]. In the latter study, tolerance to the transgene products has been formally demonstrated. For these reasons, I chose to use the rAAV2/8 system for systemic overexpression of esRAGE in mice in the present project.

1.9 Systemic overexpression of esRAGE as a strategy to block innate signalling in brain death and ischemia-reperfusion injury

The systemic overexpression of esRAGE had been proposed and shown in our lab to block innate signalling in renal IRI [170]. Our lab showed that overexpression of esRAGE significantly reduced tubular damage and reduced serum creatinine levels in IRI when compared to uninjected and sham controls. We also showed that overexpression of esRAGE significantly increased survival in severe renal IRI and preserved renal morphology. These encouraging results suggested that esRAGE expression could potentially also attenuate inflammation, improve graft function, and promote tolerance induction in kidneys transplanted from brain-dead donors, and prompted me to undertake this project.

1.10 Hypotheses and Aims

We hypothesised that donor brain death would result in increased inflammation in and hence poorer function of kidneys transplanted from brain dead donors. Our second hypothesis was that systemic overexpression of esRAGE mediated by
administration of a rAAV 2/8 vector would attenuate inflammation and preserve function of such kidneys.

In order to test these hypotheses, we aimed to:

1. Establish and characterise a murine model of brain death (Chapter 5).
2. Determine the optimum method of ureteric reconstruction for use in mouse renal transplantation from brain dead donors (Chapter 4).
3. Develop a method for systemic overexpression of esRAGE (Chapter 3).
4. Determine whether systemic overexpression of esRAGE attenuates inflammation after donor BD and renal transplantation (Chapter 6).
CHAPTER 2: MATERIALS AND METHODS

2.1 Development of a Brain Death Model in Mice

Although this model was based upon the published protocol of Atkinson et al. [23], a number of refinements were introduced throughout the development of the model. The modifications made in order to produce a functional and reproducible model of brain death in mice are described below.

2.1.1 Anaesthesia

In the initial stages of the development of the model, mice were induced and maintained under anaesthesia with inhalational Isoflurane 1.5-2.5% (Baxter Healthcare, Auckland, NZ). The anaesthetic agent is administered through a nose cone using an oxygen flow rate of 1L/min. This method of anaesthesia is well established for small animal surgery and is the standard practice within our laboratory. It soon came to our attention that using inhalational anaesthesia is not practical for this model of brain death as obstruction by the nose cone makes drilling the burr hole near impossible and tracheostomy difficult. Additionally, isoflurane induces BP fluctuation, most likely due to its cardiodepressant effects, thus, further decreasing its suitability as BP is inherently unstable following the onset of brain death and cannot be maintained in the face of this additional challenge.

The alternative we decided to use was an IP injection of a mixture of Ketamine (Hospira Australia, VIC, Australia) (81mg/kg) and Xylazine (Troy Laboratories, NSW,
Australia) (9mg/kg). Initially the mouse would be injected IP with the Ketamine/Xylazine mixture to induce anaesthesia. The depth of the sedation was monitored every few minutes and before any invasive procedure through the withdrawal reflex and the observation of the whiskers of the mouse. Movement of the whiskers upon stimulation is a sign of shallow anaesthesia and a top-up dose of half the initial dose of Ketamine (40.5mg/kg) would be injected alone. We inject only Ketamine in this first top-up dose because the effect of Xylazine last twice as long as Ketamine. For the second top-up, half the initial dose of Ketamine (40.5mg/kg)/Xylazine (4.5mg/kg) mixture is injected IP.

2.1.2 Ventilation

The Inspira advanced safety rodent ventilator (Harvard Apparatus, MA, USA) was used for ventilation with an FIO\textsubscript{2} of 60\%. The ventilator tubing was attached to a 22G blunt tipped cannula (BD Medical, MC, USA) used for insertion into the trachea. The ventilator auto-adjusts its ventilation settings according to the input weights of the mice. Pressure-controlled mechanical ventilation was substantially better than volume-controlled ventilation at maintaining good oxygen saturation in the mice. As a further refinement, a small amount of positive end-expiratory pressure (PEEP) (5-7 cmH\textsubscript{2}O) was added to the ventilator settings. Final settings for the ventilator were pressure-controlled mechanical ventilation with a PEEP of 5cmH\textsubscript{2}O with the manual input of individual mouse weight for ventilator to auto-adjust itself suitably.
2.1.3 Brain Death Induction

The induction of brain death was performed in a similar manner to that described in other protocols for induction of brain death in rodents. The method used involves inserting a 2F Fogarty catheter (LeMaitre Vascular, MA, USA) into the intracranial cavity through a burr hole. The balloon of the Fogarty catheter is slowly inflated to progressively increase intracranial pressure leading to brain stem herniation and irreversible brain death.

The burr hole was sited in the right parasagittal bone, shown Figure 9A below.

Figure 9 Creation of burr hole. A - Theoretical location of burr hole marked by “X”; B - Actual burr hole made in mice.
The initial approach we took for making the burr hole was by the use of a mechanical drill. The overlying skin over the skull would be cut open, followed by the drilling.

Despite a sufficient-sized burr hole being made, much is at risk. This method proved unsuitable as the speed of the rotary drill was difficult to control and the drill bit would often catch onto the surrounding scalp tissues, ripping them apart and causing intractable bleeding. Additionally, there is a risk of drilling beyond the thin layer of skull and into the cerebral cortex, a complication which is well documented in the Pratschke et al model of brain death in rats [16]. Because of the hazards of using this method of drilling, we decided explore to an alternative.

Due to the small size of the 2F Fogarty catheter, we decided to try use the stylet (needle) of a 14G cannula (TERUMO, Phillipines) as a hand drill. We chose 14G because its external diameter is only slightly bigger than the Fogarty catheter, allowing a good seal after insertion of the catheter. Slight downward pressure is applied to the 14G cannula stylet onto the skull whilst making a slow rotary motion with the hand. Once an impression is made on the periosteum, the stylet is angled at 45 degrees, and drilling continues until the skull, but not the dura mater, is perforated, Figure 9B above. The Fogarty catheter is then inserted and secured to the back of the mouse with a masking tape. This procedure is much gentler than mechanical drilling, and associated with less chance of causing complications like intractable bleeding and penetration of the cortical brain tissue.
The method of inflation of the Fogarty catheter has also been modified. Initially we inflated the Fogarty catheter by hand with 80µl +/- 25µl saline over a period of 15 minutes (based on Atkinson et al). Though we achieved brain death through this method, it was very difficult to maintain a constant rate of inflation, as a sudden drop in resistance of the balloon was usually accompanied by a rapid increase in volume, leading to explosive as opposed to gradual brain death. Mice would often die immediately post-BD induction. Reflecting our findings, earlier studies had shown that dogs undergoing explosive brain death experienced significantly more myocardial ischemia and arrhythmias and were more vulnerable to haemodynamic complication than those undergoing gradual brain death [21]. Based on these studies and our results, we decided it was vital to keep the inflation of the balloon constant and started using a syringe driver. The syringe connected to the Fogarty catheter was placed into an automated syringe driver (Harvard Apparatus, MA, USA) and the saline was infused into the balloon at a constant rate of 5µl/min over 20 minutes up to a maximum of 100µl. Inflation was ceased at the point where mean arterial pressure (MAP) started rising rapidly (see Figure 11 below). This was accompanied by a permanent increase in heart rate (HR), followed by a slow decline in MAP over the subsequent hours (Figure 11).

2.1.4 Fluid Support

We initially decided to use normal saline injected both IV and SC as fluid support. Mice, at the start of the experiment, would be injected with 0.5ml IV through the penile vein and 2.0ml SC at four different sites, i.e. 0.5ml SC at each site. Fluid top-
up would occur at hourly intervals from the start of the experiment for the SC injection with 1.5ml at three different sites. IV fluid top-ups of 0.5ml would occur once after the femoral artery cannulation and then at 30 minute intervals thereafter. At the beginning, we tested with mice that were ventilated alone. The fluid support seemed sufficient and mice were able to be maintained for the desired duration of 3 hours, shown in Figure 10A below. When we first attempted maintaining the BD mice, we found that this fluid support regime did not maintain intravascular volume, and mice would desaturate rapidly following the induction of BD (Figure 10B). Post mortem dissection found evidence of pulmonary oedema, with heavy, engorged lungs which sank rapidly upon placement in formalin. The pulmonary oedema observed may have been due to a number of factors including fluid overload, left ventricular failure, oncotic dilution or due to neurogenic pulmonary oedema.

To avoid reducing the plasma oncotic pressure by dilution, we decided to try using a colloid fluid instead of saline, a crystalloid. The colloid solution we chose to use was Gelofusine (B.Braun, AG, Germany), a colloidal plasma volume replacement fluid based on modified fluid gelatine which has iso-oncotic pressure, physiological pH and a low chloride content.

We started by using one bolus of 0.5ml IV Gelofusine and 2.0ml SC of normal saline at four sites. Following the initial bolus, we topped up at hourly intervals with 1.5ml of normal saline SC divided amongst three sites. Further IV boluses of 0.25ml Gelofusine were given following femoral artery cannulation if the MAP was below
55mmHg, and then as required when the MAP dropped to 40mmHg or below. This type of fluid support was able to maintain blood pressure in the mice without compromising oxygenation for up to 3 hours, shown in Figure 11 below.

Figure 10 Saline IV as fluid support for ventilated only 3 hours (A) compared to 3 hours of BD (B). A - Fluid support seemed sufficient and mice were able to be maintained for the desired duration of 3 hours in the ventilated group; B - Fluid support regime did not maintain intravascular volume, and mice would desaturate rapidly following the induction of BD.
Figure 11 Traces for optimized model of BD with Gelofusine as fluid support. BP peaked rapidly at point of BD. This was accompanied by a permanent increase in HR, followed by a slow decline in BP over the subsequent hours. Using Gelofusine maintained blood pressure without compromising oxygen saturation. Temperature well maintained between 37°C to 38.5°C.
2.1.5 Body Temperature

Core temperature was measured using a rectal probe (Columbus Instruments, Ohio, USA), and was maintained within the range of 37°C to 38.5°C (Figure 11 above) using a heating pad (Redzone Heating, Australia). Temperature was displayed on a Physiomex small animal physiological monitor (Columbus Instruments, Ohio, USA).

2.2 The Functional BD Model

All procedures performed below were done on C57BL/6 male mice. Following anaesthesia with Ketamine and Xylazine, the left thighs of the mice were shaved in preparation for femoral artery cannulation.

Once the preparation had been completed, mice were placed in a ventral position. The skin above the skull was cleaned with 70%. The skin was opened with a vertical incision. Once opened, a 14G cannula stylet was used to drill a burr hole in the bottom left corner of the right parasagittal bone of the skull, Figure 9B above. A 2F Fogarty balloon catheter was then inserted into the burr hole and secured by the use of masking tape, sticking the catheter to the mouse’s back. Brain death will be induced after mechanical ventilation and invasive BP monitoring have been established. Upon completion of the burr hole, mice were placed back into dorsal position. The mice were secured with masking tape to the working surface. A rectal thermometer was then inserted. Next, the neck skin was sprayed with 70% ethanol. A vertical midline incision was made in the anterior neck. The two halves of the sternohyoid muscle were then separated and trachea exposed. A loose 5-0 silk tie
was then placed around the trachea. The ventilator was set to pressure controlled ventilation with mouse weight entered, PEEP of 5cmH₂O and FIO₂ 60%, and gas flow was started. A small transverse incision was then made in the sub-laryngeal area between two rings of cartilage, see Figure 12A below. The 22G blunt tipped cannula attached to the ventilator tubing was then inserted into the trachea to a pre-determined mark, avoiding cannulation of either main bronchus and ensuring ventilation of both lungs. The cannula was then secured with the loose tie. An additional 5-0 silk tie was then placed around the trachea and cannula to maintain a snug fit preventing gas leakage, see Figure 12B below.

