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Kidney Injury Molecule-1 Mediated Phagocytosis and Its Therapeutic Application in Ameliorating Renal Transplant Ischemia Reperfusion Injury

Ji Yun Lee, *The University of Western Ontario*

Supervisor: Gunaratnam, Lakshman, *The University of Western Ontario*

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Abstract

Renal transplantation is a life-saving procedure for patients with end-stage renal disease. Persistent graft inflammation and fibrosis due to injury from repeated insults - both alloantigen-dependent and -independent - lead to chronic allograft dysfunction and long-term graft loss. Ischemia-reperfusion injury (IRI) to the graft is an inescapable consequence of transplantation and can result in significant delayed graft function (DGF). Tissue damage and graft dysfunction resulting from transplant-associated IRI have been correlated with acute rejection and long-term graft loss. During IRI, dying renal proximal tubular epithelial cells (TECs) release pro-inflammatory mediators, worsening tissue damage and further potentiating injury by initiating an auto-amplification loop of inflammation and cell death. Therefore, therapies that curtail this auto-amplification loop may mitigate graft dysfunction and extend the lifespan of grafts. Kidney injury molecule-1 (KIM-1) is a phagocytic receptor specifically upregulated on TECs during renal injury, enabling them to engulf apoptotic and necrotic cells during acute kidney injury. While KIM-1 can directly bind to apoptotic cells via phosphatidylserine, the clearance of necrotic cells is enhanced by the opsonin, Apoptosis Inhibitor of Macrophage (AIM) protein. The extent to which KIM-1 in the donor kidney contributes to renal transplantation has not been well-studied. In this thesis, we studied the role of KIM-1 in the donor kidney on graft outcomes following renal transplantation. We assessed the therapeutic potential of exogenous recombinant AIM (rAIM) in mitigating transplant-associated IRI. Finally, we explored the association between polymorphisms of the KIM-1 gene and DGF. Using a syngeneic murine renal transplant model, we found that KIM-1 in the donor kidney protects against renal dysfunction, inflammation, graft damage and death. Moreover, the protective effect of KIM-1

was further enhanced with the administration of rAIM. Finally, we found that common genetic variations of the coding region of human KIM-1 gene suppressed the phagocytic ability *in vitro* compared to *wild-type* KIM-1. However, none of the variations were associated with increased risk of DGF in our patient cohort. In summary, the findings reported in this thesis provide further evidence of the protective properties of KIM-1 in transplant-related IRI and the viable therapeutic potential of rAIM in renal transplantation.

Keywords

Apoptosis, Cell death, Danger Associated Molecular Patterns, Hepatitis A Virus Cellular Receptor 1, Ischemia Reperfusion Injury, Kidney Injury and Repair, Kidney Injury Molecule-1, Necrosis, Phagocytosis, Polymorphism, Renal Transplantation.

Summary for Lay Audience

A kidney transplant has the potential to prolong the life of patients with kidney failure and immensely improve their quality of life. A major limitation of transplantation is the limited lifespan of the organs in the host. This is mainly due to cumulative damage sustained by the organ during procurement/surgery (lack of blood flow to the organ) and the host immune system (i.e. rejection). At the molecular level, this is caused by dying kidney cells which "explode", releasing their inflammatory cellular contents into the body which can serve to activate the rejection process. Thus, the clearance of these dangerous remains is necessary. In this thesis, I have characterized a protein called Kidney injury molecule-1 (KIM-1), which help the remaining healthy kidney cells within the transplant to "eat" the surrounding dying cells, thereby preventing the abovementioned inflammatory cascade and help repair the damaged organ. In addition, I have documented the use of a potential therapeutic agent, "AIM", which when administered intravenously immediately following transplantation, can compound the positive effect of KIM-1, to promote even faster and more effective clearance. Finally, I translated my findings into humans and investigated whether different versions of KIM-1 in the human population would affect the primary role of KIM-1, which could have undesirable effects on its function during transplant resulting in patients requiring dialysis within the first week- a problem known as delayed graft function (DGF). I uncovered that different versions of KIM-1 protein found in the human population decreased its ability to clear dying cells compared to normal KIM-1. However, the variation of KIM-1 was not associated with increased risk of DGF. Taken together, these findings uncover a new therapeutic target (KIM-1 and AIM) that may prolong kidney transplant survival.

Co-Authorship Statement

With a great amount of guidance from Dr. Lakshman Gunaratnam (thesis supervisor), the acquisition of data, interpretation, and writing were primarily performed by Ji Yun Lee. The detailed information of co-authorship for each chapter is given below:

Chapter 2: Lee JY, Ismail OZ, Zhang X, Haig AR, Lian D, Gunaratnam L. Donor kidney injury molecule-1 promotes graft recovery by regulating systemic necroinflammation. Am J Transplant 2018;18(8):2021-2028.

Lee JY executed experiments, analyzed and interpreted data, and wrote the initial manuscript. Ismail OZ and Zhang X performed some experiments. Haig AR scored the graft pathology slides. Lian D performed all murine renal transplant surgeries. Gunaratnam L was responsible for study conceptualization and design, funding, and edited the manuscript. Funding for this project was received from the Canadian Institutes of Health Research (Gunaratnam L [nominated PI], Suri RS [co-investigator]).

Chapter 3: Lee JY, Zhang X, Lian D, Haig AR, Suri RS, Miyazaki T, Gunaratnam L. Recombinant apoptosis inhibitor of macrophage protein reduces delayed graft function in a murine model of kidney transplantation.

Lee JY executed experiments, analyzed and interpreted data, and wrote the initial manuscript. Haig AR scored the graft pathology slides. Lian D performed all murine renal

transplant surgeries. Gunaratnam L was responsible for study conceptualization and design, funding, and edited the manuscript. Funding for this project was received from the Canadian Institutes of Health Research (Gunaratnam L [nominated PI], Suri RS [co-investigator]). Miyazaki T provided the recombinant AIM. Suri RS participated in study design. Gunaratnam L was responsible for study conceptualization and design, funding, and edited the manuscript. Funding for this project was received from the Canadian Institutes of Health Research (Gunaratnam L [nominated PI], Suri RS [co-investigator]).

Chapter 4: Lee JY, Shrum B, McIntrye AD, Ban MR, Ismail OZ, Suri RS, Hegele RA, Gunaratnam L. Polymorphisms in *HAVCR1* alter KIM-1 mediated phagocytosis.

Lee JY performed the majority of experiments, participated in data analysis and, and wrote the initial manuscript. Shrum B and Ismail OZ generated the constructs of the variants. McIntrye AD and Ban MR genotyped the donor DNA and calculated the genotype and allele frequencies under the direction of Hegele RA. Gunaratnam L participated in study conceptualization, and design and editing of the manuscript. Funding for this project was received from the AMOSO Innovation Fund (Gunaratnam L [nominated PI], Suri RS [co-investigator] and Hegele RA [co-PI] and the Astellas CNTRP Innovation Grant (Gunaratnam L [nominated PI], Suri RS [co-investigator] and Hegele RA [co-investigator].

Chapter 5: Lee JY, Shrum B, Lee SH, McIntyre AD, Ban MR, Suri RS, Hegele RA, Gunaratnam L. Kidney injury molecule-1 polymorphisms and the risk of delayed graft function after deceased donor kidney transplantation.

Lee JY participated in data collection and writing of the initial manuscript (in preparation). Shrum B and Lee SH participated in collection of patient information. Shrum B designed the template for data collection. McIntyre AD and Ban MR participated in genotyping the donor DNA and data analysis. Hegele RA directed the genotyping of donors and helped with interpretation of data. Suri RS directed data analysis and interpretation, contributed to study design and edited the manuscript. Gunaratnam L participated in study conceptualization and edited the manuscript. Funding for this project was received from the AMOSO Innovation Fund (Gunaratnam L [nominated PI], Suri RS [co-investigator] and Hegele RA [co-PI] and the Astellas CNTRP Innovation Grant (Gunaratnam L [nominated PI], Suri RS [co-investigator] and Hegele RA [co-investigator].

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Abbreviations	
AKI	Acute kidney injury
AIM	Apoptosis inhibitor of macrophage
APAF-1	Apoptotic peptidase activating factor -1
APCs	Antigen presenting cells
ATN	Acute tubular necrosis
ATP	Adenosine triphosphate
BMI	Body mass index
CD5L	CD5 antigen-like
CI	Confidence interval
cIRI	Cold ischemia reperfusion injury
CKD	Chronic kidney disease
CNI	Calcineurin inhibitor
CTAB	Cetyltrimethylammonium bromide
DAMPs	Danger associated molecular patterns

DBD	Donation after brainstem death
DCs	Dendritic cells
DCD	Donation after cardiac death
DGF	Delayed graft function
DISC	Death inducing signaling complex
DSA	Donor specific antibodies
DTAB	Dodecyltrimethylammonium bromide
DV	Dengue virus
EBOV	Zaire Ebola virus
ECD	Expanded criteria donor
EHD	Ethidium homodimer
ERK	Extracellular-signal regulated kinase
ESRD	End-stage renal disease
Gα12	Guanine nucleotide binding protein alpha 12
HAVCR1	Hepatitis A virus cellular receptor 1
HCV	Hepatitis C virus

HEK 293	Human embryonic kidney 293
H&E	Hematoxylin and eosin
HLA	Human leukocyte antigen
HMGB1	High-mobility group box-1
HSP	Heat shock protein
HTN	Hypertension
IgM	Immunoglobulin M
IgV	Immunoglobulin variable
IRI	Ischemia reperfusion injury
Kim-1	Murine kidney injury molecule-1
KIM-1	Human kidney injury molecule-1
MAF	Minor allele frequencies
MAPK	Mitogen activated protein kinase
MFI	Mean florescent intensity
MHC	Major histocompatibility complex
MIC	MHC class I chain-related

MLKL	Mixed lineage kinase domain like protein
mTPT	Mitochondrial permeability transition pore
NDD	Neurologic determination of death
NF-κB	Nuclear factor -κB
NUR77	Nuclear receptor 77
PRA	Panel reactive antibody
PRRs	Pattern recognition receptors
PS	Phosphatidylserine
PTECs	Proximal tubular epithelial cells
rAIM	Recombinant apoptosis inhibitor of macrophage
RIPK	Receptor interacting protein kinase
ROS	Reactive oxygen species
RR	Relative risk
sKIM-1	Soluble kidney Injury Molecule-1
SCD	Standard criteria donors
SRCR-SF	Scavenger receptor cystine-rich superfamily

TACE	Tumour necrosis factor-α converting enzyme
TCTEX-1	T-complex testis specific protein-1
TECs	Tubular epithelial cells
TIM-1	T-cell immunoglobulin mucin domain -1
TLRs	Toll-like receptors
UW solution	University of Wisconsin solution
ZO-1	Zonula occludens-1

Preface

The following thesis takes you on a journey of the past 5 years of my tenure as a graduate student. Starting as a Master's student, I found that research into the immune and non-immune pathologies encountered in renal transplantation to be fascinating. I soon realized that there was a considerable knowledge-gap regarding the fundamental types of injuries sustained by the graft and how this impacts the lifespan of the transplanted kidney. Thus, I decided to pursue a doctoral degree in the hopes of acquiring a more in-depth understanding of the underlying mechanisms and potentially contribute to the development of novel therapeutics aimed at improving the lives of transplant patients. This thesis entails the culmination of that journey where I focused on the role of Kidney injury molecule -1 (KIM-1) in renal transplantation.