Figure 12 Depiction of tracheostomy. A - The exposure and opening of trachea; B - The securing of the ventilator tube to the trachea.
The last surgical procedure was the cannulation of the left femoral artery. This procedure was required for monitoring the MAP. The shaved left thigh was used. An incision was made in the left inguinal area. The femoral neurovascular bundle was exposed using blunt dissection with cotton buds (Figure 13A). The femoral artery was then separated from the surrounding tissues and vessels in the area between the abdominal wall and the superficial epigastric artery, see Figure 13B below. Lignocaine was used to help prevent artery vasospasm. Once separated, the distal end of the femoral artery was ligated with a 5-0 silk tie, slightly retracted with a clamp. A 5-0 silk tie was also placed around the proximal end of the artery, with a loose knot. This tie was also retracted and elevated using a clamp until the point where blood stopped running into the area in between the two ties, see Figure 14B and Step 1 of Figure 14A. A small transverse incision going half way across the femoral artery was made 2mm proximal to the distal tie (Step 2 of Figure 14A). One tip of a micro-forceps was then inserted into the artery for 10 seconds to dilate it, preparing it for cannulation. A 1.2G cannula (Solomon Scientific, TX, USA) was inserted into the femoral artery beyond the proximal tie (Step 3 of Figure 14A). The cannula was secured by tightening the proximal and distal ties (Step 4 of Figure 14A). A pulsation within the cannula should be observed at this point as a sign of success. The MAP was read and recorded via the Physiomex® data recorder system. Finally, a MouseOX® pulse oximeter (Starr Lifesciences Corporation, Oakmont, PA) with mouse tail clip was used to detect, monitor and record oxygen saturation and heart rate. Brain death was induced as described above. Fluid support was provided
as outlined above. In some instances, kidneys from the BD mice were removed and transplanted into syngeneic recipients.

Figure 13 Exposure of the femoral artery. A - Exposure of the femoral neurovascular bundle; B - Separating the artery from surround tissues and vessels.
Figure 14 Depiction of femoral artery cannulation. A - Steps to performing a successful femoral artery cannulation. Step 1 shows the ligation of the distal end of the femoral artery and retraction with suture of the proximal end to prevent bleeding when the artery is transversely slit open in step 2. Step 3 shows the cannulation of the femoral artery and step 4 shows the securing of the cannula within the artery; B - Overview of the setting when doing the procedure.

2.3 Determination of preferred method of ureteric reconstruction for mouse kidney transplantation.

Our research group has utilised two different methods for ureteric reconstruction during mouse kidney transplantation. Prior to commencing kidney transplantation from brain dead donors, we undertook a study with both retrospective and prospective arms, comparing the two methods. The results of this study are described in more detail in ‘Chapter 4: Determination of the optimum method of ureteric reconstruction for use in mouse renal transplantation’. Based on the
findings, we decided to use the ureteric implant method for all subsequent transplants. This method is described below.

2.4 Renal Transplantation

Syngeneic renal transplants were performed using male C57BL/6 mice as both donors and recipients. Some donors underwent brain death and maintenance for a period of either one or three hours prior to removal of the donor kidneys. In addition, some donors and recipients had been injected with an rAAV vector encoding esRAGE or a control protein (eGFP or human serum albumin) seven days prior to transplantation.

2.4.1 Donor Procedure

The left kidney of the BD donor was collected for transplantation. The donor procedure started with the shaving of the abdomen following application of 70% ethanol for skin sterilisation. A long midsaggital incision was then made from the xiphoid process to the pubis. A paperclip retractor was used to expose the left kidney. The left kidney and ureter were then mobilised from the surrounding tissues, and the ureter was cut distally, close to the bladder. The aorta and IVC together with the left renal artery and vein and surrounding vessels were then dissected and mobilized. A loose 5-0 silk tie was placed around the vascular bundle of infra-renal aorta and IVC. The SMA was then tied off with a 5-0 silk tie followed by the ligation of the supra-renal aorta with 5-0 silk. Once completed, the supra-renal IVC was transected, followed by immediate perfusion distal to the loose tie
through the infra-renal aorta. 2ml of heparinised saline at 4°C was slowly injected using a 30 gauge needle connected to a syringe. After successful perfusion, the loose tie previously placed around the infra-renal aorta and IVC was tied firmly. The supra-renal aorta distal to the previously placed tie was then cut. The left kidney was mobilised by transecting the vascular bundle of aorta and IVC distal to the tie, see Figure 15. The donor kidney, with the aorta and IVC segment, was then stored in sterile saline at 4°C whilst preparing the recipient for transplantation.

Steps 1-7 of kidney harvesting:
1. Tying off SMA
2. Tying aorta
3. IVC transection
4. Perfusion
5. Tying IVC & aorta bundle
6. Aorta transection
7. Transection IVC & aorta bundle

Figure 15 Steps to procurement of donor kidneys. The steps to harvesting the donor kidneys are outlined here (left). On the right hand side is what a successful procurement of the kidney should look like.
2.4.2 Recipient Transplant

Anaesthesia of the recipient mouse was induced and then maintained using 1.5-2.0% of inhalational isoflurane evaporated with 0.3L/min of oxygen delivered to the mouse through a nose cone. After shaving and skin sterilisation, an abdominal midsaggital incision was made, as for the donor procedure. Paperclip retractors were used to expose the field around the left kidney. Gauze soaked in normal saline was used as a sponge to keep the bowel moist and retract it to the upper right side of the abdomen to expose the aorta, IVC and left kidney. 5-0 silk ties were placed around the left renal artery, vein and ureter. The left kidney was then removed, see Figure 16 below. The aorta and IVC were then mobilised from the left renal artery and vein down to the iliac bifurcation, the associated lumbar arteries and veins were divided. Two small vessel clamps were then placed on the aorta and IVC segment both distally and proximally. A vertical incision was made in the anterior wall of the aorta with microsurgical scissors to match the calibre of the donor aorta, see Figure 16 below. The donor kidney was then removed from the saline storage solution and placed at the recipient site, covered with a cold saline sponge. The donor aorta was anastomosed, end to side, to the recipient aorta using 10-0 continuous micro-sutures. A similar anastomosis was then made with the donor to recipient IVC, see Figure 17 below. The two vessel clamps were released to allow blood flow to the revascularised graft, see Figure 18 below. A small piece of Gel-foam was placed around the anastomosis with cotton buds to control any bleeding. The kidney was placed to the left side of the abdomen and the ureter was
orientated to ensure there was no twisting or kinking. A 5-0 needle and silk suture (Ethicon) was used with the suture tied to the end of the ureter. The needle was then pulled through the recipient bladder from the back wall to the front wall, followed by the suture and attached ureter. Two to three stitches of 10-0 nylon (Ethicon) were applied to fix the adventitial tissue surrounding the ureter to the bladder where it entered at the back wall. Excess ureter was cut at the bladder front wall and after retraction of the ureter, the bladder defect was closed with one 10-0 suture (Figure 19, below). The method for ureteric implantation described here has been developed in our research group, and differs from the previously published methods [171] in that it avoids the requirement to pull the ureter through the bladder with forceps. This modification results in less trauma to the bladder, allows easy manipulation and fixation of the ureter, and prevents the ureter from slipping out of the bladder following implantation. Following ureteric anastomosis, the abdomen was washed and moistened with room temperature saline and the kidney graft and bowel returned to their anatomical positions. Abdominal closure was achieved using a 5-0 absorbable suture for muscle followed by skin. At the end of the recipient operation, the animal was injected with buprenorphine IM for analgesia and Ampicillin for infection prophylaxis, and then allowed to recover in a heated box.
Figure 16 Preparation of renal recipient for transplantation. The recipient left kidney had been removed and the aorta and IVC had been prepared for renal transplantation.

Figure 17 Implantation of donor kidney into recipient. The anastomosis of the artery is done first followed by the anastomosis of the vein.
Figure 18 Depiction of successful renal transplantation. The appearance and colour of the kidney (right) upon successful transplantation.

Figure 19 Steps involved in the ureteric implant method. A suture is tied to the end of the ureter and pulled through the bladder from the back (step 1). A few stitches are used to fix the adventitial tissue surrounding the ureter to the bladder where it entered at the back wall (step 2). Excess ureter was cut and allowed to shrink back into the bladder (step 3). And finally, a few stitches are used to close the front opening of the bladder (step 4).
2.5 Sample Harvesting

2.5.1 Serum

Whole blood was collected from the IVC with a 21G needle and syringe, then transferred into an eppendorf tube. The blood was allowed to clot at RT for 1 hour prior to centrifugation at 3,000rpm for 15 minutes. The upper clear layer (serum) was transferred to a new eppendorf and then spun for another 15 minutes at 14,000rpm. The top layer (serum) was then transferred into separate eppendorf tubes in 50µL aliquots.

2.5.2 Organs

Heart, lungs, liver, kidneys, spleen and pancreas were collected into OCT for frozen sectioning, formalin for paraffin sectioning, and liquid nitrogen as snap frozen pieces for RNA extraction and analysis. The kidneys and spleen were cut transversely prior to embedding. For mice that had undergone kidney transplants, only the serum, kidneys (both native and transplanted) and spleen were harvested as described above.

2.6 Cloning and subcloning of esRAGE cDNA

A cDNA encoding human esRAGE was obtained in two different ways. Firstly, esRAGE was amplified from normal human lung explanted in the course of resecting a pulmonary tumour. Amplification primers were forward CACCATGGCAAGCCGAACAGCGGAGC and reverse GCGGATATCTTTTACCCACGTCCCCAG. PCR products were run on 1% agarose gel
to confirm that a band of expected size was amplified (see Figure 22 in Chapter 3), then sequenced using CACCATGGCAGCCGGAACACGCAGTTGGAGC.

In parallel, a codon-optimised cDNA sequence for esRAGE had been ordered from GeneArt (Life Technologies, NY, USA). 5µg of the pMA plasmid containing this cDNA was resuspended into 50µL of ultrapure H₂O and then transformed into SURE-2 competent E. coli (Stratagene, CA, USA) and allowed to grow on agar plates. A few colonies from the plate were selected for Mini-preparation and colony PCR using Quick Mini-prep Kit (Invitrogen, CA, USA) to verify that plasmids contained the esRAGE cDNA. Once the inserts were verified, a colony was selected for Maxi-preparation. Plasmid DNA concentration was estimated by measuring absorbance at 260nm on a nanodrop spectrophotometer (Thermo Scientific, MA, USA). Restriction enzyme digestion NotI (New England Biolabs, MA, USA) and EcoRV (New England Biolabs, MA, USA) and agarose gel electrophoresis confirmed the presence of a band running at the expected size of 1113 base pairs (bp). The esRAGE insert was amplified from the maxi-prepped plasmid DNA using esRAGE-specific primers as above, then both the GeneArt sequence and the native sequence were TOPO cloned into pcDNA3.2 (Invitrogen, CA, USA), a mammalian expression vector. The pcDNA3.2 plasmids containing each of the esRAGE cDNAs were then transformed into one shot Top-10 E. coli. Correct sequencing was confirmed once again with Mini-preparation and PCR. Following this, expression of the esRAGE protein from these constructs was tested in vitro by transfecting the mammalian cell line HEK293D with the pcDNA 3.2-esRAGE plasmid, collecting the supernatant at 48 and
72 hours after transfection and quantitating esRAGE using the commercially-available human esRAGE sandwich ELISA (B-Bridge International, CA, USA). The ELISA method is described below at ‘2.8.1 esRAGE ELISA’. Protein expression in vitro was slightly higher from the GeneArt than from the native construct, and so the codon-optimised GeneArt construct was used in all further experiments.

2.7 Production and Packaging of an rAAV 2/8 vector encoding human esRAGE

Packaging of recombinant rAAV 2/8 vectors requires that the packaging cell line HEK293D is triple-transfected with the following plasmids: pAM2AA (encoding the gene of interest and the inverted terminal repeats of the AAV serotype 2 genome) p5E18VD2/8 encoding the rep gene from AAV2 but the viral capsid protein from AAV serotype 8, and pXX6 (encoding adenoviral helper factors).

![Diagram](image)

**Figure 20** Triple transfection with pAM2AA, pXX6 and p5E18VD2/8, yields rAAV2/8 virions after purification.
To produce complementary sticky ends and ensure rapid directional ligation, the template expression vector pAM2AA-eGFP and PCR fragments of esRAGE were simultaneously digested with EcoRV and NotI to release esRAGE fragments as well as to linearize the pAM2AA-eGFP vector.

The digested pAM2AA and esRAGE fragments were run on a 1% agrose gel and extracted using Wizard Gel Extraction Kit (Promega, WI, USA). These were the ligation products. The amount of PCR product used in the ligation was set at a 3:1 vector: insert ratio, and the ligation was performed using Quick T4 DNA Ligase (New England Biolabs, MA, USA) in a 5µl reaction at 5 minutes room temperature.

The products of the ligation were transformed into SURE-2 Supercompetent cells (Stratagene, CA, USA). The transformed cells were plated onto agar plates with ampicillin (100µg/mL) at 2 different concentrations of 20µL and 200µL. A resistant colony was randomly selected for Mini-preparation and colony PCR to verify sequence. Once verified, correct clones underwent Maxi-preparation and then sequence confirmation as described above in ‘2.6 Cloning and subcloning of esRAGE cDNA’.