This thesis may be of interest for transplant surgeons and nephrologists, or researchers interested in ischemia reperfusion injury and transplantation. The overall aim of this thesis is to understand the importance of renal transplantation in improving the long-term survival. However, numerous limitations to renal transplantation remain, a major one being the limited lifespan of kidney grafts. Hence, efforts to prolong graft lifespan and increase the likelihood of post-transplant success, is dependent on understanding and targeting ways to minimize the injury to the kidney graft. Using both mice kidney transplant model and human transplant data, this thesis reveals the KIM-1 protein as a potential therapeutic target to prolong kidney transplant survival.

The aim of this thesis is to understand the pathophysiological mechanisms underlying ischemia-reperfusion injury in renal transplantation and developing strategies to improve the long-term survival of the transplanted kidneys.

Chapter 1

1 Introduction

1.1 End-stage Renal Disease

Kidney damage and scarring (fibrosis) affects the ability of the kidney to filter and remove waste and manage excess fluid. Persistent (and irreversible) kidney damage, also known as chronic kidney disease (CKD), can lead to the gradual failure of the kidneys. There are 5 progressive stages of chronic kidney disease where end-stage renal disease (ESRD) is the final stage of CKD where both kidneys have irreversibly failed with less than 15% of the kidneys functioning [1]. In the United States alone, there are approximately 750,000 people suffering from ESRD and over 2 million people affected worldwide. Alarmingly, the incidence of ESRD has been rising steadily at a rate of 5-7% per year [2].

Kidney failure could manifest in the host in a variety of different ways including but not limited to: protein energy malnutrition, abnormal metabolism of bone and mineral, anemia, fluid retention, and uremia (accumulation of nitrogenous waste in the body) [1]. Thus, renal replacement therapy is vital for patients with ESRD. Currently, the two available treatments are dialysis and kidney transplantation; the 5-year survival rates can vary depending on the treatment type. Specifically, the 5-year survival rate was approximately 42-52% among dialysis patients whereas transplantation conferred 77-84% survival rates in recipients [2-5]. Furthermore, compared to dialysis patients, successful transplantation recipients experience an increase in the quality of life, and are faced with dramatically reduced health care costs [6-9]. Therefore, renal transplantation is considered the optimal treatment choice for patients suffering from ESRD.

1.1.1 Renal Transplantation

Allo-, syn- and xeno-transplantations comprise of three theoretical forms of transplantation. Xenogeneic or xeno-transplantation involves organ or tissue grafting between two members of different species and is usually experimental in the context of renal transplantation, whereas syngeneic transplantation involves grafting between individuals that are genetically identical (i.e. identical twins). Allogeneic transplantation, which is the most commonly performed type of transplantation in humans, is the grafting of organs or tissues between 2 genetically different individuals from the same species. In 2019 alone, over 23.000 kidney transplantation was performed in the United States [2, 10] .

For a successful transplantation, a preoperative procedure needs to be conducted where the recipients and the donors undergo ABO blood group matching and human leukocyte antigen (HLA) compatibility assessment, as HLA mismatched transplant patients exhibited considerably worse graft survival due to alloreactive responses in solid organ transplantation [11, 12]. Upon successful matching, recipients will usually receive a solitary donor kidney that is placed into their iliac fossa, a different location from the native kidneys- this is termed a heterotopic transplant. This is because recipients usually do not undergo bilateral nephrectomy except under specific circumstances (e.g. large polycystic kidneys). Following the successful anastomoses of the donor artery to the recipient external iliac artery and subsequent donor vein to the external iliac vein, the donor ureter is then connected to the bladder of the recipients [13].

There are 2 types of renal transplantation performed worldwide: living donor vs. deceased donor transplants. Live donor recipients receive a single kidney from a healthy donor and generally live longer and experience better graft survival compared to deceased donor recipients (Table 1-1) [5]. Living donation can take place between related or unrelated individuals and can be directed (e.g. mother to son) or undirected (altruistic donation). Deceased donor transplantations on the other hand, have 2 categories: donation after circulatory death (DCD) and neurologic determination of death (NDD, or also known as donation after brainstem death, DBD). DCD involves the donor kidneys being retrieved from an individual who died naturally from cardiovascular causes, where the heart stops beating resulting in the cessation of blood supply to the body. In contrast, NDD involves the donor kidneys being acquired from individuals following determination of neurologic death, where the heart continues to provide blood supply, however no neurological activity is detected. Alternatively, expanded criteria donors (ECD) are donors (DCD or NDD) that are above 60 years of age, or donors that are between the ages of 50 and 59 with at least two of the following comorbidities including: history of hypertension, death due to cerebrovascular accident, and/or terminal creatinine (a metabolite of muscles which usually gets filtered by the kidneys) greater than 1.5mg/dl. Transplant recipients that received kidneys from ECD exhibited 70% increased risk of graft failure compared to those who received kidneys from standard criteria donors (SCD) [14].

Table 1-1. Canadian kidney graft survival rates in renal transplant patients, 2004-2013.

		2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Deceased Donor	N	517	509	616	639	643	678	653	689	717	711
	3 Months	95.0	96.1	95.5	96.4	95.6	95.3	97.4	97.0	97.4	96.6
	1 Year	91.7	92.3	93.2	93.1	92.1	92.5	94.8	92.9	94.8	—
	3 Years	85.5	85.6	86.2	87.0	86.6	87.9	90	—	—	—
	5 Years	78.1	79.9	81.2	81.4	82.6	—	—	—	—	—
Living Donor	N	345	370	415	413	409	403	413	404	392	437
	3 Months	98.5	97.8	97.6	98.8	97.8	98.8	98.1	98.3	98.7	99.3
	1 Year	98.3	95.7	96.4	96.6	96.3	97.5	96.1	97.5	97.7	—
	3 Years	94.5	91.9	93.3	92.7	93.2	94.5	93.9	—	—	—
	5 Years	89.8	88.9	88.0	87.9	89.2	—	—	—	—	—

Table adapted from CORR 2015 [5].

Numerous studies have shown that renal transplantation is the treatment of choice for patients with kidney failure for many reasons including improved long-term survival, lower overall cost when compared to dialysis and enhanced quality of life [7, 9, 15]. For instance, successful transplant recipients no longer have to depend on dialysis for survival, which can be as many as 3 times a week lasting 3-4 hours per session. However, a major limitation of renal transplantation is that there is a tremendous shortage of donor kidneys compared to the growing demand for organs [16]. Moreover, studies have suggested that the demand is steadily rising by 8% each year [2] . In Canada, the number of patients on the kidney transplant waitlist continue to increase, but due to limited number of available donor organs, the number of deaths while waiting for a transplant show a similar trend (Table 1-2) [5]. Likewise in the USA, there were more than 100,000

ESRD patients on transplant waitlist in 2016 with only 20% of the patients able to receive transplant surgery [2].

Table 1-2. Number of Canadians on waiting list and deaths on waiting list, 2004-2013.

	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Waiting List	2,872	2,759	2,962	2,963	2,892	2,902	3,362	3,406	3,428	3,227
Deaths on Waiting List	55	66	70	46	58	76	82	80	84	88

Table adapted from CORR 2015 [5].

Aside from the scarcity of donor kidneys, another complication with transplantation is the relatively finite lifespan of the graft. On average, the lifespan of kidney transplants are 12-20 years for living donor grafts and 8-12 years for deceased donor grafts [17]. Strikingly, approximately 30% of patients on the waitlist are those with failed transplants [18]. Therefore, each available donor kidney is a precious commodity and steps should be taken to ensure the success of each transplant procedure. This has led to the “one transplant for life” movement [19]. One essential factor in ensuring donor kidneys remain in optimal condition is to minimize the injury occurred during and after the transplantation procedure. Unfortunately, preventing injury to the donor tissue remains a challenging task as renal transplants are subjected to a series of injuries from both alloimmune and non-alloimmune (both in the donor and upon transplantation) insults which can culminate to chronic allograft dysfunction and graft loss [20, 21].

1.1.2 Alloimmune Injury

Due to the highly polymorphic nature of major histocompatibility complex (MHC) genes, or HLA genes in the humans, the physiological phenomenon of alloreactivity remains a major obstacle to successful renal transplantation [22]. Over 15,000 different HLA alleles have been discovered to date [23]. This is not to say that other non-conventional molecules, such as the polymorphic MHC class I chain-related (MIC) genes, do not contribute to alloimmunity. Allogeneic permutations between the donor and recipient can trigger a vast array of physiological responses via the activation of alloreactive T and B cells leading to graft inflammation and persistent infiltration of other immune cells such as macrophages [24-26]. Alloimmune injury, which can be largely categorized as T cell- (cellular) or antibody- (humoral) mediated, significantly contributes to persistent graft damage over time [27]. Allograft rejection requires the activation of alloreactive $CD4^+$ T cells, which have been shown to be necessary and sufficient for rejection in animal models. For example, mice deficient in $CD4^+$ T cells were protected from renal dysfunction, tissue damage and infiltration of neutrophils following renal IRI [28]. Dendritic cells (DCs), arising from both donor and/or recipient tissues, have been identified as one of the key cell types involved in initiating alloimmune injury, whereby upon activation, DCs migrate to secondary lymphoid tissues where they can further activate alloreactive T cells and B cells [29-32]. Activated B cells can produce donor specific antibodies (DSA) that can bind to the surface of the allogeneic HLA antigen leading to graft rejection, also known as antibody-mediated rejection [22]. B cells can also serve as antigen presenting cells (APCs) for cardiac allograft rejection [33]. Notably, prior sensitization to alloantigens in the recipient, via blood transfusion, pregnancies

and/or previous transplantations, results in an even greater humoral alloresponse [22, 34]. In the event that the triggered alloresponse also targets the one or more of the donor HLA molecules expressed by the graft, the existence of donor-specific antibodies confers a poor prognosis on prospective recipients [35]. The culmination of persistent alloimmune injury to the graft from both effector T cells and/or donor specific antibodies, can lead to acute or chronic rejection of the graft [21, 22] .

1.1.3 Non-alloimmune Injury

Non-alloimmune mechanisms of injury are another major component to graft injury that can limit the success of a transplant, and comprises of multiple factors, including drug toxicity, infection following transplantation, autoimmunity against cryptic epitopes in the donor kidney, ischemic injury in the donor and/or recipient [27, 36]. Solid organ transplantation recipients require to be on lifelong immunosuppression as it decreases the likelihood of graft rejection. The most prescribed immunosuppressants include: calcineurin inhibitors (CNI) (i.e. cyclosporine and tacrolimus), mycophenolic acid, sirolimus, prednisone and basiliximab. Cyclosporine and tacrolimus have helped to drastically improve graft outcomes [37-39] but are associated with acute and chronic nephrotoxicity in renal and non-renal transplantation [39-41]. The pathophysiologic mechanisms underlying CNI nephrotoxicity include direct and indirect effects (e.g. via vasoconstriction, thrombotic microangiopathy) [41]. In addition, the suppression of the host's immune effector cells is the desired outcome, however this inevitably renders the host more susceptible to viral infections and malignancies, which secondarily, can also cause further damage to the graft [27, 42-44].

Recipient factors that may curtail the lifespan of the renal transplant include the host's underlying comorbidities such as diabetes, lipid disorders, hypertension, and/or the recurrence of the primary kidney disease (e.g. glomerulonephritis) [27, 45]. As a whole, these are referred to as recurrence of original renal disease which includes primary renal diseases (membranous glomerulonephritis), systemic diseases (e.g. amyloidosis) and metabolic diseases (e.g. diabetic nephropathy). Various factors are included in the viability of the organ category such as, donor age, living versus deceased donor, ischemia time, and delayed graft function, all of which can contribute to promoting injury to the graft [27, 46-48]. Thus, comprehensive therapies that target and address a plurality of mechanisms of alloimmune and non-alloimmune pathologies may pave the way in mitigating graft damage and ultimately prolonging the lifespan of the transplanted kidneys, and its effect in the recipients.

1.1.3.1 Ischemia Reperfusion Injury

Ultimately, all donor kidneys are inescapably subjected to a specific type of injury during the transplantation process, known as ischemia reperfusion injury (IRI), which is yet another major hurdle to transplantation success. Ischemia is defined as the reduction of blood flow whereas reperfusion is defined as the restoration of blood flow [49]. From the retrieval of the donor organ to the implantation and successful anastomosis in the recipients, the donor kidney is faced with varying degrees of IRI. For example, when donor kidneys are obtained from a DCD donor, the kidneys will face a series of varying periods of ischemia from the time the life support is withdrawn allowing for cardiac arrest (and natural death) until and including the transplantation procedure. Specifically,

during procurement while the kidney is still inside the body, the transplant tissue experiences a brief period of warm ischemia when the clamp is placed prior to nephrectomy. Donor kidneys subsequently undergoes a prolonged period of cold ischemia during storage, while submerged in the universal University of Wisconsin (UW) solution, at 4°C until time for transplant. More recently, pulsatile hypothermic perfusion was shown to mitigate the effects of DGF compared to static cold storage [50]. Next, the donor kidney is then exposed to warm ischemia again during the period of implantation and successful anastomosis inside the host. Finally, the reintroduction of the host's blood introduces reperfusion injury (Figure 1-1) [51].

In the case of kidneys obtained from DBD donors, the organ is faced with similar types of cold and warm ischemia and reperfusion injury during procurement to successful transplantation. One distinction, however, is because DBD donors do not have ceased blood flow, they do not experience prolonged period of warm ischemia before procurement, but rather experience a brief period warm ischemia because neurologic death still alters the hemodynamics (Figure 1-1) [51, 52]. It is noteworthy that the major consequence of IRI is DGF following transplantation which can severely impact the health and longevity of the graft as well as negatively impact overall survival [47, 48, 53-55].

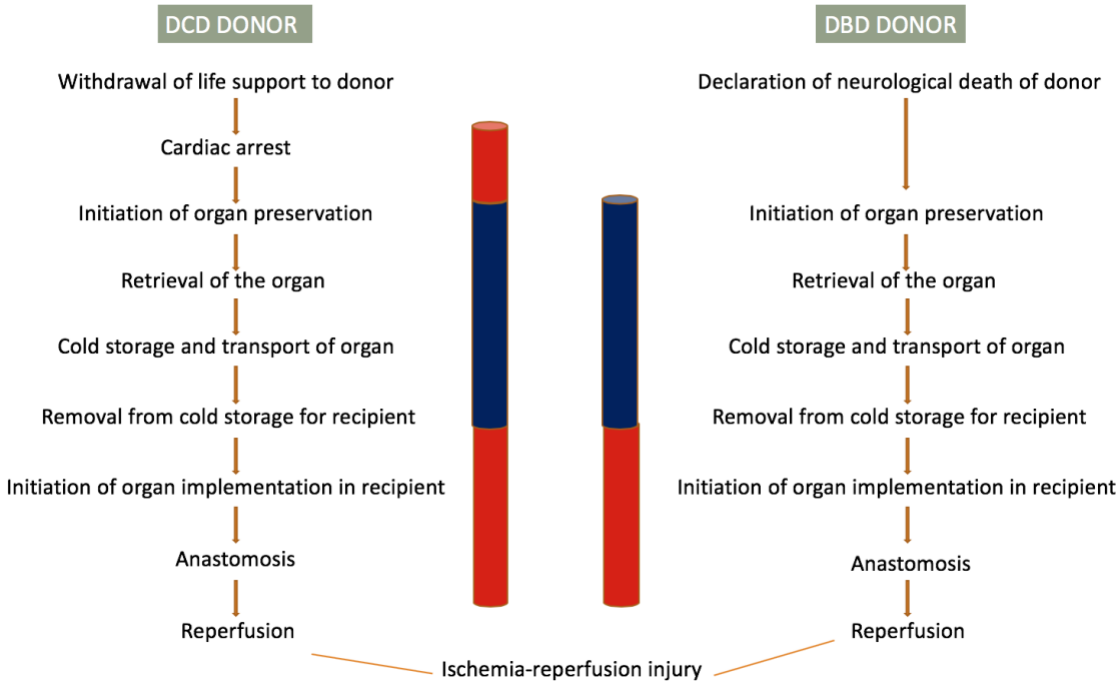

Figure 1-1. Flow diagram representing the types of ischemia and reperfusion injury the donor organ experiences from retrieval to implantation.

Flow diagram representing each event and type of ischemia that the donor graft endures from the death of the donors (both DCD and DBD) until the restoration of blood flow in the recipients. The red bar represents warm ischemia, and the blue bar represents cold ischemia.

Figure adapted from Aitken et al. [51]

1.1.3.2 Delayed Graft Function

The incidence of DGF is higher in patients receiving donor kidneys from DCD donors compared to DBD donors (44% vs. 24%) due to prolonged warm ischemia time in DCD kidneys [56]. Primarily caused by IRI, DGF is a complication where the transplanted kidney fails to function readily following transplantation, and is clinically defined as the need for dialysis within the first postoperative week [57, 58]. Some other clinical risk factors influencing DGF include: donor/recipient sex, donor/recipient body mass index, donor history of hypertension, donor age, and donor terminal serum creatinine [59, 60].

DGF often necessitates prolonged hospitalization while patients undergo multiple rounds of dialysis which have tremendous financial considerations to both the health care system and the patients [60, 61]. Additional consequences of DGF are increased morbidity and mortality [60, 62-64]. In addition, the requirement to continue multiple rounds of dialysis even after transplantation can have a negative psychological impact on the patients [65]. Moreover, recipients experiencing complications due to DGF are at a greater risk of developing acute rejection, graft failure and development of chronic graft dysfunction, which can lead to early death [60, 62, 66, 67]. Therefore, minimizing IRI and subsequent DGF could tremendously improve both patient and graft outcomes following transplantation.

1.1.4 Animal Models of Renal Transplantation

Many animal models have been implemented to study renal transplantation, including the use of large animals, such as pigs, to small animals like rats and mice [68, 69]. The advantages of large animals, specifically pigs, to study renal transplantation include: the close phylogenetical homology to humans compared other species, simplicity of breeding, and comparable kidney size with humans. Notably, pig kidneys have been shown to have more favorable outcomes when used in xenografts, compared to kidneys from non-human primates [70, 71]. Unfortunately, the limitation of using pigs to study renal transplantation is that the cost is exceedingly high compared to using small animals.

Small animal models especially mice are the most commonly utilized by researchers. Although mice are genetically distant from humans compared to pigs, murine models are of great use since sufficient information is already known about their immune system, breeding is simple, they are economical, extensive reagents are already available to study murine genes and proteins, and mice can be genetically modified allowing for mechanistic studies [69]. For these reasons, this thesis focused on mouse models of renal transplantation.

Considering the diverse etiologies that can give rise to the pathology associated with transplantation, the genetic background of donor and host mice play a vital role in establishing the pathogenesis of kidney injury during kidney transplantation. To study the effect of non-alloimmune injury (i.e. ischemia reperfusion injury), syngeneic donor and the recipient mice are necessary to eliminate any MHC mismatches and subsequent alloimmune injury. In contrast, fully MHC mismatched (allogeneic) donor and recipient

mice are required when assessing the effect of alloimmunity [72, 73]. Additionally, the degree to which allogeneic mice are subject to MHC mismatch can be correlated with post-transplant survival rates [72].