Once the sequence was verified, p5E18 and pXX6 were used along with pAM2AA-esRAGE for packaging. HEK293D cells were cultured at 37°C in 5% CO₂ with standard medium: DMEM supplemented with 10% fetal calf serum (FCS). These cells were seeded into 40x10cm² tissue dishes. The following day, the medium was refreshed in the morning and a triple transfection using calcium phosphate was performed 3 hours later. At 16 hours post-transfection, standard medium was replaced with
DMEM + 2% FCS. Cells were then harvested 48 hours post-transfection and immediately stored at -80°C.

Virions were separated from cell lysate using a cesium chloride gradient purification, and then concentrated. Viral quantification was performed using quantitative real time PCR and aliquotted at 5x10^{11} viral genome copies for use.

2.8 ELISAs and ELISA-based assays

2.8.1 esRAGE ELISA

The esRAGE Human ELISA Kit (Cat # 1009-1, B-Bridge International) was used for quantitative determination of mouse esRAGE concentrations in tissue culture supernatant and in mouse serum.

The kit utilises the sandwich ELISA format. A microplate is pre-coated with the capture antibody, which is specific for the unique 20 amino acids at the C-terminus of esRAGE. The assay was carried out according to the manufacturer’s protocol. 20µl of sample was added to each well with 100µl of esRAGE antibody HRP conjugated and incubated for a 20 hour period. After incubation plates were washed in PBST with BSA (provided in kit). Tetramethylbenzidine and hydrogen peroxide were added as a substrate for colour development and reaction was stopped after 30 minutes. Absorbance was measured at 450nm on a FLUOstar Omega plate reader (BMG Labtech, Germany). This assay is intended for detection of human esRAGE in the physiological range in human subjects. Overexpression of human esRAGE in mouse liver resulted in serum levels that were sometimes over
5,000-fold greater than the upper limit of the dynamic range of the assay (0.05ng/ml to 3.2ng/ml). For this reason, tissue culture supernatants and sera were diluted in kit diluent between 1:2500 and 1:62,500 prior to assay.

2.8.2 Lipocalin-2 / NGAL ELISA

Quantikine® mouse lipocalin-2/NGAL immunoassay (Cat # MLCN20, R&D Systems Inc.) was used for quantitative determination of mouse lipocalin-2/NGAL concentrations in mouse serum.

According to the user manual: “This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse Lipocalin-2 had been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any mouse Lipocalin-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for mouse Lipocalin-2 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the colour measured is in proportion to the amount of mouse Lipocalin-2 bound in the initial step. The sample values are then read off the standard curve” [172]. The assays were carried out according to the manufacturer’s protocol.
The only aspects not determined by these protocols are the serum dilutions. The following dilutions were prepared:

- **Dilution1:** 1:100, 2µl serum + 198µl calibrator diluent
- **Dilution2:** 1:1000, 10µl Dilution1 + 90µl calibrator diluent
- **Dilution3:** 1:10000, 10µl Dilution2 + 90µl calibrator diluent
- **Dilution4:** 1:100000, 10µl Dilution3 + 90µl calibrator diluent

### 2.8.3 S100B ELISA

Enzyme-linked Immunosorbent Assay Kit for S100 Calcium Binding Protein B (S100B) (Cat # E90567Mu, Uswn Life Science Inc.) was used for quantitative determination of mouse S100B concentrations in mouse serum. The assay was carried out according to the manufacturer’s protocol. 100µl of sample was incubated for 2 hours and then 100µl of detection antibody was added. Plate was washed and tetramethylbenzidine and hydrogen peroxide were added as a substrate for colour development and reaction was stopped after 30 minutes. Absorbance was measured at 450nm on a FLUOstar Omega plate reader (BMG Labtech, Germany). Samples were diluted with 0.02M PBS at 1:2 and 1:10 for values to lie within the detection limits of 23.5pg/ml up to 1500pg/ml.

### 2.8.4 Proinflammatory Cytokines (Multiplex)

MILLIPLEX® xMAP Mouse Cytokine / Chemokine magnetic bead panel kit (Cat # MCYTOMAG-70K, Millipore™) was used to simultaneously quantify the following 25 murine cytokines and chemokines: G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5,
IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IP-10, KC, MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES and TNFα.

The MILLIPLEX® xMAP is based on the Luminex® xMAP® technology. According to information from the manufacturer “This technology uses (proprietary) techniques to internally colour code microspheres with two fluorescent dyes. One hundred distinctly coloured bead sets are able to be created, each of which is coated with a specific capture antibody. After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. The microspheres are allowed to pass rapidly through a laser which excites the internal dyes marking the microsphere set. A second laser excites PE, the fluorescent dye on the reporter molecule. Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay based on fluorescent reporter signals” (adapted from the MILLIPLEX® MAP user manual [173]).

The assays were carried out on mouse sera according to the manufacturer’s protocols. The only aspect not predetermined by these protocols are the serum dilutions. The samples were diluted 1:2 by adding 13µL of the mouse’s serum to 13µL of assay buffer. Some samples were also diluted 1:4 by adding 7µl of serum with 21µl of serum matrix.

Samples were incubated at 4 °C with antibody-coated beads overnight. The following day, 25µl of detection antibody was added and the plate was further
incubated at RT for 1 hour. Then a detection reagent was added to each well and incubated at RT for 30 minutes. The plate was then washed and beads resuspended in sheath fluid and run on the Luminex 200 (Millipore).

### 2.9 Frozen Sections for Staining and Immunohistochemistry

6 µm sections were cut from frozen OCT blocks using a Cryostat (Shandon Cryotome E, Thermo Scientific, MA, USA). Sections were guided onto pre-coated slides (SuperFrost®, MENZEL-GLÄSER, Berlin, Germany), then allowed to air dry for 1 hour at RT followed by an acetone fix of 8 minutes at RT. Once fixed, the slides were allowed to air dry at RT for 10 minutes and then either wrapped in foil and stored at -80°C or stained immediately. If the slides were stored at -80°C prior to staining, the slides were allowed to thaw (wrapped in foil) at RT for at least 15 minutes and then unwrapped and allowed to air dry for 30 minutes at RT. A wax pen (Dako, Denmark) was used to draw around the sections to help retain the solutions on the sections during incubations. The amount of solution added to sections varied from 50-100µL depending on the size of the section. During the staining process, slides were kept moist in a humidified chamber until the final step. Staining was carried out at room temperature unless otherwise indicated.

#### 2.9.1 Staining for Macrophages

##### 2.9.1a CD 68

Slides were initially washed with 3 changes of PBS buffer for 3 minutes each and then incubated in 0.06% H2O2/PBS buffer for 10 minutes to block endogenous
peroxidase activity. The slides were washed again in 3 changes of PBS buffer for 3 minutes each and then incubated with avidin solution (Dako, Denmark) for 10 minutes to block endogenous biotin. The slides were then briefly rinsed in PBS buffer and incubated with biotin solution (Dako, Denmark) for 10 minutes to block residual avidin. The slides were rinsed in PBS buffer and then the sections were blocked in 20% normal horse serum/PBS buffer for 20 minutes.

The primary antibody (rat anti-mouse-CD68, FA-11, Cat # MCA1957, AbD Serotec, North Carolina, USA) at a dilution of 1:400 in 1% normal horse serum/PBS buffer was added to the sections and allowed to incubate at RT for 1 hour. Following the incubation, the slides were once again washed 3 times in PBS buffer for 3 minutes each. The secondary antibody (biotinylated goat anti-rat, polyclonal, Cat # 559286, BD Pharmingen, CA, USA) at a dilution of 1:200 in 1% normal horse serum/PBS buffer) was then added and incubated at RT for 1 hour.

The slides were washed in 3 changes of PBS buffer for 3 minutes each followed by the addition of a detection agent to the sections for 30 minutes. The detection agent was prepared 30 minutes before use by mixing 1:100 reagent A and 1:100 reagent B (Vector Laboratories, Peroxidase standard, Cat # PK 4000) in 1% normal horse serum/PBS buffer. After this incubation, the slides were washed 3 times for 3 minutes each in PBS buffer followed by a quick rinse in water.

Chromogen solution was then added to each section for 2-3 minutes. The chromogen solution was prepared by adding one drop of DAB (Diaminobenzidine) per 1 ml of DAB substrate buffer (Dako North America Inc). The slides were then
washed 3 times for 3 minutes in distilled water. The slides were counterstained and coverslipped as described below under ‘2.11 Counterstaining and coverslipping’.

**2.9.1b F 4/80**

Initially, the primary antibody (anti-mouse F4/80 antigen FITC, BM8, Cat # 11-4801-82, eBioscience, CA, USA) at a dilution of 1:400 in IP diluent was added to the sections and allowed to incubate for 30 minutes at RT. The slides were then washed in TBST twice for 5 minutes each. The secondary antibody (rabbit anti-FITC-HRP, polyclonal, Cat # 18-783-77622, GenWay, CA, USA) at a dilution of 1:200 in IP diluent with 20% heat-inactivated normal mouse serum was added to the sections and incubated for 30 minutes at RT. The slides were washed again in TBST twice for 5 minutes each followed by the addition of the chromogen solution for 6 minutes at RT. The chromogen solution was prepared by adding one drop of DAB (Diaminobenzidine)(Dako North America Inc) per 1 ml of DAB substrate buffer (Dako North America Inc) with 15µl of 18% sodium azide per 1ml of the solution. The slides were then washed 3 times for 3 minutes in water, and counterstained as described below.

**2.9.2 Staining for regulatory T cells (FoxP3)**

Slides were initially rinsed briefly in TBST and then incubated in cold 0.1% v/v Triton-X-100 in 0.1% w/v Na citrate for 2 minutes on ice. The slides were then washed in TBST for 5 minutes twice and the sections were blocked with IP diluent containing 20% normal mouse serum (NMS) for 20 minutes. A tapping/shaking
motion was used at the end of the block to remove the blocking solution and the primary antibody (anti-mouse/rat FoxP3 FITC, FJK-16s, Cat # 11-5773-82, eBioscience, CA, USA) diluted in IP diluents at a ratio of 1:50 was added and allowed to sit for 45 minutes. The isotype control used was rat IgG2a diluted in IP diluent at a ratio of 1:50. The samples were washed twice in TBST for 5 minutes each and then the secondary antibody (rabbit anti-FITC-HRP, polyclonal, Cat # 18-783-77622, GenWay, CA, USA) diluted in IP diluent with 20% heat inactivated NMS at a ratio of 1:200 was added for 30 minutes.

The samples were washed twice in TBST for 5 minutes each and DAB solution with the addition of 18% sodium azide was applied to the sections for 6 minutes. Once colour development was complete, slides were transferred immediately into tap water. The slides were rinsed in 3 changes of water and then allowed to either air dry overnight at RT or counterstained immediately while the slides were still wet.

2.10 Paraffin Sections for Staining and Immunohistochemistry

For determination of morphology by Haematoxylin and Eosin (H&E) and Periodic Acid Schiff (PAS) stains, and for some immunohistochemical staining, formalin-fixed tissues were embedded in paraffin wax and then cut into thin slices. Three steps were used in this process, dehydration, clearing and paraffin wax infiltration. The dehydration process is used to replace the water in tissues with alcohol. Dehydration facilitates wax infiltration and sectioning. The dehydration step was performed by placing the tissue into graded alcohols 70%, 80%, 95%, 100% and 100% for two hours each. The clearing process then removes the alcohol from the tissue
and replaces it with an intermediate solvent that is miscible with paraffin wax. This was done by placing the dehydrated tissue in two changes of xylene for 1.5 hours each. The final step was to replace the clearing agent with molten paraffin wax. The wax infiltrates the specimen to fill out all cavities in the tissue and to support the delicate structure of the tissue against the shearing forces of sectioning. This process required four changes of molten wax to ensure complete removal of the clearing agent, each step lasting one hour.

A microtome sectioning instrument was used to obtain 5µm sections of the kidney specimens which were then mounted onto microscope slides. After cutting, the sections were floated onto a 42°C water bath and then picked up and placed onto a pre-coated slide (SuperFrost®, MENZEL-GLÄSER, Berlin, Germany). The slides were then placed in a 45°C oven overnight to fix the specimens onto the slides. The first step in each staining process was to deparaffinise the sections, this was done by rehydrating the slides by placing them in two changes of xylene for 15 minutes each, followed by two changes of 100% alcohol, two changes of 95% alcohol, one change of 70% alcohol and then washing in water (two minutes each). All incubations were carried out at ambient temperature using room temperature reagents unless otherwise stated.