In mice, renal transplantation could be performed in one of two ways: 1) bilateral nephrectomy of the native kidneys following transplantation or 2) nephrectomy of one native kidney but leaving the contralateral native kidney in place with a transplanted kidney. Bilateral nephrectomy allows the mice to solely depend on the transplanted kidney for survival. Nephrectomy of only one native kidney following transplantation has both advantages and limitations. This method is often used in long-term longitudinal studies and chronic allograft injury models because mortality is not considered a risk factor due to the presence of the healthy native kidney. On the other hand, because the single native kidney is sufficient for maintaining adequate kidney function (adequate glomerular filtration rate for sustaining health), potential deficiencies in the function of transplant kidney cannot be delineated [72]. As such, the use of these animal models should be tailored to the scientific question and potential translation of findings should be made with the understanding of the limitations of each model.

1.2 Pathophysiology of Ischemia Reperfusion Injury

Tissue injury that invariably follows from ischemia is caused by the cessation of adequate blood flow to the organ. The arrest or limitation of blood supply leads to deprivation of oxygen (hypoxia) and nutrients, and the build-up of metabolic substances and carbon dioxide [74]. This triggers a cascade of pathways leading to a significant reduction of adenosine triphosphate (ATP) production and availability, and subsequent depressed

activity of Na^+/K^+ ATPase and calcium pumps [75, 76]. The disruption of the ion balance between the intra- and extra-cellular space and the build-up of lactic acid can lead to acidosis, and ultimately causes the activation of phospholipases, proteases and dysregulation of the cellular actin cytoskeleton [77-79]. Moreover, the disturbances of cellular homeostasis, during ischemic injury, leads to breakdown of tight junctions, loss of brush borders, and an influx of calcium leading to the overall impairment of kidney function [80-82].

Upon reperfusion, following successful anastomosis, increased levels of oxygen and pH is observed along with restored blood flow [83]. Reperfusion introduces additional injury to the graft as excess influx of oxygen leads to the dysfunction of the mitochondrial electron transport chain, and the activation of xanthine oxidase activity which causes production of large amounts of harmful reactive oxygen species (ROS) [83, 84]. Consequently, ROS can damage and compromise the integrity of the cellular membrane and cytoskeleton. Additionally, ROS increases mitochondrial calcium which forms the mitochondrial permeability transition pore (mTPT) leading to cell death through various mechanisms (Figure 1-2) [85] [86]. Taken together, both ischemia and reperfusion injury during transplantation result in a cascade of pathways that contribute to cellular dysfunction or death. Cell death from IRI can take on many forms including apoptosis or necrosis contributing to renal dysfunction via different mechanisms including obstructing the tubular lumen and triggering tissue-destructive inflammation [87, 88]. In addition, loss of tight junction integrity can lead to back-leak of filtered toxins (and creatinine) into the circulation.

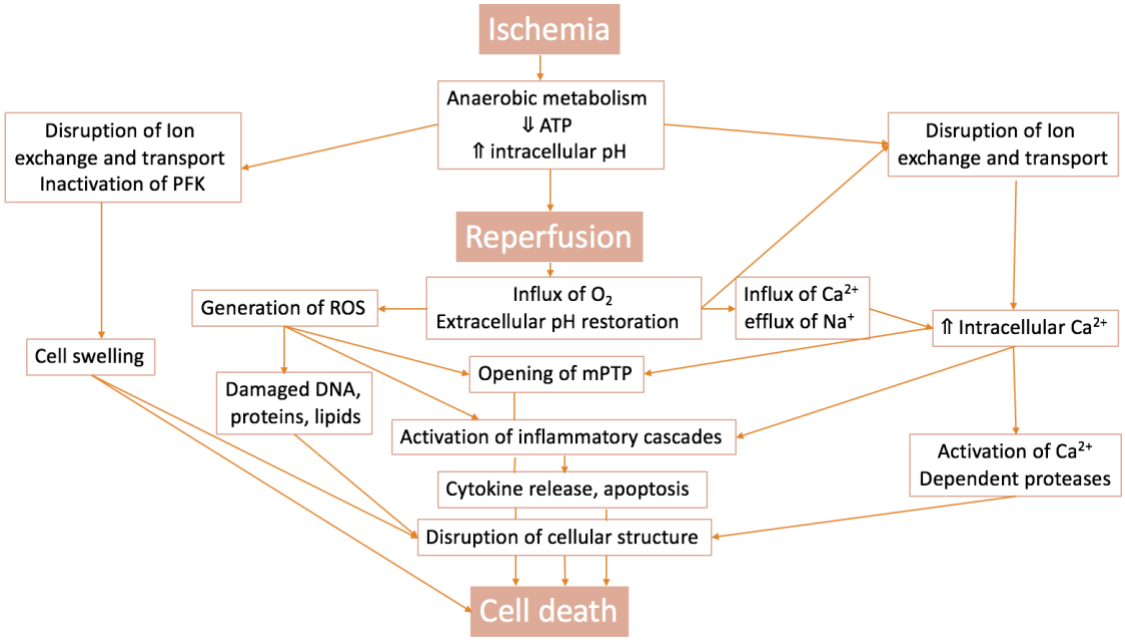

Figure 1-2. Flow diagram outlining major pathophysiology of ischemia reperfusion injury leading to cell death.

Flow diagram representing each pathological event that a graft experiences during ischemia reperfusion injury, which eventually leads to the death of tubular cells.

Figure adapted from Kalogeris et al. [85]

1.2.1 Cell Death

Proximal tubular epithelial cells (TECs) comprise of more than 75% of renal parenchymal cell mass, making it the most abundant renal cell type [89]. Due to their nature of being hypo-perfused under homeostatic conditions, coupled with their high energy demands while exhibiting poor glycolytic capacity, TECs are particularly more susceptible to IRI compared to other cells types in the kidney [90-92]. Thus, IRI to the TECs can trigger various forms cell death including but not limited to apoptosis, necroptosis and necrosis.

Apoptotic cell death is a programmed cell death and can be triggered via intrinsic or extrinsic pathways [85]. DNA fragmentation into discrete fragments of about 180-200 base pairs by specific nucleases is a distinctive characteristic of apoptosis [93]. Following a sufficient stressor, intrinsic apoptotic cell death can be induced which involves the translocation of the pro-apoptotic Bcl2 protein family, such as Bax and Bak, into the outer membrane of the mitochondria. Consequently, the mitochondrial outer membrane becomes permeabilized and results in the release of pro-apoptotic proteins such as cytochrome *c*, endonuclease-G, and Smac/DIABLO [94, 95]. Cytoplasmic cytochrome *c* can interact with apoptotic peptidase activating factor -1 (APAF-1), forming the apoptosome which activates caspase-9 and subsequently caspase-3. Caspase-3, the master regulator of apoptosis, proteolyzes numerous cellular proteins which ultimately results in DNA fragmentation [96].

The extrinsic apoptotic pathway is triggered upon activation of TNFα, Fas and TRAIL receptors. Trimerization of these receptors occur once they are activated, which then

forms the death-inducing signaling complex (DISC) by recruiting a number of proteins including death domain containing proteins like FADD and TRADD. This complex then activates caspase-8 and caspase-3 downstream [97, 98]. Cytoplasmic and nuclear condensation and fragmentation as a result of caspase-3 leads to formation of apoptotic bodies with externalization and exposure of phosphatidylserine (PS) on the outer, intact membrane, which is the hallmark of apoptotic cells [99].

Unlike apoptosis, the morphological hallmarks of necrosis are characterized by their cellular and organelle swelling, disruption of the plasma membrane, and the release of intracellular contents [100]. Initially, necrotic cell death was considered to be a random, unprogrammed processes in response to stress. However, growing evidence suggests that necroptosis, programmed necrosis, which is the predominant from of cell death during IRI, is regulated by activation of receptor interacting protein kinases (RIPK) [101-104]. Activation of RIPK, through various signaling cascades, phosphorylates mixed-lineage kinase domain like protein (MLKL) resulting in the loss of plasma membrane integrity and death by necroptosis [101, 105, 106]. Furthermore, studies have shown that blocking RIPK signaling conferred a protective effect in a murine model of IRI [101, 107-109].

Aside from necroptosis, other regulated necrotic cell death pathways include pyroptosis and ferroptosis. Of these 3 pathways, pyroptosis is considered to be the most immunogenic cell death via the activation of caspase-1 [110]. Caspase-1 is responsible for cleaving inflammatory cytokines including pro-IL-18 and pro-IL-1β into their active forms, resulting in an enhanced proinflammatory environment compared to other regulated cell death pathways [111]. Ferroptosis is less immunogenic than pyroptosis and is an iron-dependent pathway of regulated necrotic cell death [112]. During ferroptosis,

cystine deprivation results in glutathione depletion leading to the inhibition of glutathione peroxidase 4 and lipid peroxidation, ultimately resulting in cell death by plasma membrane rupture [110, 113]. Numerous studies that have targeted downstream of these regulated cell death pathways exhibited successful protection against murine models of IRI [114-117]. As such, necroptosis, pyroptosis, and ferroptosis are the most studied regulated cell death pathways in ischemia reperfusion injury and transplantation.

1.2.2 Damage Associated Molecular Patterns

In contrast to apoptosis which is also referred to as “clean cell death”, necrosis is known to be the most immunogenic form of cell death due to the rupturing of the plasma membrane and leakage of intracellular contents into the extracellular milieu [118]. The intracellular contents could include several highly immunogenic compounds such as, high-mobility group box-1 (HMGB1), heat shock protein (HSP), histones, s100 proteins, and uric acid. Although the aforementioned molecules serve a vital purpose when in their natural intracellular location, these endogenous molecules can trigger proinflammatory responses once spewed into the extracellular milieu or circulation. As a result, these molecules have been identified as damage associated molecular patterns (DAMPs) or danger signals [118, 119]. Extracellular DAMPs can be recognized by renal parenchymal cells and innate immune cells via pattern recognition receptors (PRRs), whose activation in turn triggers the downstream production of proinflammatory cytokines and mediators [120]. The signaling pathway which links the extracellular DAMPs to the proinflammatory response in cells in termed danger signaling. Moreover, DAMPs can be released either passively or actively for instance during sepsis [121].

HMGB1 is a nuclear protein that acts as a DNA chaperon to enhance chromatin folding and transcriptional activation in all nucleated cells [122]. However, once released (passively) by necrotic cells and uncleared apoptotic cells (undergoing secondary necrosis) [123], HMGB1 can trigger activation of inflammation through binding to toll-like receptors-2, -4, and -9 (TLR2, TLR4 and TLR9) [124]. Through the activation of the MyD88 signaling cascade, nuclear factor -κB (NF-κB), a master regulator of inflammation, is activated leading to production of inflammatory cytokines (i.e. IL-1, IL-6 and TNF-α) and chemokines and subsequent recruitment of immune cells [125-127]. Importantly, HMGB1 has been shown to mediate kidney IRI and neutralizing HMGB1 protected against renal IRI in mouse models [128-131].

DAMPs, particularly HMGB1 has been shown play a key role in necroinflammation [129, 131]. Necroinflammation describes the phenomenon of an auto-amplification loop of cell death which describes the process of progressive inflammation initiated by cell death begetting more cell death [113]. Specifically, necroinflammation is initiated through the interactions of DAMPs released by few necrotic cells and the immune response, resulting in inflammation which triggers progressively more necrotic cell death which in turn elicit a greater immune response. The pathological consequence of necroinflammation is tissue damage which manifests as graft damage and organ dysfunction or failure [20, 113, 132]. Therefore, endogenous pathways that promote the clearance of dying cells can mitigate necroinflammation and protect from tissue damage in IRI. Agents that enhance such processes might be a novel therapeutic strategy in renal transplantation where acute and chronic tissue injury is common.

1.3 Kidney Injury Molecule-1

Kidney injury molecule-1 (KIM-1 in humans, and Kim-1 in mice), also known as T-cell immunoglobulin mucin domain -1 (TIM-1), is a member of T-cell immunoglobulin mucin (TIM) family [133, 134]. In humans, the TIM family consists of TIM-1, TIM-3, and TIM-4, whereas in mice, the TIM family includes TIM-1 to TIM-8 [135-137]. KIM-1 is encoded by the gene hepatitis A virus cellular receptor-1 (HAVCR1), which encodes a receptor for hepatitis A virus [138]. Thus, depending on the anatomical location and cell type where this protein is expressed, it is referred to as HAVCR1 (in liver), TIM-1 (on immune cells including T cells, regulatory B cells, and NKT cells) or KIM-1 (in kidneys) [133, 139].

Activated CD4+ T cells, predominately Th2 cells, express TIM-1, where its ligation acts as a co-stimulatory signal resulting in the promotion of cytokine production, T cell proliferation, and the inhibition of tolerance [134, 140, 141]. TIM-4, a ligand for TIM-1, regulates CD 4+ T cell activation and differentiation upon interaction by modulating the Th1/Th2 cytokine balance [142]. Interestingly, the TIM-1: TIM-4 axis was reported to exacerbate injury following IRI, and blocking this interaction ameliorated renal damage [137].

Originally, KIM-1 was discovered as HAVCR1, a receptor for hepatitis A virus in African green monkey cells [143]. The initial characterization of HAVCR1 described a port of entry for hepatitis A virus into the cell [144]. Later, humans were found to possess a homolog of HAVCR1 [145] which regulated the cellular entry of many viruses including: Dengue virus (DV), Human immunodeficiency virus, hepatitis C virus, and

Zaire Ebola Virus (EBOV) [146-149]. Shortly after the discovery of human HAVCR1, a mouse homolog named KIM-1, was discovered on injured kidney tubular cells [150]. Therefore, the conserved nature of KIM-1 provides translational power across the species barrier.

1.3.1 Polymorphism of KIM-1

The gene for KIM-1, *HAVCR1*, is highly polymorphic with thousands of variants identified. This effect of the variability of *HAVCR1* results in a highly heterogenous human population where numerous different forms of KIM-1 can be observed [133, 141]. Indeed, previous studies have shown that susceptibility to a wide range of autoimmune disorders and viral infections are linked with differential KIM-1 variant expression [142-145]. Interestingly, in humans that expressed a select variant of KIM-1 which has a 6 amino acid insertion in the mucin domain producing a "longer variant", exhibited greater risk of susceptibility to severe hepatitis A [143]. The pathogenesis mediated by the longer variant of KIM-1 in hepatitis A infection was found to be specific for both hepatocytes and NKT cells. First, hepatitis A virus were able to more proficiently bind to hepatocytes that expressed the longer variant of KIM-1 which increased the likelihood of infection. Secondly, NKT cells which also express the *HAVCR1* gene (known as TIM-1 in T cells), were more responsive to virally infected hepatocytes and targeted them for destruction by cellular-mediated cytotoxicity which exacerbated liver damage [143, 146]. In contrast however, longer KIM-1 variants were found to be protective against atopy in some individuals [147]. Moreover, individuals that exhibited that KIM-1 variant that caused lower surface level expression exhibited delayed Human immunodeficiency virus

infection [146]. Taken together, these and other studies, demonstrate the association between genetic variations in *HAVCR1* and the risks of disorders of immune dysregulation such as asthma, rheumatoid arthritis, systemic lupus erythematosus, and allergic rhinitis differs [145, 148-151]. Whether genetic variants of KIM-1 affect KIM-1 function in the kidney or are linked with susceptibility to renal injury following IRI has not been studied to date.

1.3.2 Structure and Expression

HAVCR1 encompasses 14 exons and is located on chromosome 5p33.3 [139]. With a length of 1095 bp, HAVCR1 mRNA encodes a type 1 cell surface glycoprotein that consists of an extracellular immunoglobulin variable (IgV) domain, a mucin domain, a transmembrane domain, and a short intracellular domain [150]. Extracellular compartments of KIM-1 include N-terminal six-cystine IgV domain, and a highly glycosylated mucin domain rich in threonine, serine and proline [162]. Intracellularly, KIM-1 has a short C-terminal cytoplasmic tail that has a tyrosine kinase phosphorylation motif (QAEDNIY) which is essential for signaling (Figure 1-3) [150, 163-165].

KIM-1 can be found on the apical surface of renal proximal TECs [150], however, a healthy kidney does not express KIM-1 but transiently upregulates its expression following an injury to the kidney [166]. Basal expression of Kim-1 has been reported in Balb/c mice kidneys [167]. Upon expression on TECs, KIM-1 undergoes spontaneous ectodomain cleavage and shedding, releasing the extracellular portion of soluble KIM-1 (sKIM-1) into the urine (or conditioned media in cultured TECs) [168-170]. The cleavage of KIM-1 is considered to be performed by tumor necrosis factor-α converting enzyme

(TACE), also referred to as ADAM 17 [171]. At the protein level, a full-length human KIM-1 has an apparent molecular weight of 104 kDa, but once cleaved, it generates a soluble form of KIM-1 that is about 90 kDa in size and a small membrane bound form of KIM-1 that is approximately 14 kDa [168]. KIM-1 is predicted to generate a 34 kDa protein in the absence of any post-translational modifications. Murine Kim-1 is smaller in size at ~60 kDa, where its size discrepancies with human KIM-1 may be explained by the reduced number of glycosylation sites observed in mouse Kim-1 [168, 172].

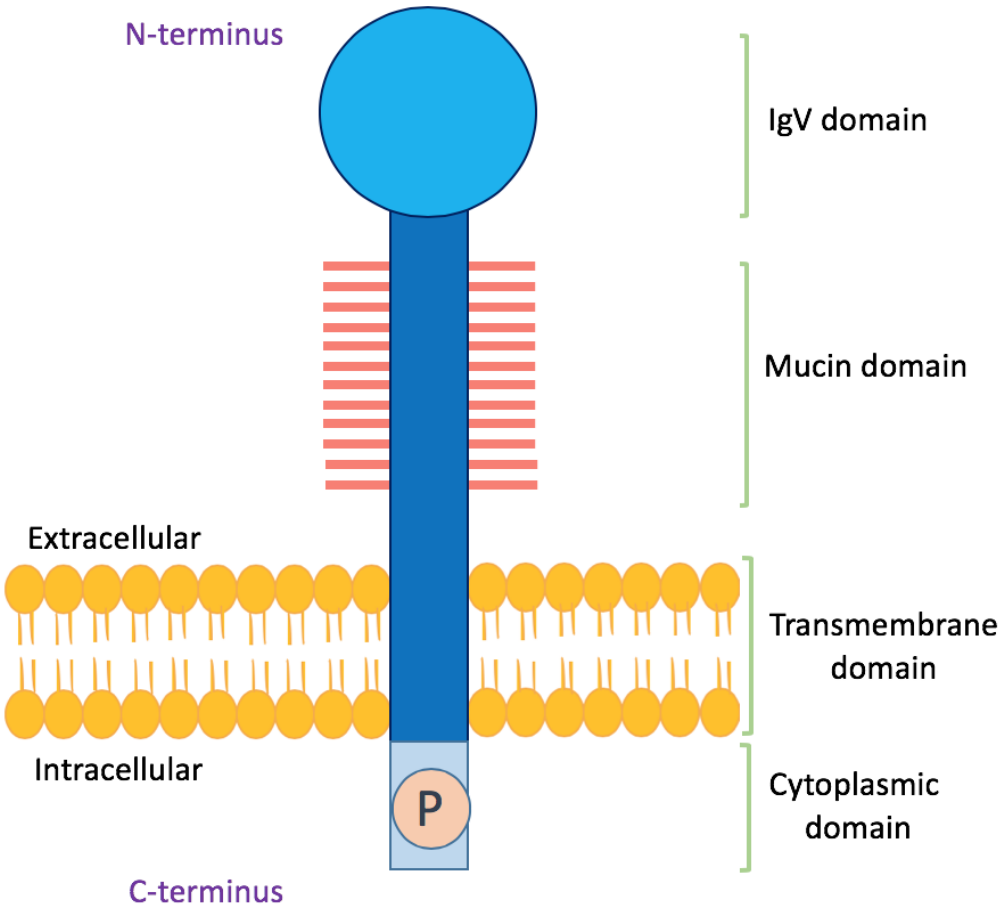

Figure 1-3. Structure of Kidney Injury Molecule -1

A schematic representation of the structure of KIM-1 which consists of an extracellular, a transmembrane and an intracellular domain. The extracellular domain entails an IgV domain which is a site for phosphatidylserine binding, and a highly glycosylated mucin domain. The intracellular cytoplasmic domain contains tyrosine kinase phosphorylation motif required for signaling.