2.10.1 Haematoxylin and Eosin (H&E) staining

The slides were placed in Harris’s haematoxylin for two minutes and then washed in water to remove excess haematoxylin. To differentiate the nuclei and cytoplasm, excess stain is removed by quickly dipping the slides in acid alcohol ten times and
then washing in water again. The slides were then placed in Scott’s blueing solution for 30 seconds and then washed in water once again. The slides were examined under a microscope to assess whether the nuclei were clearly stained and that the cytoplasm was not. If the staining was not adequate then the slides would have to be passed through the same steps from the acid alcohol once more.

If the staining of the nuclei was acceptable, the slides would be placed in 70% ethanol for 30 seconds. This was followed by staining the slides in two changes of eosin, 40 seconds each. Slides were then dehydrated and coverslipped as described below.

2.10.2 Periodic Acid Schiff (PAS) Staining

After deparaffinisation and hydration, the sections were placed into 1% periodic acid solution for 15 minutes followed by a brief wash in tap water. The sections were then rinsed in distilled water for 5 minutes followed by a 30 minute incubation in Schiff’s solution. The sections were washed in tap water for 15 minutes and then counterstained, dehydrated and coverslipped as outlined below.

2.10.3 Staining for Neutrophils

The initial step in this staining process was antigen retrieval. This process is performed to expose epitopes which have become masked by the tissue fixation process. We did this by placing the slides in a plastic specimen container containing a citrate buffer (10mM trisodium citrate dehydrate pH 6.0). The container was then placed on top of a heat shield within a pressure cooker pan containing 500ml of
water. The pressure cooker used was a Digital decloaking chamber (BioCare Medical) and the settings used for the pressure cooker were as follows: no delayed start, preheat for 30 seconds at 80°C, auto-continue function on, P1 125°C, P2 fan on 95°C, P2 fan off 90°C.

Once the specimens had cooled down to room temperature they were washed in TBST buffer three times for three minutes each. After the washing, a wax outline was drawn around each of the sections with a Dako pen and then 80 µL of blocking solution was placed on the sections for 20 minutes. The blocking solution comprised 20% normal goat serum in a diluent TBST buffer.

After the blocking step the sections were incubated with the primary antibody for one hour. The primary antibody used was a Rat Anti Mouse Ly-6B.2 Alloantigen (AbD Serotec), this was diluted 1:100 in 1% normal goats serum/TBST diluent. Two control stains were used for this step, the negative control stain used was 1% NGS in TBST diluent, the isotype control (no specificity for neutrophils) used was FITC Rat IgG2a k Isotype control (BD Bioscience) 1:50 in 1% normal goat serum/TBST diluent. The specimens were then washed three times for three minutes in TBST wash buffer.

Following this, the slides were incubated in 3% H₂O₂/methanol for 5 minutes. This step was done to block any endogenous peroxidase activity. The slides were then washed again three times for three minutes in TBST wash buffer. All sections, including those stained with the control antibodies, were then incubated with the secondary antibody. The secondary antibody used was biotinylated goat anti-rat
(BD Bioscience) diluted 1:150 with 1% normal goats serum/TBST diluent. After incubation the slides were washed three times in TBST wash buffer for three minutes each. Detection was performed using the Vectastain ABC system as for CD68 above. The slides were then counterstained, dehydrated and coverslipped as below.

2.10.4 Staining for Proliferating Cell Nuclear Antigen (PCNA)

The staining for PCNA was similar to that of neutrophils. The only differences were that, for PCNA, slides were place in a plastic specimen container containing Tris buffer (pH 9) for antigen retrieval instead and that PCNA utilised different primary and secondary antibodies at different concentrations. For PCNA staining, the primary antibody (rabbit anti-PCNA, polyclonal, Cat # ab2426, abcam, England) was added at a dilution of 1:500 and the secondary antibody (biotinylated goat anti-rabbit, polyclonal, Cat # E0432, Dako, Denmark) was added at a dilution of 1:300.

2.11 Counterstaining and coverslipping

Both immunohistochemistry from frozen sections and paraffin sections were counterstained using the same process. The slides were counterstained in Harris’ haematoxylin for 5-10 seconds and then washed in water to remove excess staining. To differentiate the nuclei and cytoplasm staining, excess stain was removed by dipping slides in acid alcohol 1-3 times and then washing in water. The slides were then placed in Scott’s blueing solution for 30 seconds and washed in water once more. The slides were examined under a microscope to assess whether the nuclei
were clearly stained and that the cytoplasm was not. If the staining was not
datequate then the slides would have to undergo the above steps again.

The next step in the process was to dehydrate the slides. This was done by placing
the slides in graded alcohols 70%, 95%, 95%, 100%, 100% for two minutes each and
then finally in two changes of histolene/xylene for two minutes each.

The final step in the process is to coverslip the slides. This is done by placing DPX
(dibutylphthalate polystyrene xylene) on microscope slide coverslips and then
placing the slides on top of this solution. Air bubbles were then removed from
between the coverslip and the slide with a probe and the slides were left to dry.

2.12 RT-PCR

2.12.1 RNA Extraction

RNA was extracted with an RNAqueous-Mini™ kit (Ambion, Texas, USA) following a
modified protocol. Lab coat, face mask and gloves were worn throughout the
procedure. All steps were carried out on ice unless mentioned otherwise. 4 Falcon
tubes of 50ml of triple distilled water (TDW) at RT were used to wash the
homogeniser (Polytron@ PT2100) initially and in between samples, 700µL of lysing
buffer (RNAqueous-Mini™ kit, Ambion, Texas, USA) was placed into a RNase-free
2ml Eppendorf tube on ice for each sample. A frozen tissue specimen of approx 3m³
was placed inside a tube with the lysis buffer and homogenized with the
homogeniser. Repeated aspiration and expulsion with a 26G gauge needle and
syringe was used to shear DNA and decrease the viscosity of the lysate.
70μL of 2M sodium acetate (Sigma) was then added to the tube and mixed briefly, followed by the addition of 700μL of RNA grade phenol solution (Sigma). The tube was then vortexed thoroughly for 30 seconds and left on ice for 5 minutes to settle. 140μL of chloroform (BDH AnalaR, Kilsyth, Australia) / isoamyl alcohol (BDH AnalaR) was then added, vortexed thoroughly for 30 seconds, and allowed to sit on ice for 15 minutes. The tube was then centrifuged at 11,000rpm at 4°C for 45 minutes using a centrifuge (5415R, Eppendorf International, Germany).

Once the centrifugation was completed, the aqueous (upper) phase of the solution was transferred into a new 2mL Eppendorf tube, taking care not to transfer any of the interphase (middle) or phenol (lower) phase as they contain contaminating DNA and protein.

800μL of 65% ethanol (RNAqueous-Mini™ kit, Ambion, Texas, USA) was added to the removed aqueous phase. This was then purified on an RNA affinity column (RNAqueous-Mini™ kit, Ambion, Texas, USA) following the manufacturer’s protocol. To the 180μL of eluted RNA, 2μL of glycogen, 12μL of 7.5M ammonium acetate and 390μL of 100% ethanol were added and left to precipitate overnight at -20°C before being centrifuged at 11,000rpm at 4°C for 45 minutes.

The supernatant was removed carefully with gentle pipetting as to not disturb the pellet. The RNA pellet was then washed with 1.5mL of 70% ethanol and centrifuged at 11,000rpm at 4°C for 10 minutes. The supernatant was once again removed carefully, as above. The pellet was then allowed to air-dry at RT for 10 minutes before dissolved and mixed thoroughly in 30μL of DEPC-water.
Absorbance of 1µL of the extracted RNA in DEPC-water was measured, using a Nanodrop Spectrophotometer (ND-1000, Thermo Scientific, MA, USA) to determine its concentration. The RNA was diluted down to 0.2µg/µL, and stored in liquid nitrogen.

2.12.2 Analysis of RNA quality using the Agilent Bioanalyser

Agilent RNA 6000 Nano Kit (Cat # 5067-1511, Agilent Technologies) was used to analyse the integrity of the extracted RNA. The analysis was carried out according to the manufacturer’s protocol. The instrument generates an absorbance spectrum (Figure 21 below) which is used to calculate the RNA integrity number (RIN). All samples had a RIN greater than 7.5, providing evidence of high quality RNA without degradation.

![Absorbance spectrum generated to calculate RIN](image)

**Figure 21 Absorbance spectrum generated to calculate RIN.** This an example of the spectrum used to calculate RIN.
2.12.3 cDNA Synthesis

All steps were carried out on ice unless mentioned otherwise. 1μg of RNA (5μL of the stored product) was made up to 10μL with (5μL of) DEPC-water in a 0.2mL tube. 3μL of Oligo dT was added to the sample and then mixed briefly before being heated for 5 minutes at 70°C. The sample was then spun briefly again and cooled on ice. To the sample, 4μL of 5x reaction buffer, 2μL 0.1M DTT and 1μL of 10mM dNTP were added. After the additions, the sample was mixed by pipetting up and down and then heated for 2 minutes at 50°C. 1μL of Superscript III (Invitrogen, CA, USA) enzyme was added before heating for 60 minutes at 50°C followed by 15 minutes at 70°C. The final product (cDNA) was then diluted 1:5 with 80μL of DEPC-water and aliquotted in 4μL volumes into PCR trays. Caps were placed tightly over the wells and the trays were stored at -80°C until amplification.

2.12.4 Analysis of Genes / PCR

A master mix was prepared for each reaction. The master mix comprised 10μl of 2x Universal buffer (SensiMix, BIOLINE, USA), 0.2μl of 100x Primers (FOR+REV) (30μM), 0.2μl of 100x Probe (20μM), and 5.6μl of TDW making up a total volume of 16μl. The master mix was mixed thoroughly and 16μl of the master mix was placed into each of the stored cDNA wells. The wells were capped and centrifuged for 1 minute at 1,000rpm at RT temperature in a centrifuge (Allegra X-12R, Beckman Coulter, USA) and then the plate was run on the LightCycler® 480 (Roche Diagnostics, Indiana, USA).
The Primers and Probes used were either ordered as a whole product from Applied Biosystems or individually designed using Primer Express and purchased from Sigma. The genes purchased as a completed product from Applied Biosystems were CXCL10 (Mm00445235), IL10 (Mm00439616), TGF-β (Mm00441724), IL6 (Mm00446190), HSP2 (Mm00434069), TNF-α (Mm00443258) and TLR4 (Mm00445274). For a list of the genes we designed and ordered from Sigma, refer to Table 1 below.

**Table 1 Designed sequences of Primers and Probes for genes of interest.** The sequences for the genes of interest were designed using Primer Express and then purchased from Sigma.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TGCACCACCACTGCTTAGC</td>
<td>GGCATGGACTGTGTCATG</td>
<td>CCTGCCAAGGTACCATCCATGAC</td>
</tr>
<tr>
<td>KC</td>
<td>TTGGTTCAGAAAAATTGTCCAAAAG</td>
<td>CAGGTGCCATCAGACAGCT</td>
<td>TGCTAAAAGGTGTTCCCCCAAGTA</td>
</tr>
<tr>
<td>MCP1</td>
<td>GAGCATCCACGTGTTGCCAT</td>
<td>TGGTAATGAGTAGACAGCA</td>
<td>AGCCGATGCAGTAAAGCCCC</td>
</tr>
<tr>
<td>MIP2</td>
<td>GCCCCAGGACCACA</td>
<td>CTTTTCAGCCCTTTCGA</td>
<td>TGGACCAGACAGAAGTCATA</td>
</tr>
<tr>
<td>CCL17</td>
<td>GGATGCCATCGTTTTCTG</td>
<td>CCAATCTGATGCGCTCTTCTC</td>
<td>TCCAGGGCAAGCTCATCCTGTC</td>
</tr>
<tr>
<td>CCL22</td>
<td>GGAGTTCTTCTGGACCTCAAAATC</td>
<td>TCTCGGTTCCTGACAGTTATCA</td>
<td>CAACGCCAGGCTTGCGGCA</td>
</tr>
<tr>
<td>IL1β</td>
<td>GCACACCCACCTGCAG</td>
<td>AACCCTTTTCTACATTCCTTTTCTT</td>
<td>TGGAGAGTGTTGGATCCCAAGC</td>
</tr>
<tr>
<td>RAGE</td>
<td>GGACCCTTACGGCACCCTTGA</td>
<td>GAGTCCGGCTTCAGGGTGTC</td>
<td>ATCCCGATGGCAAAGAACAAACA</td>
</tr>
</tbody>
</table>
2.13 Statistical Analysis

Different methods of statistical analysis were used in different chapters of the thesis.

2.13.1 Chapter 3

In Chapter 3, one-way ANOVA, followed by a post-hoc T-test with Bonferroni correction was used. Results were presented as mean ± SEM.

2.13.2 Chapter 4

Here, comparison of complications (listed in Table 2) was performed using Fisher’s exact test. The survival analysis utilized the log-rank (Mantel-Cox) test.

2.13.3 Chapters 5 and 6

In Chapters 5 and 6, where distribution of data is parametric, it is displayed as mean ± SEM. Where data appears to be non-parametric, it is displayed as individual data points with the median indicated.

Comparison of multiple groups of parametric data was performed using one-way ANOVA, followed by a post-hoc T-test with Bonferroni correction. Pairwise comparisons of cell counts at a single time point (Figures 50 and 51) were undertaken using Student’s T-test.
CHAPTER 3: CLONING AND EXPRESSION OF esRAGE

AND CONTROL CONSTRUCTS

3.1 Creation and Amplification of rAAV esRAGE

esRAGE was amplified from resected lung tissue, and agarose gel electrophoresis of the PCR products yielded a band of expected size (Figure 22 below).

![Amplification of esRAGE from human lung tissues.](image)

**Figure 22 Amplification of esRAGE from human lung tissues.** Primers specific for esRAGE amplified a PCR product of the expected size 1113 base pairs (bp) from resected human lung tissue. The position of the 1031bp fragment of the DNA ladder is indicated.
The nucleotide sequence was subsequently confirmed to be identical with that of the NCBI reference sequence for esRAGE (accession number AB061668). In parallel, a codon-optimised cDNA sequence for esRAGE was designed by GeneART (Life Technologies, NY, USA), using a proprietary algorithm. Codon optimisation is a process which exploits the redundancy of the genetic code to replace certain nucleotides with others which will not result in a change to the amino acid sequence of the translated protein product, but will create a more favourable secondary structure for high-level expression of the transgene product. The chromatogram and sequence is shown in Figure 23.
Figure 23 Codon-optimised nucleotide sequence of esRAGE designed by GeneART.
To verify that both esRAGE constructs would yield the correct secreted protein product, the native and codon-optimised GeneArt sequences were each subcloned into the mammalian expression vector pcDNA 3.2 TOPO as described in ‘Chapter 2’. HEK293D cells were transfected with this construct using Lipofectamine (Life Technologies, USA), and the supernatants collected at 48 and 72 hours post-transfection. Concentration of esRAGE in supernatant was estimated using the B-Bridge esRAGE ELISA kit. High levels of secreted esRAGE were detected in the culture supernatants of HEK293D cells transfected with plasmids containing either native or codon-optimised constructs (Figure 24). Expression appeared slightly stronger from the codon-optimised construct, and this was used for packaging into rAAV2/8 and all subsequent experiments.

esRAGE was packaged into an rAAV2/8 vector as described in ‘Chapter 2’. A schematic diagram of the vector genome is shown below (Figure 25).
Figure 24 ELISA plate showing expression of esRAGE from both constructs (yellow wells). Wells 1-8 of rows D and F represent dilution series of TCS for cells transfected with codon-optimised (D) and native (F) constructs. Expression was stronger from the codon-optimised construct.

![ELISA plate](image)

Figure 25 Schematic representation of esRAGE expression cassette. esRAGE is packaged into rAAV2/8. The inverted terminal repeats are the only elements remaining from wild-type AAV2. An ApoE/hAAT promoter/enhancer confers hepatocyte specificity.
3.2 in-vivo expression of esRAGE

Following the packaging of rAAV-esRAGE, we performed a dose-response experiment to determine the most appropriate vector dose. Male C57BL/6 mice aged 8-10 weeks received doses of rAAV-esRAGE of $1 \times 10^{10}$, $5 \times 10^{10}$, $1 \times 10^{11}$, and $5 \times 10^{11}$ vector genomes (VG) via intraperitoneal injection (IP). The concentration of esRAGE was then measured in the serum of the injected mice at 10 days post-injection. Systemic expression of esRAGE was dose-dependent, and the dose of $5 \times 10^{11}$ VG resulted in the highest esRAGE serum concentration (Figure 26). This vector dose did not result in liver inflammation as measured by serum ALT concentration or the presence of an inflammatory infiltrate upon H and E staining of the liver (Figure 27 A and B), and this dose was therefore chosen for use in later experiments.
**Figure 26 Dose-response curve for rAAV-esRAGE.** Mice were injected IP with esRAGE at doses ranging from $1 \times 10^{10}$ to $5 \times 10^{11}$ VG. Levels of esRAGE measured in serum by ELISA were dose-dependent and many thousand-fold greater than physiological concentration in humans (0.1-1 ng/mL). n=4-6 in each group.

**Figure 27 Administration of rAAV-esRAGE does not result in liver inflammation.** Neither serum ALT levels (A) nor the appearances of liver on H and E staining (n=4-6 in each group) (B) suggested liver inflammation with the highest dose of $5 \times 10^{11}$ VG rAAV-esRAGE.
We next conducted a time-course experiment using $5 \times 10^{11}$ VG intraperitoneally (IP) to determine the kinetics of rAAV2/8-mediated esRAGE expression. Murine esRAGE is undetectable in serum, and physiological concentrations of esRAGE in humans are in the range 0.1-1 ng/mL. By d2 following IP injection, levels around 5000-fold greater than physiological concentrations in humans had already been achieved. esRAGE levels continued to increase until d42 post-injection, then started to decline. Nonetheless, durable high-level expression was demonstrable throughout 100 days after injection. For practical reasons, it was determined that sufficient levels of esRAGE for our experimental purposes were reached by 7 days after IP injection. Procedures of brain-death induction and renal transplantation were therefore performed 7 days post-inoculation.

Figure 28 Kinetics of expression for rAAV-esRAGE injected intraperitoneally (IP) at $5 \times 10^{11}$ VG. Serum esRAGE expression was already substantial by d2, and reached a peak at day 42 followed by a slow decline. n=4-6 in each group.
Finally, to verify our assumption that esRAGE secreted by the liver would be distributed throughout the body, and would appear in high concentration within transplantable organs, we collected liver, spleen, kidney and heart from two C57BL/6 mice at 5 days following IP injection with $5 \times 10^{11}$ VG rAAV-esRAGE, and measured the concentration of esRAGE per gram of total protein in tissue lysates. Not surprisingly, the highest levels were detected in liver, where esRAGE is produced, followed by kidney where esRAGE is reabsorbed from the urine. Spleen and heart contained lower, but still substantial levels of esRAGE (Figure 29). No human esRAGE was detected in organs from control C57BL/6 mice.

![esRAGE level in tissues d5](image)

**Figure 29 Levels of esRAGE in various organs.** Concentrations of esRAGE in relation to total protein in tissue lysates were greatest for liver and kidney, and less, though still substantial for spleen and heart. No human esRAGE was detected in organs from control C57BL/6 mice. n=4-6 in each group.
3.3 Control vectors

Two control vectors were used in subsequent experiments. A vector encoding Human serum Albumin was produced by PhD student Miriam Habib using albumin cloned from explanted human liver. Vector production and packaging were as outlined here for esRAGE, and a dose of $5 \times 10^{11}$ VG yielded a concentration of human albumin of $90\mu$g/mL. An rAAV vector encoding green fluorescent protein (eGFP) was the generous gift of Professor Ian Alexander. We performed a timecourse experiment to determine kinetics of expression by injecting the vector at a concentration of $5 \times 10^{11}$ VG IP, then detecting green fluorescence of hepatocytes in two ways. Macroscopically, we used a fluorescence stereomicroscope (M165FC, Leica, Germany) with illumination at 395-455nm and an FITC filter to photograph the liver and surrounding tissues at days 0, 2, 5, 7, 10, 21, 42 and 100. Specimens were also collected into OCT for sectioning and fluorescence microscopy.

Macroscopic images obtained at each sampling time are shown in Figure 30 below. No fluorescence was observed on day 0. From day 2 onwards, liver fluorescence was detected, reaching peak intensity between days 21 and 42, with a modest decline by day 100. No fluorescence of other organs was detected.
Figure 30 Kinetics of expression of control vector rAAV-GFP. The left panel for each sampling time shows the abdomen illuminated with white light at the same time as blue light of 395-455nm. The right panel shows liver fluorescence alone. Strong liver fluorescence was detectable from d5 to d100 post-injection. Fluorescence of other abdominal organs was not detected.
CHAPTER 4: DETERMINATION OF THE OPTIMUM METHOD OF URETERIC RECONSTRUCTION FOR USE IN MOUSE RENAL TRANSPLANTATION.

4.1 Study Rationale

Our research group has utilised two different methods for ureteric reconstruction during mouse kidney transplantation. Prior to commencing kidney transplantation from brain dead donors, we undertook a study with both retrospective and prospective arms, comparing the two methods. The methods are bladder anastomosis using a bladder patch, and ureteric implantation into the bladder. This second method is a refinement of previously published implant procedures.

For the retrospective analysis, there were a total of 62 cases in which the bladder patch method was used and 101 cases where the implant method was used. Animal survival, and in cases of mortality, post-mortem findings are summarized in Table 2 and Figure 31. For the patch method, 13 out of 62 animals died or were euthanized within 3 post operative days with a success rate of 79%. Using the implant method, 14 out of 101 animals died or were euthanized within 3 post operative days with a success rate of 86.1%, which was not significantly different compared to that of the patch method (p=0.28). The reasons for early animal death included vessel complications which often resulted in hind limb paralysis and the incidence of these complications was similar in both groups. Most other early deaths were of indeterminate cause. From day 4 to day 14, 7 out of 49 animals (14%) died of
ureteric complications with the patch method, while 10 out of 87 animals (11%) with the implant method suffered ureteric complications. The implant method led to apparently higher levels of ureteric stenosis (Table 2), although the result was not statistically significant (p=0.33). In contrast, the patch method was more likely to cause bladder necrosis and leakage (confirmed by the presence of urine in the abdominal cavity and direct visualization of the ureter/bladder patch anastomosis site at post-mortem) (p=0.02). The majority of animals dying during this period succumbed to graft rejection rather than operative complications. After day 14, most transplanted animals survived long term with some surviving greater than 300 days in the retrospective series (Figure 31A). Varying degrees of hydronephrosis were noted in some long-term surviving animals at the time of death or when sacrificed for tissue collection. The proportion of animals with hydronephrosis was equivalent in each treatment group. Median survival with the patch method was 7 days compared to 35 days with the implant method. A higher proportion of animals died before day 15 in the patch group than in the implant group but this difference did not quite reach statistical significance (p=0.06). Overall survival assessed at 300 days post-transplant was not different between the two methods (p=0.09) (Figure 31A).

The prospective analysis is shown in Figure 31B and confirmed the findings of the retrospective analysis. There was no significant difference in survival between the two groups (p=0.82) or between the survival in the retrospective versus prospective analysis for the patch method (p=0.29) or the implant method (p=0.51). For the
patch method, one animal died at day 10 with urine leak, one at day 5 with ureteric and bladder patch necrosis (Figure 32), one at day 4 with renal artery thrombosis and one at day 79 with hydronephrosis. In the implant method, one animal died at day 7 with possible lymphatic leakage and one at day 35 with hydronephrosis. The causes of death for the remaining animals in both groups could not be determined.

The operation times for the two methods differed. The operation time for the donor procedure for the implant method (14.8 ±2.2 min) (n=16) was significantly shorter than that for the patch method (28.3±2.4) (n=16) (p<0.001). Similarly, the recipient operation time using the implant method (61.4±4.7) was shorter than that using the patch method (77.8±5.5) (p<0.001). There was no difference between the two methods in the time to complete the arterial (p=0.07) or venous (p=0.70) anastomoses, or in the graft ischaemia time (p=0.71). The average total operation time (donor plus recipient times) was 76 minutes/transplant for the implant method, while 106 minutes/transplant for the patch method.