1.3.3 KIM-1 Function

The IgV domain of KIM-1 contains a metal ion dependent ligand binding site, where phosphatidylserine, an “eat-me” signal on apoptotic cells, can bind [173]. Upon binding, the KIM-1 expressing renal TECs are transformed into semi-professional phagocytes and engulf apoptotic cells through a process known as, efferocytosis [153, 164, 174]. Using murine models, our lab found that total genetic ablation of KIM-1 predisposed them to more severe kidney dysfunction and damage following native renal IRI compared to *wild-type* mice [175]. Similar results were reported when mice exhibiting mutation in the KIM-1 mucin domain (KIM-1 Δmucin) which impairs binding to PS [164]. KIM-1 Δmucin exhibited greater renal dysfunction, renal inflammation, and mortality following native renal IRI. The abovementioned would suggest that the primary function of KIM-1 in the kidneys is the removal of apoptotic cells which thereby limiting further inflammation.

A secondary equally important function of KIM-1 is in the repair of denuded tubular epithelium following acute injury. KIM-1 expression activates the extracellular signal regulated kinase/ mitogen-activated protein kinase (ERK/MAPK) signaling pathway, which in turn facilitates the migration and proliferation of TECs, promoting repair [176]. During an ischemic insult, KIM-1 was also shown to protect against cell death by interacting with pro-apoptotic nuclear receptor 77 (NUR77) via the IgV domain [177]. An *in vitro* study has shown that following depletion of ATP and glucose to mimic ischemic injury, the lack of KIM-1 expression resulted in higher levels of NUR77, and increased amount of cell death [177]. Therefore, the clearance of dying cells during

kidney injury, as well as promoting tissue repair by the neutralization of pro-apoptotic factors, KIM-1 plays a crucial role in mitigating inflammation and tissue damage during kidney injury.

1.3.4 KIM-1 Signaling

The conserved tyrosine kinase phosphorylation motif in the cytoplasmic tail of KIM-1 is involved in downstream signaling pathways via engagement with protein kinases [163]. Upon binding of apoptotic cells to KIM-1, the intracellular tyrosine kinase of KIM-1 is phosphorylated, leading to the interaction with p85. Subsequently, modulated by PI3K, phosphorylation and activation of NF-κB signaling is inhibited, thereby negatively regulating inflammation (e.g. production of IL-6) (Figure 1-4) [140, 164].

Considering that phagocytosis is the primary function of KIM-1 in the kidney, it is of no surprise that KIM-1 would be involved with cytoskeletal remodeling during the internalization of apoptotic bodies. Previously, our group has shown that KIM-1 interacts with T-complex testis specific protein 1 (Tctex-1), a dynein light chain protein, to mediate the internalization of apoptotic cells via KIM-1 dependent efferocytosis [178]. In addition, our laboratory has shown that during renal IRI, intracellular domain of KIM-1 directly interacts with alpha subunit of heterotrimeric G protein 12 (Gα12) directly and suppresses its activation [179]. ROS produced during IRI activates Gα12 disrupting the formation of tight junctions and zonula occludens-1 (ZO-1) on renal TECs during renal repair through Src tyrosine kinases [180, 181]. Upon activation, KIM-1 acts to negatively regulate Gα12 to limit its activity spurred on by ROS [179]. Hence, the molecular

mechanisms involved in KIM-1- mediated protection against ischemia reperfusion injury involves several signaling pathways and multiple intracellular proteins.

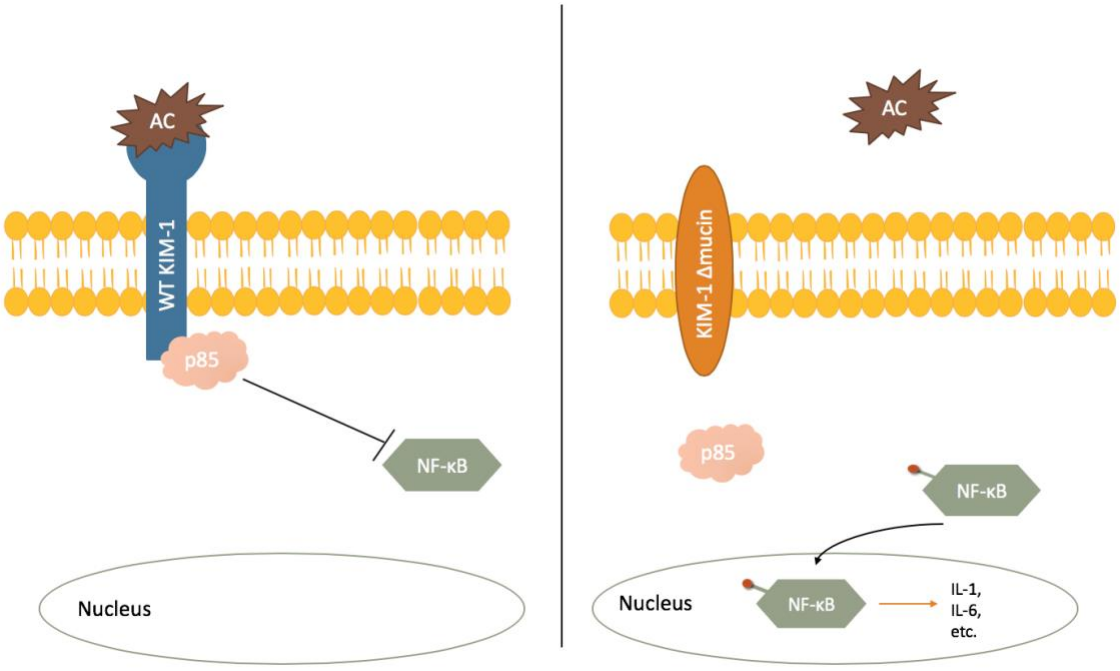

Figure 1-4. Diagram of the downstream signaling cascade pathway of KIM-1 inhibition of NF-κB upon apoptotic cell binding.

Upon binding of apoptotic cells to KIM-1 via phosphatidylserine, p85 is recruited which leads to the inhibition of NF-κB activity which limits the production of inflammatory cytokines such as IL-6, IL-1 and RANTES. KIM-1 Δ mucin are defective in binding to apoptotic cells leaving NF-κB unperturbed, resulting in inflammation.

Figure adapted from Yang et al. [164]

1.3.5 Clinical relevance of KIM-1

Following injury, soluble KIM-1 is shed into the urine upon cleavage of its ectodomain. Because KIM-1 is upregulated during proximal tubule injury and not present in the healthy human kidney, KIM-1 is regarded as a specific and sensitive biomarker for renal acute kidney injury [182, 183]. KIM-1 is also aberrantly expressed in renal cell carcinoma tumours and multiple kidney diseases [182, 184-186]. Urinary KIM-1 levels in patients with chronic heart failure can be used as a prognostic clinical marker of worsening renal function [187]. Of note, KIM-1 can also be detected in the blood upon kidney injury which is thought to be due to the disruption of tight junctions and tubular back-leaking [188]. In addition, growing evidence has shown that soluble KIM-1 is also detected in the blood, which serves as a useful biomarker for acute and chronic kidney disease and has been used to predict progression to ESRD in Type 1 diabetes patients [189].

Studies have shown the utility of tissue expression of KIM-1 in numerous clinical diseases. For example, KIM-1 expression, examined in the biopsies of transplanted kidney of patients who developed graft dysfunction, is used to determine the degree of injury [190]. Both urinary and membrane-bound KIM-1 expression can be detected in patients suffering from chronic kidney disease (CKD) [139, 191] Surprisingly, transgenic mice that were generated to expresses KIM-1 constitutively in TECs, developed spontaneous CKD exhibiting significant amounts of kidney inflammation and fibrosis. This result indicates that sustained expression of KIM-1 may be pathogenic, leading to renal fibrosis and kidney failure [192, 193]. During the early stages of acute kidney injury

(AKI), KIM-1 upregulation is protective due to its beneficial role of clearing dying cells, initiating TECs repair, and limiting inflammation. However, sustained chronic expression of KIM-1 may be harmful as could promote fibrosis, ultimately leading to CKD. Therefore, modulating KIM-1 expression or function for therapeutic purposes for renal injury must be done with caution and careful evaluation using representative animal models.

1.4 Apoptosis Inhibitor of Macrophage

Apoptosis inhibitor of macrophage (AIM) protein (also referred to as CD5-antigen like, CD5L), as the name suggests, is produced by tissue macrophages to aid in their survival by inhibiting apoptosis [194]. AIM is a member of the scavenger receptor cystine-rich domain superfamily (SRCR-SF) [195], and circulates in high concentrations in the blood (~5μg/ml in both humans and mice) bound to the Fc region of pentameric immunoglobulin M (IgM) [196, 197]. A pentameric IgM forms a 50° groove where one molecule of AIM can bind [197]. Although the size of AIM is relatively small (42 kDa in mice and 37 kDa in humans), the assembly of AIM with IgM precludes AIM from renal excretion due its large molecular size (~1000kDa) [198]. During AKI, IgM-bound AIM dissociates where it can perform its function [196].

Human and murine AIM share considerable homology (~78%) consisting of conserved protein sequences, however, they diverge in the degree of glycosylation. Human AIM does not contain N-glycans, whereas mouse AIM bears heavy glycosylation. Interestingly, modification of N-glycosylation in AIM has been shown to alter its secretion and activity [199].

1.4.1 Clinical relevance of AIM

Similar to KIM-1, AIM is also subject to cleavage allowing for the dissociation from IgM which allows for renal excretion, and consequently detection in the blood and urine. Although AIM cleavage is performed by an unknown protease, cleaved AIM is smaller in size by 10 kDa which allows for the successful filtration through the glomerulus and secretion into the urine [200]. The ability to detect AIM in the blood and urine makes it a powerful biomarker and has been used to detect numerous diseases including: patients with kidney injury, liver fibrosis in hepatitis C patients, atopic dermatitis, cirrhosis and liver cancer [196, 201-203]. Once cleaved however, AIM cannot re-associate with IgM, leading to its destabilization and subsequent activation where it can be free to be involved in multiple disease states [196, 200].