Because the implant method was a significantly faster operation, with outcomes at least as good as those of the bladder patch method, we decided to use the implant method for all subsequent mouse kidney transplant experiments. The ureteric implant procedure is described in detail in ‘Chapter 2’.
Table 2 Results of mouse kidney Tx with patch and implant ureteric anastomosis:

<table>
<thead>
<tr>
<th>Survival / Reason for Death</th>
<th>Patch (n=62)</th>
<th>Implant (n=101)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 3 Days (n) (%)</td>
<td>13 (21.0%)</td>
<td>14 (13.9%)</td>
<td>0.28</td>
</tr>
<tr>
<td>→ Limb Paralysis</td>
<td>3 (4.8%)</td>
<td>5 (5.0%)</td>
<td>1.00</td>
</tr>
<tr>
<td>→ Unknown</td>
<td>10 (16.1%)</td>
<td>9 (8.9%)</td>
<td>0.21</td>
</tr>
<tr>
<td>4 - 14 Days (n) (%)</td>
<td>23 (37.1%)</td>
<td>30 (29.7%)</td>
<td>0.39</td>
</tr>
<tr>
<td>→ Ureteric Stenosis</td>
<td>2 (4.1%)*</td>
<td>9 (10.3%)*</td>
<td>0.33</td>
</tr>
<tr>
<td>→ Bladder Leak</td>
<td>5 (10.2%)*</td>
<td>1 (1.1%)*</td>
<td>0.02**</td>
</tr>
<tr>
<td>→ Rejection</td>
<td>16 (32.7%)*</td>
<td>20 (23.0%)*</td>
<td>0.23</td>
</tr>
<tr>
<td>&gt; 14 Days (n) (%)</td>
<td>26 (41.9%)</td>
<td>57 (56.4%)</td>
<td>0.08</td>
</tr>
<tr>
<td>→ Hydronephrosis</td>
<td>4 (15.4%)*</td>
<td>5 (8.8%)*</td>
<td>0.45</td>
</tr>
</tbody>
</table>

* Expressed as the percentage of surviving animals at each time point.

** There are significant differences between the data compared.
Figure 31 Animal survivals with the two different ureteric reconstruction methods.

A. Retrospective data showing that early mortality from nonspecific causes appeared to be higher in the mice transplanted using the patch method than in those transplanted using the ureteric implant method, but thereafter survival curves were parallel. B. Prospective study survival data showing that there is no difference between the two different methods for long-term survival.
Figure 32 Bladder patch necrosis is an important cause of early death of mice following renal transplantation using the bladder patch anastomosis method. Ureteric reconstruction with patch method (4 days post transplantation) showing necrosis of the donor bladder patch and distal end of the ureter due to inadvertent damage to the blood supply during the operation. The vertical arrow points to the urine leakage site, whilst the horizontal arrow shows the necrotic patch and ureter.
CHAPTER 5: ESTABLISHMENT AND CHARACTERISATION OF A MURINE
MODEL OF BRAIN DEATH INCLUDING EFFECTS UPON SYNGENEIC
RENAL TRANSPLANTATION

5.1 Establishment of a mouse model of brain death

Steps in the establishment of the murine brain death model are described in
‘Chapter 2’. Once established, the model was characterised with respect to the
physiological parameters of the mouse, release of DAMPs such as S100B and
HMGB1, release of cytokines and chemokines into the systemic circulation, and
histological and biochemical markers of organ damage.

5.2 Physiological parameters

Many physiological parameters were measured before, during and after the
induction of brain death. Mean arterial pressure (MAP) was measured via a femoral
artery cannula and BP transducer. A rectal probe was used to detect the
temperature of the mouse and a mouse pulse oxymeter with tail clip was used to
monitor the heart rate (HR) and oxygen saturation (SO2). During brain death, a
Cushing response was observed with a spike in MAP accompanied by tachycardia.
The tachycardia persisted, whereas MAP slowly declined. Oxygen saturation was
maintained throughout the experiment at close to 100%. The body temperature of
the mice were maintained between 36.5-38°C. Recordings taken during a typical BD
experiment are shown in Figure 33 below.
Figure 33 Physiological traces of a mouse maintained for a duration of 3 hours after the induction of brain death. At the point of BD, a peak in blood pressure (MAP) occurs, concurrent with an increase in heart rate (HR). MAP slowly drops over time, whereas tachycardia persists. IV boluses of 0.25ml Gelofusine were given whenever the MAP reached 40mmHg. Oxygen saturation was maintained close to 100% throughout the maintenance period. Core temperature of the mice was kept between 36.5-38°C.
5.3 Release of Damage-associated molecular patterns (DAMPs)

Inflammatory ligands released from tissues during ischaemia and reperfusion include HMGB1 and S100B. We determined whether these ligands were released into the systemic circulation at 30 and 60 minutes, and 3 hours following the induction of brain death in our model, as well as in ventilated, non-BD controls.

5.3.1 S100B

Serum S100B is a biomarker for CNS and other tissue damage. By 30 minutes after the induction of brain death, an increase above baseline measurements in serum S100B could be observed (Figure 34). Peak levels of serum S100B (10,163+/−4775 pg/ml) were reached three hours after the onset of brain death (P<0.05 compared with baseline). Serum S100B was also elevated above baseline in the mice ventilated for three hours (1731+/−1114 pg/ml) but the levels attained in these mice were significantly less than those in the BD group (P<0.05).
Figure 34 Timecourse of systemic S100B release after induction of brain death.

S100B was detectable in the serum at levels above baseline from 30 minutes after the induction of brain death. Peak levels were reached at 3 hours after BD induction, and a more modest elevation in levels of S100B was also noted in the serum of ventilated controls at this time. n=4-6 in each group.
5.3.2 HMGB1

In contrast to S100B, serum levels of HMGB1 were not appreciably increased until three hours after the induction of brain death (26.53+/−6.38ng/ml v/s 3.83+/−0.98ng/ml, P<0.001) (Figure 35). While serum levels of HMGB1 increased slightly above baseline in ventilated controls, levels of HMGB1 in BD mice were significantly greater than those in control animals (8.12+/−2.1ng/ml, P<0.01).

Figure 35 Timecourse of systemic HMGB1 release after induction of brain death.

HMGB1 levels were significantly increased at 3 hours after the induction of brain death, compared with baseline levels and with levels in animals ventilated for three hours. n=4-6 in each group.
5.4 Release of cytokines into the systemic circulation

Increased systemic levels of a number of pro-inflammatory cytokines and chemokines including IL-6, TNF-α and IL-8 have been well-documented following the induction of brain death. Here, we used a multiplex assay to determine levels of a panel of cytokines at various times following BD induction. Most of these soluble factors displayed a similar pattern of systemic release, with maximal or near maximal levels being reached by 60 minutes after BD induction (Figure 36).
Figure 36 Systemic release of cytokines after the induction of brain death. n=4-6 in each group.
5.5 Histological and biochemical evidence of organ damage and inflammation during brain death

5.5.1 Lungs

Given that ventilator-induced lung injury can occur, even with gentle mechanical ventilation, and that this can result in systemic inflammatory manifestations [174], we assessed the lungs histologically. In these mice which received colloid fluid support intravenously, there was little evidence of pulmonary oedema (data not shown). However, a very marked neutrophil infiltration was present in both the lungs from ventilated controls and was even more pronounced in brain-dead animals (Figure 37).
Figure 37 The effect of ventilation and brain death on neutrophil infiltration in mouse lungs. A is a normal mouse lung stained for neutrophil infiltrates. Few neutrophils are present; B is lung from a mouse which had undergone ventilation for 3 hours. Neutrophils are plentiful; C is lung from a BD mouse after 3 hours of maintenance in the BD state. Heavy neutrophil infiltration can be observed.
5.5.2 Kidneys

Damage to kidneys was assessed histologically, as well as by measuring the expression of neutrophil Gelatinase-associated lipocalin, an early biomarker of renal damage. The expression of genes for a range of cytokines and chemokines, as well as for the pattern-recognition receptors RAGE and TLR4 was measured, and inflammatory infiltrates of neutrophils and macrophages were detected by immunohistochemistry.

5.5.2a Serum Neutrophil Gelatinase-associated Lipocalin (NGAL)

NGAL is a sensitive biomarker for renal injury, and able to detect damage in the acute phase of renal injury before a rise in serum creatinine has occurred [175]. By 60 minutes after the induction of brain death, the serum NGAL levels have clearly increased above the baseline of to a level >1000µg/ml (1142+/−220µg/ml, P=0.0026) (Figure 38). The maximum serum NGAL level was reached at three hours after the onset of brain death (5990+/−7035µg/ml). Levels of NGAL are also elevated above baseline in the ventilated controls after three hours (455+/−345µg/ml) but this rise is modest in comparison to that in BD animals (P=0.015).
Figure 38 Timecourse of serum NGAL after induction of brain death. Serum levels of NGAL had risen substantially by 60 minutes after the induction of brain death and were further elevated at the 3-hour sampling point. A modest elevation in serum NGAL was found in ventilated controls after 3 hours. n=4-6 in each group.
5.5.2b Expression of pattern-recognition receptors

Increased expression of pattern recognition receptors in kidneys following ischaemia reperfusion injury has been widely reported [42, 44]. We used RT-PCR to determine whether upregulation of RAGE and TLR4 could be detected in mouse kidneys soon after induction of brain death. We were surprised to find that, if anything, mRNA expression for these genes appeared to be decreased by three hours after the induction of BD. A similar decrease was seen for ventilated controls, suggesting that this effect may have resulted from prolonged anaesthesia or mechanical ventilation (Figure 39 below).

Figure 39 Gene expression for pattern-recognition receptors in kidneys during brain death. n=3-6 in each group.
5.5.2c Expression of cytokines and other soluble mediators

Gene expression (mRNA levels) for the chemokines CXCL10, KC, MCP1, MIP2, and CCL22 follows a similar pattern whereby the peak of expression is reached by 1 hour after the onset of brain death. Expression of CCL17 started to rise earlier than the other chemokines (Figure 40 below). Cytokines with pro-inflammatory activity, such as IL-6, IL-1β and TNF-α had attained maximum expression by 60 minutes after the induction of brain death, as had those with reported immunomodulatory activity (IL10, TGF-β) (Figure 41).
Figure 40 Chemokine gene expression in kidneys following the induction of brain death. The following genes were analysed within the kidneys: CXCL10, KC, MCP1, MIP2, CCL17 and CCL22. All had reached peak expression within one after the induction of BD. n=3-4 in each group.
Figure 41 Cytokine gene expression in kidneys following the induction of brain death. The following genes were analysed within the kidneys: IL10, TGF-β, IL6, IL1β, HSP2 and TNF-α. Similar to the chemokine genes examined, all had reached peak expression within one after the induction of BD. n=3-4 in each group.
5.5.2d Neutrophil infiltration within the kidneys of mice following brain death

Histological changes lag behind changes in gene expression, and whereas the neutrophil chemokine KC was upregulated in renal tissue and in serum at 60 minutes after the onset of BD, neutrophils were not detected in the kidneys of mice at times earlier than 3 hours post-BD. It can be seen from Figure 42 that there are significantly more neutrophils in kidneys from mice at 3 hour post-BD (8.8+/−5.6 cells per high powered viewing field) than ventilated controls kidneys (1.9+/−1.6 cell per high powered viewing field, P=0.03).

![Neutrophil cell count graph](image)

**Figure 42 Neutrophil infiltration of kidneys at 3 hours after induction of BD.** There are significantly more neutrophils in kidneys from BD mice at 3 hours than in ventilated controls and normal controls. n=4-6 in each group. * indicates significant differences between the compared groups (P-value <0.05).
5.5.2e Macrophage infiltration within the kidneys

No one epitope is able to identify all subsets of macrophages in mice. Antibodies recognizing two different epitopes (CD68 and F4/80) were used for macrophage detection. Normal kidneys have a substantial population of resident macrophages (13.7+/- 6.9/HPF, Figure 43A, and 16.3+/-2.8/HPF, Figure 43B). A modest increase in macrophage numbers above this baseline was noted in kidneys after 3 hours of BD using either antibody (F4/80: 23.3+/-10.1/HPF, P=0.03; CD68: 25.3+/-7.6/HPF, P=0.005). CD68 but not F4/80 staining also identified a small increase in macrophage numbers in the kidneys from ventilated control mice (22+/-4.8/HPF, P=0.005) (Figure 43B).
Figure 43 Macrophages within the kidneys of mice after brain death. A and C: staining with F4/80, B: staining with CD68. Based on F4/80 staining, there is a modest increase in macrophages in the kidneys of BD mice compared with the normal baseline. CD68 identified a slight increase in renal macrophage numbers in ventilated controls as well as in BD mice. n=4-6 in each group. For A and B, * indicates significant differences between the compared groups (P-value <0.05).
5.5.2f Kidney Morphology

H&E stained sections of kidneys were examined for morphological evidence of damage to kidneys during brain death. Kidneys from BD mice after 3 hours of maintenance showed evidence of some tubular vacuolation and a mild increase in interstitial inflammatory infiltrate, when compared with normal mouse kidneys (Figure 44 A and B). Because it is not feasible to attempt to maintain mice for longer than 3 hours after the induction of brain death, kidneys from BD mice were collected after 3 hours of maintenance, then transplanted into syngeneic recipients. At D4 after syngeneic transplantation, these kidneys show features of acute tubular necrosis (ATN), with loss of tubular nuclei and a mixed infiltrate including neutrophils and mononuclear cells, Figure 44D. The extent of renal damage in these kidneys appeared substantially greater than that in kidneys transplanted from control donors into syngeneic recipients (Figure 44C), making it uncertain whether these kidneys would be able to support life in later planned experiments following the removal of the contralateral native kidney on post-operative d4.
Figure 44 Histological appearances of normal kidney, and kidney at various stages after BD induction and/or renal transplantation. A: normal kidney; B: kidney at 3 hours after induction of BD shows some evidence of tubular vacuolation and a mild increase in interstitial inflammatory infiltrate; C: kidney at day 4 after syngeneic transplantation from a control donor shows tubular dilatation and occasional protein cast formation; D: kidney at day 4 after syngeneic transplantation from a brain dead donor (maintenance period 3 hours) displays features of ATN, with loss of tubular nuclei and a mixed infiltrate including neutrophils and mononuclear cells.
Because of the rapid kinetics of inflammatory cytokine release we had observed in BD mice, and because the early rise in NGAL provided evidence that the process of renal damage had already commenced by one hour after BD onset, we modified our experimental protocol to collect and transplant kidneys at one hour after BD induction. This change resulted in many more viable renal tubules in kidneys collected at d4 after syngeneic transplantation (Figure 45 panel C v/s panel B). In contrast, the extent of neutrophil infiltration was similar between kidneys transplanted after 3 hours of maintenance or one hour of maintenance and in both instances greatly exceeded neutrophil infiltration in syngeneic transplants from control donors (Figure 46), suggesting that the proinflammatory effect of brain death upon renal transplantation would still be observed with the shorter maintenance period.
Figure 45: Appearances of syngeneic kidney transplants from BD donors following either 1 hr or 3 hours of maintenance.

A- Syngeneic KTx D4

B- Syngeneic KTx D4 + BD Donor 3hrs

C- Syngeneic KTx D4 + BD Donor 1hr
Figure 46 Neutrophil infiltration of syngeneic renal transplants from BD donors maintained for 1 hour or 3 hours following induction of brain death.
5.6 Effects of brain death combined with syngeneic renal transplantation

For these experiments, mice were maintained for one hour following induction of brain death. Kidneys were collected and transplanted into syngeneic recipients, and tissues were harvested one or four days after renal transplantation.

5.6.1 Upregulation of pattern-recognition receptors within the kidneys

TLR4 expression following syngeneic transplantation was increased in kidneys from BD donors when compared to those from control donors on both d1 (median 61.63 v/s 34.21) and d4 (median 140.6 v/s 59.7) post-transplantation. In the case of RAGE, an increase in mRNA levels in kidneys from BD donors was only noted on d4 (median 110.8 v/s 24.5) (Figure 47).

![Figure 47](image_url)

**Figure 47** Expression of pattern recognition receptors following brain death and syngeneic renal transplantation. n=4-6 in each group.
5.6.2 Upregulation of chemokine gene expression following brain death and syngeneic transplantation.

Expression of all the chemokines examined was increased in kidneys following syngeneic renal transplantation from control donors, when compared with levels in normal mouse kidneys. In most instances, there was a further increase in expression levels when kidneys from brain dead donors were transplanted. This was particularly marked for CXCL10 (median 104.9 v/s 14.39, P=0.02) and CCL22 (median 39.1 v/s 3.35, P=0.04) on d1, and MCP-1 (median on 250 v/s 32.43, P=0.02) d4 (Figure 48). CCL22 was the only chemokine where mRNA levels were not increased in kidneys from BD donors above those in control kidneys on d4 post-transplantation.
Figure 48 Expression of chemokine genes after brain death and syngeneic renal transplantation. n=4-6 in each group.
5.6.3 Expression of genes for cytokines and other soluble mediators following donor brain death and syngeneic renal transplantation.

IL-6 mRNA expression increased considerably by d1 post-transplant in syngeneic kidneys transplanted from control donors (median 7.6 v/s 0.21, P=0.03), with a further increase noted in kidneys transplanted from brain-dead donors (median 13.56). The pattern of expression for TNF-α was similar, whereas levels of TGF-β, IL-1β and HSP2 did not change substantially in grafts from control donors, but were upregulated on both days 1 and 4 post-transplant in kidneys from BD donors. IL-10 was unusual in that expression in BD donor kidneys on d4 was no greater than that in control kidneys (Figure 49).
Figure 49 Expression of cytokines and other soluble mediators after brain death and syngeneic renal transplantation. n=4-6 in each group.
5.6.4 Neutrophil infiltration within control kidneys and those from BD donors

Normal mouse kidneys contained no neutrophils. A mild neutrophil infiltrate was present in control kidneys (0.8+/−1.0 cells/HPF on d1 and 1.4+/−1.1 cells/HPF on d4 post transplantation). The extent of neutrophil infiltration was dramatically increased in kidneys from BD donors on d4 post-transplantation (23.1+/−19.0, P=0.006) (Figure 50).

![Neutrophil cell count](image)

**Figure 50 Neutrophil infiltration following brain death and syngeneic renal transplantation.** Numbers of infiltrating neutrophils in kidneys from BD donors were significantly increased compared to those from control donors on d4 post-transplantation. n=4-6 in each group. * indicates significant differences in the compared groups (P-value <0.05).
5.6.5 Macrophage infiltration in control kidneys and those from BD donors

As above, both CD68 and F4/80 were used to identify macrophages within transplanted kidneys. Using F4/80, the number of resident macrophages in normal kidneys was 13.7+/-6.88 cells/HPF. Whereas there appeared to be a slight increase in the number of macrophages present within the kidneys after syngeneic transplantation from control donors (18.5+/-7.7 at d1 and 23.7+/-16.4 at d4, P=NS), many more infiltrating macrophages were detected in the kidneys transplanted from BD donors (38.2+/-12 cells/HPF on d1, P=0.02 and 65.1+/-24.1 on d4, P=0.03) (Figure 51A). These results were paralleled by those obtained with CD68 staining. Representative sections are shown below (Figure 51B).
Figure 51 Macrophage infiltration following brain death and syngeneic renal transplantation. Increased numbers of macrophages are detected in kidneys from BD donors compared with those from control donors at both d1 and d4 after syngeneic renal transplantation using either F4/80 (A) or CD68 (B). n=4-6 in each group. For A, * indicates significant differences between the compared groups (P-value <0.05).
CHAPTER 6: MODULATION OF INFLAMMATORY RESPONSES AFTER BRAIN DEATH AND SYNGENEIC RENAL TRANSPLANTATION BY SYSTEMIC OVEREXPRESSION OF esRAGE.

Having determined that donor brain death resulted in increased expression of inflammatory markers and increased infiltration of kidneys by neutrophils and macrophages both prior to and following subsequent syngeneic renal transplantation, we then evaluated whether systemic overexpression of esRAGE in the kidney donor and recipient could modulate inflammation in this setting.

6.1 Systemic esRAGE expression reduces circulating levels of HMGB1 after brain death.

Figure 52 shows the serum HMGB1 concentration for mice at 3 hours following the onset of brain death, ventilated controls and mice which had been injected with either rAAV-esRAGE or the control vector rAAV-HAS 7 days prior to BD induction. HMGB1 concentrations are significantly elevated (>25ng/ml) at 3 hours after induction of BD, compared to those in ventilated controls (<10ng/ml). BD mice injected with esRAGE had serum HMGB1 levels (~10ng/ml) approximating those in ventilated controls. However, we noted that levels of HMGB1 were reduced by a comparable amount in mice injected with the control vector rAAV-HSA. Albumin binds many substances in plasma, and because these may include ligands for RAGE and Toll-like receptors, rAAV-HSA may not be the most appropriate control vector for rAAV-esRAGE. In the later series of experiments examining the effects of
esRAGE expression upon kidneys after BD and syngeneic transplantation, we decided to use rAAV-GFP as an alternative control for rAAV-esRAGE.

**Figure S2 Serum HMGB1 levels.** n=4-6 in each group.
6.2 Effect of esRAGE on the expression of genes for the pattern recognition receptors RAGE and TLR4 in kidney.

Reduction in availability of RAGE ligands has been reported to reduce RAGE expression [85]. High-level systemic expression of esRAGE modestly reduced mRNA expression of both RAGE (9.38+/−1.4 cf 19.8+/−6.6, P=0.048) and TLR4 (18.46+/−4.83 cf 39.35+/−10.41, P=0.02) (Figure 53 below). mRNA levels for RAGE and TLR4 remained low in uninjected mice at 3 hours after induction of BD, and this was not affected by esRAGE expression (data not shown).

Figure 53 Systemic expression of esRAGE is associated with a modest reduction in mRNA expression for RAGE and TLR4 in kidney. n=4-6 in each group. * indicates significant differences between the compared groups (P-value <0.05).
6.3 The effect of esRAGE upon mRNA expression for chemokines, cytokines and other soluble mediators in kidney after brain death.

There was a wide scatter in the results for gene expression of chemokines, cytokines and other soluble mediators in kidneys at 3 hours following induction of brain death, including those from mice treated with esRAGE or control vector. Although there was a trend towards reduction in the median levels of expression for many of these mediators in mice treated with esRAGE, none of the differences between treatment groups reached statistical significance (Figures 44-55).
Figure 54 Cytokine gene expression in kidneys of BD with/without esRAGE. n=3-5 in each group.
Figure 55 Cytokine gene expression in kidneys of BD with/without esRAGE. n=3-5 in each group.
6.4 Expression of esRAGE reduces renal neutrophil infiltration after brain death

No neutrophil infiltrate was observed in normal C57BL/6 mice, nor in normal C57BL/6 injected with esRAGE or control vector, (Figure 56). Numbers of infiltrating neutrophils were significantly reduced in BD mice expressing esRAGE (4.1+/-2.5 cells/HPF), compared with uninjected BD mice (8.8+/-5.6 cells/HPF, P=0.035).

Figure 56 Renal neutrophil cell counts with/without brain death and esRAGE.

There are significantly less neutrophils in the kidneys of mice injected with esRAGE than in those of their uninjected counterparts. n=4-6 in each group. * indicates significant differences in the compared groups (P-value <0.05).
6.5 esRAGE expression reduces macrophage infiltration of kidneys following brain death.

Both F4/80 and CD68 were used for the identification of macrophages in the kidneys from normal mice and those which had undergone brain death and a three-hour maintenance period. F4/80-positive macrophages/HPF increased from 13.7+/−6.9 in the kidneys of normal mice to 23.3+/−10.3 at 3 hours after BD. Numbers were similar in BD mice treated with control vector (28.00+/−12.7, P=NS) but were reduced to approximate those in normal mice (13.10+/−7.8, P= 0.008) compared with untreated or control (vector-treated BD) by expression of esRAGE (Figure 57A, below). The same pattern was observed when CD68 was used for macrophage detection (see representative sections below (Figure 57B).
Figure 57 esRAGE expression reduces renal macrophage infiltration following brain death. (A) results obtained using F4/80 for macrophage detection, (B) parallel findings when CD68 is used to identify macrophages. n=4-6 in each group. For A, * indicates significant differences between the compared groups (P-value <0.05).
6.6 The effects of esRAGE upon donor kidneys under conditions of brain death and syngeneic renal transplantation.

The ability of esRAGE expression to attenuate inflammation in donor kidneys following brain death and syngeneic renal transplantation was next determined.

6.6.1 mRNA expression for pattern-recognition receptors within the kidneys

mRNA expression levels for RAGE and TLR4 were examined at d1 and d4 following syngeneic renal transplantation. Again, a wide scatter in results was observed. There was a trend towards reduction of expression of these receptors in mice treated with esRAGE vector which was more prominent at d4 post-transplantation (RAGE median 110.8 v/s 59.83, TLR4 median 140.6 v/s 80.4). These changes did not reach statistical significance (Figure 58).