The role of AIM in the progression of various diseases is an area of active research. Typically, AIM exerts its effect on disease in its IgM-free form as AIM is inactivated when associated with IgM. With respect to function, free AIM can be endocytosed into adipocytes and hepatocytes through CD36 where it acts to inhibit the synthesis of cytoplasmic fatty acids. Ultimately, this causes the decrease of the deposition of triglycerol in cells which has been reported to prevent fatty liver progression and obesity [204, 205]. Likewise, by inhibiting fatty acid deposition, AIM has been shown to have a protective role in obesity related liver diseases and hepatocellular carcinoma [206]. Alternatively, AIM has also been shown to exert significant antimicrobial activity during bacterial infection by it enhances the clearance of bacteria and fungi through opsonization [207, 208]. Moreover, AIM has been studied in the context of multiple other diseases

including chronic obstructive pulmonary disease, multiple sclerosis, inflammatory bowel disease, and atherosclerosis [204, 209-212]. Finally, the modulatory function of AIM has also shown promise in kidney injury following renal IRI [213].

1.4.2 AIM in Acute Kidney Injury

During AKI, AIM is released from its IgM pentamer bound form and is filtered through the glomerulus where it can interact with healthy and injured tubular epithelial cells [213]. To date, the specific mechanism behind the dissociation between AIM and the IgM pentamer is yet to be determined. However, one may speculate that during IRI, the homeostatic balance of ions in the body is altered, reducing the affinity of AIM for IgM. Nevertheless, the phenomenon of AIM dissociation is essential for its protective role in IRI. This is supported by studies showing that cats subjected to IRI are highly prone to severe kidney injury because feline AIM is unable to dissociate from IgM due to a particularly high affinity [214, 215].

Immunohistological staining of kidneys taken from both mice and humans suffering from AKI revealed that IgM-free AIM predominately accumulates on intraluminal necrotic debris [196, 200]. The primary role of AIM during renal injury is to prevent renal tubular obstruction by coating dead cell debris promoting clearance, thereby reducing inflammation and promoting repair. Dead cell clearance mediated by AIM is dependent on KIM-1. AIM coating the intraluminal debris acts as an opsonin where KIM-1 can recognize and directly bind necrotic cells conferring an enhanced ability of TECs to phagocytose and clear the necrotic debris. This process excludes PS interactions with KIM-1 as KIM-1 recognition of PS was not disturbed by AIM [213]. Although the direct

interaction of KIM-1 and AIM enhances KIM-1 mediated phagocytosis, the specific mechanism regarding their binding remains unknown. Furthermore, when mice suffering from native renal warm IRI were administered with recombinant AIM (rAIM), kidney function and survival was markedly rescued [213]. Hence, testing the therapeutic benefits of rAIM in renal transplantation is timely and has the potential to improve graft survival given the impact of DGF (and IRI) on the kidney graft.

Taken together, rapid removal of cellular debris is vitally important in mitigating graft damage and inflammation following renal transplantation. Large bodies of work support KIM-1 as a potential target for therapeutic intervention in mitigating tissue injury following native renal warm IRI. However, the extent to which targeting KIM-1 and or AIM in a setting of renal transplantation where both warm and cold ischemia occurs still remains to be explored.

1.5 Rationale and Objectives

1.5.1 Rationale

Renal transplantation remains the optimal treatment option compared to dialysis for patients suffering from kidney failure evidenced by marked improvements to health, quality of life, and overall survival [7]. However, the major limitation is the paucity of donor kidneys while the demand for kidney transplants is increasing. In 2013, out of the 3,300 Canadians on the kidney transplant waiting list, approximately 90 patients died while waiting [5]. Moreover, due to the finite lifespan of the graft, about 30% of transplant recipients require more than one transplant within their lifetime [18]. Taken together, in the face of a constricted supply pool, coupled with a steadily rising demand of donor kidneys, therapies that seek to minimize graft damage and ultimately prolong the longevity of kidney grafts are vitally important.

Current knowledge describes that IRI, an avoidable consequence of kidney transplantation process, results in DGF which ultimately imposes a negative impact of the graft longevity and overall survival [47, 54]. Apoptotic and necrotic cell death is a hallmark of IRI due to depletion of ATP and production of ROS resulting in deleterious effects on the graft via necroinflammation [107, 113, 216]. Thus, one key strategy to maximize transplantation success is to mitigate IRI-induced tissue injury, or promote tissue repair following IRI [57].

Upregulation of KIM-1, a biomarker of renal tubular injury, on proximal TECs protect against native renal warm IRI, restoring renal dysfunction, injury and overall mortality in

mice via the clearance of dying cells [164, 175]. However, the role of donor KIM-1 in renal transplantation where both warm and cold IRI are present has not been explored. The objective of my thesis was to understand the multifactorial roles of KIM-1 in injury, tissue repair, and inflammation following renal transplant IRI and to test whether KIM-1 can be targeted for potential therapeutics. Finally, I also sought to translate our findings to clinical scenario by determining the functional consequences of naturally occurring genetic variants of KIM-1 and their association with clinical DGF.

1.5.2 Objectives

Objective 1. Determine whether donor KIM-1 expression is protective during syngeneic renal transplantation where severe cold and warm IRI is present using a murine model of renal transplantation (alloantigen-independent i.e. IRI).

Objective 2. Determine whether targeting the KIM-1 pathway to enhance the clearance of dead cell/debris via administration of recombinant AIM ameliorates IRI-induced pathology in a murine model of renal transplantation.

Objective 3. Investigate whether 3 coding variants of KIM-1 that occur in high frequency in the human population alter KIM-1 expression and/or its phagocytic function.

Objective 4. Determine whether the 3 KIM-1 variants (Objective 3) are associated with increased risk of delayed graft function in human deceased donor kidney transplantation.

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Chapter 2

2 Donor kidney injury molecule-1 promotes graft recovery by regulating systemic necroinflammation

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2.1 Introduction

Renal transplantation offers improved survival and quality of life compared to staying on dialysis [1]. However, cold-ischemic reperfusion injury (cIRI) is unavoidable with renal transplantation and contributes to overall length of hospitalization, risk of acute rejection, and premature graft loss [2, 3]. Renal proximal tubular epithelial cells (PTECs) are highly susceptible to ischemic injury as they comprise more than 70% of renal parenchymal cells; PTECs also have high energy needs and possess poor glycolytic capacity [4]. Initial injury is caused by ATP depletion, but subsequent activation of innate and adaptive immune pathways leads to secondary graft damage [5]. Though kidneys have tremendous capacity for repair, severe injury and/or unregulated inflammation can hinder this ability [6].

The passive release of danger-associated molecular patterns (DAMPs, e.g. HMGB1) from uncleared apoptotic (that undergo secondary necrosis) and necrotic cells activate renal parenchymal and innate immune cells via pattern recognition receptors (PRRs) [7]. This can lead to the production of pro-inflammatory cytokines (i.e. IL-1α and IL-1β) and other mediators that further propagate tissue damage [8] and cell death by initiating an auto-amplification loop [9, 10]. In addition, tubular obstruction from extrusion of dying PTECs into the lumen can further contribute to renal dysfunction in IRI [11]. HMGB1 is released into the circulation from many types of allografts after transplantation and inhibiting its function has been shown to dampen alloresponses and even prolong graft survival in several pre-clinical models [12-14].

Kidney Injury Molecule-1 (KIM-1, also known as TIM-1 [15]) is a type 1 transmembrane glycoprotein that is upregulated on renal TECs following injury [16]. We and others have previously reported that KIM-1 is a phosphatidylserine (PS) receptor expressed on PTECs, transforming them into semi-professional phagocytes for neighboring apoptotic and necrotic cells [17-19]. Importantly, primary PTECs are entirely dependent on KIM-1 for efferocytosis [19]. While KIM-1 can directly bind and clear apoptotic cells expressing PS [17], the binding and clearance of necrotic cells by PTECs requires the serum protein (opsonin), Apoptosis Inhibitor of Macrophages (AIM/CD5L), which is freely filtered by the kidney during acute kidney injury (AKI) [18]. KIM-1 expression, or lack thereof, likely does not contribute to renal pathology in native conditions, as no differences in kidney function or pathology between *wild-type* and KIM-1 deficient mice were observed prior to IRI induction [20]. Work by our group and that of Bonventre confirmed that, compared to wild-type mice, both KIM-1 deficient mice [20] and mice expressing a mucin domain deletion (mutant) [21] are more prone to ischemic acute kidney injury owing to two major mechanisms triggered by KIM-1 signaling upon engagement of KIM-1 (on PTECs) by apoptotic cells or debris: (1) Initiation of an anti-inflammatory program in PTECs mediated via interaction of KIM-1 with p85 and subsequent PI3K-dependent down-modulation of NF-κB [21]; (2) Inhibition of harmful Gα12 activation by reactive oxygen species [20]. These studies suggested that KIM-1 primarily protected mice from AKI by mediating intracellular signaling in PTECs.

Here we present new evidence of an essential role for donor-KIM-1 in regulating systemic and local inflammation by preventing the release of HMGB1 from necrotic cells and thereby protecting against transplant-related cIRI. The pathophysiology of tissue

damage and its consequences to the host in the setting of renal transplantation is fundamentally different from that of native kidney IRI (e.g. cold storage, multiple episodes of warm ischemia, alloimmunity) [22, 23]. In addition, renal transplantation affords a unique opportunity for targeted therapy for AKI because: (1) The timing of initial injury is known in advance; (2) there are multiple stages of injury and delivery for therapeutics (in the donor, storage solution, and/or the recipient). Therefore, we investigated the role of graft KIM-1 in a syngeneic (life-sustaining) murine renal transplantation model [24] with the ultimate aim of translating our findings to the clinic.

2.2 Materials and Methods

2.2.1 Animals

Wild-type C57BL/6 mice were obtained from the Charles Rivers Laboratory. C57BL/6 KIM-1 deficient mice (KIM-$1^{-/-}$) were obtained from Dr. Andrew N. J. McKenzie (MRC laboratory of Molecular biology, Cambridge, UK). KIM-1 deficient mice were generated by targeted disruption of exon 2 of mouse *havcr1*$^{-/-}$ gene in mouse embryonic stem cells as described previously [25]. All animal procedures were pre-approved by Western University animal use subcommittee in accordance with the regulations of the Canadian Council on Animal Care.