![Figure 58](image)

**Figure 58** Expression of pattern-recognition receptors on d4 following renal transplantation. n=4-6 in each group.
6.6.2 The effect of esRAGE upon mRNA expression for chemokines, cytokines and other soluble mediators in kidney after syngeneic renal transplantation from brain dead donors.

esRAGE expression did not result in significant reduction of mRNA levels for most of the chemokines and cytokines examined in kidneys at either one or four days following syngeneic renal transplantation from brain dead donors (Figures 59 A, B, C, D below) though as noted above there was a broad scatter of results.
Figure 59 No cytokine reduction following syngeneic renal transplantation from brain dead donors following the injection of esRAGE (A, B, C and D). n=4-6 in each group.
6.6.3 esRAGE expression reduces neutrophil infiltration in kidneys on d4 after syngeneic renal transplantation from brain dead donors.

Figure 60 shows that the mild neutrophil infiltration was not reduced by esRAGE expression in kidneys from BD donors at d1 following syngeneic renal transplantation (Figure 60A). A more intense neutrophil infiltrate was present on d4 post-transplantation, and the density of this infiltrate was reduced by esRAGE expression (23.1+/−19 cf 9.1+/−8.7, P=0.027).

Figure 60 Neutrophil cell counts for kidneys from BD donors at d1 (A) and 4 (B) after syngeneic renal transplantation. n=4-6 in each group. * indicates significant differences between the compared groups (P-value <0.05).
6.6.4 esRAGE expression reduces macrophage infiltration in kidneys from brain dead donors at days 1 and 4 after syngeneic renal transplantation.

Both F4/80 and CD68 were used to enumerate macrophages in the kidneys from mice in these treatment groups. The number of F4/80-positive macrophages infiltrating kidneys was reduced significantly by esRAGE expression on both d1 post-transplantation (18.8+/−3.5 cf 38.2+/−14, P=0.019, Figure 61A) and d4 post-transplantation (27.3+/−7.9 cf 65.1+/−24.1, P=0.005, Figure 61B). Results obtained with CD68 paralleled these changes (representative sections from d4 post-transplantation shown in Figure 61C below)
Figure 61 Numbers of infiltrating macrophages in kidneys from brain dead donors following syngeneic renal transplantation are reduced by esRAGE expression. A - F4/80, d1 post-transplantation; B - F4/80 d4 post-transplantation; C - CD68 d4 post-transplantation. n=4-6 in each group. For A and B, * indicates significant differences between the compared groups (P-value <0.05).
6.6.5 Hypercellular appearance of kidneys from mice expressing esRAGE is likely to be the result of renal cell proliferation rather than inflammatory cell infiltration.

Although infiltrating neutrophils and macrophages were significantly less common in the kidneys of mice expressing esRAGE at d4 following syngeneic transplantation from a BD donor, than in those from uninjected mice, the kidneys of both groups of mice had a similar hypercellular appearance on H and E stained sections (Figure 62, below). Further stains were performed to try and determine whether this appearance was the result of infiltrating CD4+ or CD8+ T cells, or of proliferation of intrinsic renal cells. Proliferating cells were identified using an antibody against proliferating cell nuclear antigen (PCNA). From Figure 70, it can be seen that there are significantly more PCNA-positive cells in the esRAGE-injected group when compared to its non-injected counterpart (7.6+/−1.1 cf 4.2+/−2.7, P=0.047). This difference is even more striking when cell counts from esRAGE-expressing mice are compared with those from mice treated with the control vector (0.23+/−0.06, P=0.0008).

Figure 62 Similar H&E appearance of the kidneys of mice expressing esRAGE and uninjected control at d4 following syngeneic transplantation from a BD donor.
Figure 63 esRAGE expression significantly increases the number of proliferating cells in kidneys at d4 after syngeneic renal transplantation from BD donors. There are significantly more PCNA-positive cells in the esRAGE-injected group than in the non-injected group or the control-injected group. n=4-6 in each group. * indicates significant differences between the compared groups (P-value <0.05).
In contrast, Figure 64 shows that there are no significant differences in CD4 cell counts in kidneys between these groups.

![CD4 cell count](image)

**Figure 64** CD4 cell counts for kidneys at d4 following syngeneic renal transplantation from BD donors. There are no significant differences between the groups. n=4-6 in each group.

Immuochemical staining for FoxP3 and CD8a cells was negative for all samples. The positive controls for both FoxP3 and CD8a were stained positive.
CHAPTER 7: DISCUSSION

In this project, we have established a mouse model of brain death and subsequent renal transplantation. Whilst it is only possible to maintain mice for a relatively short period after the induction of brain death, this extension of the brain death model more closely resembles the setting of clinical transplantation than models of warm IRI utilizing vascular clamping and release. We showed that brain death in this model was accompanied by the expected physiological changes [176] and that as reported for other similar models [16, 176], a systemic pro-inflammatory state ensues, with release of alarmins including S100B and HMGB1, and rapid elevation in circulating levels of cytokines and chemokines such as IL-6, TNF-α, IL-1β, MCP-1 and KC. Structural changes in kidneys at this stage include some tubular vacuolation and a mild inflammatory infiltrate, and biochemical evidence of kidney damage is provided by increased serum concentrations of NGAL, an early biomarker of acute kidney injury [177]. Release of HMGB1 followed a different timecourse to that which is described following warm renal or liver ischaemia and reperfusion [43, 77, 170]. In those settings, HMGB1 release peaks at 10-15 minutes after organ reperfusion, whereas in this model, peak levels were not attained until 3 hours after BD induction. This difference in tempo may be due to the fact that while perfusion of transplantable organs is erratic following BD, it is not completely interrupted and then restored as occurs in vascular occlusion models. Conversely, increased levels of S100B are noted in the systemic circulation within 30 minutes after the onset of BD, although they continue to rise through the three-hour
maintenance period. This may be reflective of the dual origin of S100B. The CNS is particularly rich in S100B, which is released into the systemic circulation as the brain is being injured by the progressive rise in intracranial pressure which accompanies intracranial balloon inflation in this model. When brain death occurs, cerebral blood circulation ceases, and S100B release is then the consequence of the supervening damage to other organs and tissues.

After determining the optimum method for ureteric reconstruction, we collected kidneys from BD donors, and performed syngeneic renal transplants to examine how donor brain death amplifies the ischaemia-reperfusion injury which inevitably accompanies any transplantation procedure. As anticipated, we found that donor brain death resulted in upregulation of a number of pro-inflammatory cytokines and chemokines in transplanted kidneys, and in substantially increased infiltration with neutrophils and macrophages. As distinct from mouse models of warm renal ischaemia and reperfusion, where ischaemia is achieved through clamping the renal pedicles [44], neutrophil infiltration was greatest on d4 post-transplantation, similar to the tempo of infiltration with macrophages. These findings parallel the timecourse of gene expression for the neutrophil chemokine KC and the macrophage chemokine MCP-1, where peak expression of both chemokines was achieved at d4 post-transplantation (Chapter 5 Figure 48).

Signalling through pattern-recognition receptors such as TLR2 and TLR4 is critical for the propagation of inflammation following an ischaemic insult to the kidneys [42, 44]. esRAGE is a naturally-occurring soluble isoform of RAGE [178]. esRAGE can
exert anti-inflammatory effects via the binding and sequestration of circulating ligands such as S100B and HMGB1. Because HMGB1 is a common ligand for TLRs 2, 4 and 9, as well as for RAGE [50, 54, 55], HMGB1 binding by esRAGE can interfere with signaling through all these receptors. In addition to its activity as a decoy receptor, esRAGE can also directly block RAGE signaling by forming heterodimers with cell-surface RAGE and thus preventing RAGE dimerization which is a necessary precursor to signal transduction [85, 89].

In order to study the effects of esRAGE upon IRI in brain-death and subsequent syngeneic renal transplantation, we generated an rAAV vector encoding esRAGE. Intraperitoneal injection of this vector resulted in strong, liver-specific expression of esRAGE. Secreted esRAGE protein was detected at high levels in the systemic circulation from d2 until at least d100 following IP injection, and was well tolerated with no evidence of liver inflammation. Substantial concentrations of human esRAGE were present in the heart, kidneys and spleen, as well as the liver and serum of transduced animals. The rAAV2/8 vector system is an efficient means of gene transfer to liver [162]. The vector is minimally immunogenic, and immunological tolerance to the transgene product has been formally demonstrated on a number of occasions, including in a stringent skin transplant model [169]. The achievement of long-lived, high-level esRAGE expression using this system is in line with these previous reports.

The most striking effect of esRAGE expression in mice undergoing brain death and syngeneic renal transplantation was a dramatic reduction in the numbers of
infiltrating neutrophils and macrophages within the kidneys. Several factors may contribute to this. Expression of KC (the murine orthologue of IL-8) and of MCP-1, was reduced modestly, if at all, in the kidneys of esRAGE–injected mice after BD and renal transplantation. However, RAGE is a known binding partner for the leucocyte integrin MAC-1 [84, 102], and reduction of leucocyte infiltration in the presence of esRAGE may represent an effect of direct inhibition of this interaction. Neither TNF-α nor IL-6 levels were reduced by esRAGE expression in kidneys from BD donors after syngeneic transplantation, although a modest reduction was apparent in the kidneys of BD mice treated with esRAGE vector. Although these cytokines are pro-inflammatory, TNF-α also has an important pro-survival effect on ischaemic organs, reducing the numbers of cells undergoing apoptosis and increasing proliferation as measured by the number of nuclei positive for PCNA [92]. Administration of recombinant soluble RAGE to mice undergoing warm liver ischaemia and reperfusion resulted in a survival advantage which was accompanied by increased mRNA transcripts for TNF-α, and by increased proliferation of parenchymal cells following the injury [92]. Similarly, substantially increased numbers of proliferating cells were demonstrated in the kidneys of esRAGE-treated mice at d4 after syngeneic transplantation from BD donors, compared with those of uninjected mice and particularly with those of mice receiving the control vector (Chapter 6, Figure 63). Given that the grafts from esRAGE–treated mice had reduced numbers of infiltrating leucocytes compared to those in the other groups, it is likely that this represents proliferation of renal parenchymal cells in the transplanted kidneys.
In conclusion, we have developed a clinically-relevant model for the study of the early inflammatory events which accompany deceased donor renal transplantation. The ability of rAAV-mediated systemic expression of esRAGE to attenuate this inflammation was assessed during brain death and after transplantation of the kidneys from BD donors, and the principal findings were that esRAGE reduced infiltration by neutrophils and macrophages and increased the proliferation of renal parenchymal cells in the graft post-transplantation. These effects are likely to be translated into improved graft function, and augmented ability of the graft to sustain life. RAGE signaling on T cells contributes to adaptive immune responses, including alloresponses [179]. Testing of esRAGE at later timepoints when the transplanted kidneys are life-sustaining and in allotransplant models where its effects upon the adaptive immune response are predicted to be additive to those on non-antigen-specific graft inflammation is the logical next step for this line of investigation, and will shortly be undertaken.
BIBLIOGRAPHY


APPENDIX: COMPOSITION OF SOLUTIONS

1x PBS

1x PBS was used for the experiments, however, the stock solution was made up to 10x and then diluted down before use. For 10x PBS, 80g of NaCl (UNIVAR), 2g of KCl (UNIVAR), 14.4g of Na$_2$HPO$_4$ (Sigma), 2.4g of KH$_2$PO$_4$ (AnalaR) were added to 1L of TDW and adjusted to pH 7.4.

1x TBS/Tween

1x TBST was used for the experiments, however, the stock solution was made up to 10x and then diluted down before use. For 10x TBST, to 1L of TDW, 13.9g of Tris base (Sigma), 60.6g of Tris HCl (Sigma), 60.6g of NaCl (UNIVAR) and 5ml of Tween 20 (AMRESCO) was added and adjusted to pH 7.4.

IP diluent

IP diluent was made by mixing together 47.25ml of 1x TBST, 2.5ml of heat-inactivated swine serum, 0.25ml of 18% sodium azide (AnalaR), and then filtered through 0.22µm, and stored at 4°C for use.

Tris buffer

1.39g of Tris base (Sigma), 6.06g of Tris HCl (Sigma), 6.06g of NaCl (UNIVAR) were mixed in 1L of TDW and adjusted to pH9.
Citrate Buffer

2.94g of tri-sodium citrate (dihydrate) (Sigma) was added to 1000ml of TWD and then adjusted to pH6.

DEPC water

The DEPC water used was 0.1% DEPC water. It was made by adding 0.5ml of DEPC solution (Sigma) to 500ml of TDW and treated by autoclaving or heating at 70°C for 1 hour or 60°C overnight.

0.1% v/v Triton-X-100 in 0.1% w/v Na citrate

5ml of 2% sodium citrate (2g in 100ml TDW) (Sigma) was added to 95ml TDW and 100µl of Triton-X-100 (Sigma).