2.2.2 Renal transplantation

Male KIM-$1^{+/+}$ C57BL/6 recipient mice were bilaterally (sequentially) nephrectomized and transplanted with a single donor kidney from male KIM-$1^{+/+}$ or KIM-$1^{-/-}$ C57BL/6

mice in a single procedure [26]. This is a survival model. Donor kidneys were exposed to 35 min of cold ischemia. Mice with weight loss of 15% or clinical deterioration were euthanized according to animal care protocols. Animals were followed 7 days post-transplant. Survival and renal function (serum creatinine) were monitored at 1, 3, and 7 days post-transplant. For each group, mice were euthanized at each time point to observe and evaluate the graft.

2.2.3 Primary tubular epithelial cell culture

Primary tubular epithelial cells were cultured at 37°C in 5% (vol/vol) CO_2 by only collecting the renal cortex of KIM-1 deficient and WT C57BL/6 mice. These cells were maintained in DMEM (Invitrogen, Carlsbad, CA) and F12 (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen, Carlsbad, CA), 1% Penicillin-streptomycin (Invitrogen, Carlsbad, CA), 0.1% ITS (Invitrogen, Carlsbad, CA), 0.1% EGF (Peprotech, Rocky Hill, NJ) and hydrocortisone (Thermo Fisher Scientific, Rockford, IL). Primary TECs were used up until one passage. Hallmark appearance of TECs were confirmed by visual analysis.

2.2.4 *In vivo* HMGB1 detection

Post-transplant serum was collected and serum HMGB1 was quantitatively determined using Sandwich-enzyme immunoassay kit in accordance with its protocol (Shino-Test Corporation, Tokyo, Japan). Multiskan GO software was used for quantification (Thermo Fisher Scientific, Rockford, IL).

2.2.5 Phagocytosis assay/ *In vitro* HMGB1

Thymocytes were isolated from C57BL/6 mice at 3-6 weeks old. To induce apoptotic cells, thymocytes were placed under UV light for 5 minutes followed by overnight incubation at 37°C in 5% (vol/vol) CO_2 in DMEM media containing 1% Penicillin-streptomycin and 10% FBS. To induce necrotic cells, thymocytes were heat-killed by incubating the cells at 65°C for 16 minutes in PBS. For the phagocytosis assay, approximately $1x10^6$ primary TECs from Kim-$1^{-/-}$ and Kim-$1^{+/+}$ C57BL/6 mice were plated and subsequently co-cultured with either $1x10^6$ healthy, apoptotic, or necrotic thymocytes. After 24 hours of incubation at 37°C in 5% (vol/vol) CO_2, the conditioned media were collected and analyzed for HMGB1 using western blots.

2.2.6 Western blot

Tissue sections were stored in 4% SDS. Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL) was used to quantify isolated proteins from whole tissue sections. We incubated the blots with mouse polyclonal KIM-1 (R&D Systems Inc., Minneapolis, MN) and mouse monoclonal GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Western blots were developed and quantified using the FluorChem M system (ProteinSimple, San Jose, CA).

2.2.7 RNA isolation and polymerase chain reaction

Tissue sections were collected in TriPure isolation reagent (Roche Diagnostic, Basel, Switzerland) and total RNA was extracted. qSCRIPT cDNA SuperMix (Quanta

Biosciences, Gaithersburg, MD) was used to generate cDNA. Real-time polymerase chain reaction (RT-PCR) was completed using StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA) and SYBR Green (Thermo Fisher Scientific, Rockford, IL) detection was used to quantify relative expression. Primers (Integrated DNA Technologies, Coralville, IA) used were: IL-6: F-5'-TACTCCTTCCTACCCCA ATTTCC-3' R- 5'- TTGGTCCTTAGCCACTCCTTC-3'; MIP-2α: F-5'-CAAAGGCA AGGCTAACTGACC-3' R- 5'ACATCAGGTACGATC CAGG C-3'; TNF-α: F-5'-AGCCCACGTCGTAGCAAAC-3' R- 5'ACAAGGTACAACCCATC GGC-3'. GAPDH: F- 5' TCAGCATCTCTAAGCGTGGT-3' R-5'-ATGTTGTCTTC AGCTCGGGA-3' was used as the housekeeping gene.

2.2.8 Histology, immunohistochemistry

Tissue sections were stored in 10% formalin before sectioning. A renal pathologist, blinded to the groups, scored the tissue sections that were stained with hematoxylin and eosin (H&E) using a previously described semi-quantitative method [20]. Scoring included: acute tubular necrosis (ATN) and tubular obstruction. For ATN, sections were assessed for the presence of tubular necrosis, pyknotic nuclei, formation of proteinaceous casts, brush border damage, proximal dilation and interstitial widening. Each section was given an ATN score out of 5; 0= 0%, 1= <10%, 2= 11-25%, 3= 26-45%, 4= 46-75%, 5= > 75% ATN [18]. Degree of tubular obstruction was measured by looking at intraluminal debris of dead PTECs. Similarly, each section was given a score out of 5, with the same scoring margins as ATN. Immunohistochemistry was performed on sections to visualize apoptotic cells using anti-cleaved caspase-3 (Abcam, Cambridge, MA), infiltration of

neutrophils using anti-myeloperoxidase (Abcam, Cambridge, MA), and macrophages using anti-CD68 (Abcam, Cambridge, MA).

2.2.9 Immunofluorescence

Ethidium homodimer (EHD; Invitrogen, Carlsbad, CA) was used to quantify necrosis in kidney graft sections. 5μM EHD was injected into the renal artery for 10 minutes at a speed of 1mL/min, followed by another 10 minutes of PBS at the same speed [14]. Frozen sections were used, and areas of necrosis were quantified using a FLUOVIEW X831 confocal microscopy (Olympus, Tokyo, Japan). A total of 5 random non-overlapping sections of the outer cortex were taken per slide. Quantification was done using ImageJ software (National Institutes of Health, Bethesda, MD).

2.2.10 Statistical analysis

Log-rank test was used to determine the graft survival. One-way ANOVA was used for multiple comparisons whereas t-test was used for unpaired values. Mann-Whitney test was used when comparing nonparametric data. All these tests were carried out using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). All data are presented as means ± SEM, and $p < 0.05$ was used for significance.

2.3 Results and Discussion

We characterized the expression of KIM-1 following transplantation. Graft KIM-1 expression was persistently upregulated even after 7 days post-transplantation, peaking by day 1 post-transplant (Figure 2-1A, 1B). We then observed that absence of KIM-1 expression in the donor kidney resulted in significantly greater renal dysfunction as indicated by higher serum creatinine values on day 3 (161.8 ± 39.8 vs. 15 ± 3.082 μmol/L, p=0.0104; Figure 2-1C). Recipients of KIM-$1^{-/-}$ grafts also exhibited significantly reduced 7-day survival compared to mice receiving *wild-type* grafts (5/5 vs. 3/12, p=0.0105; Figure 2-1D).

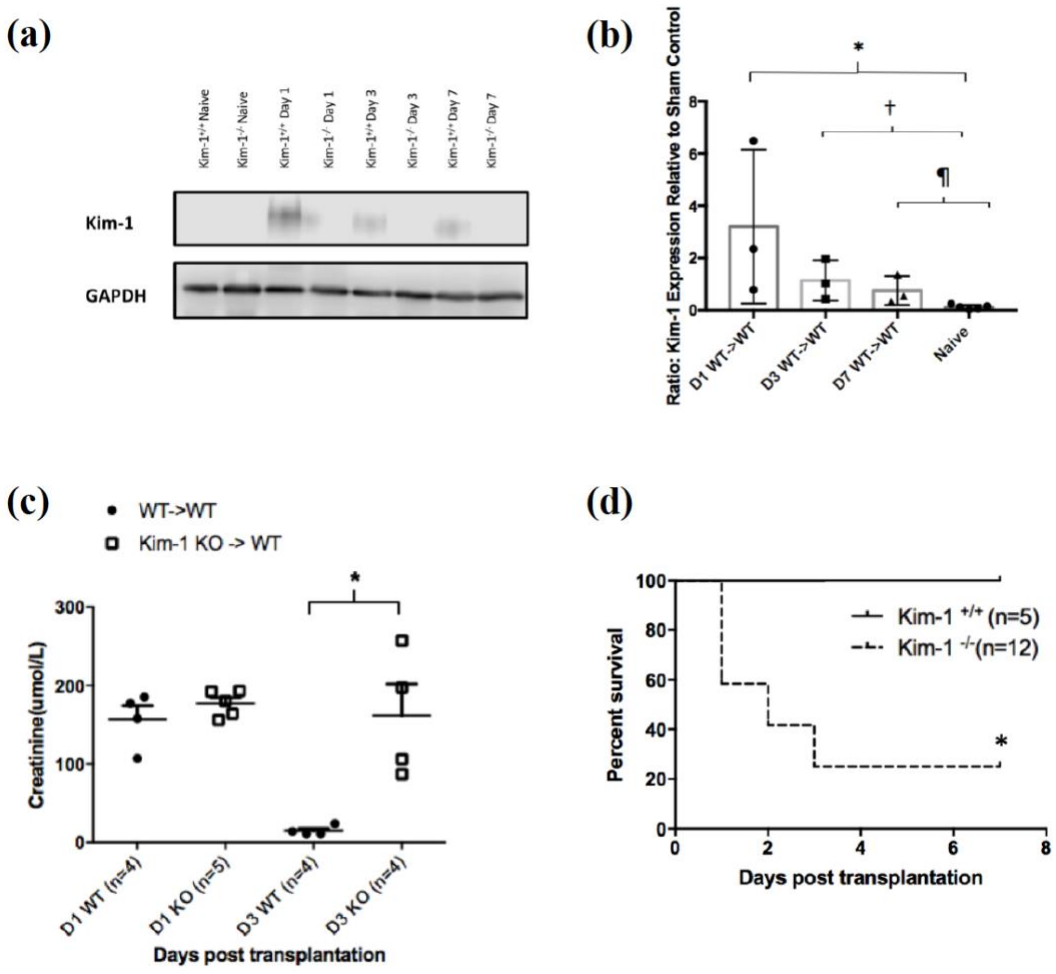

Figure 2-1. KIM-1 expression on PTECs improves early graft function and survival.

C57BL/6 KIM-1 deficient or *wild-type* (WT) donor kidneys subjected to cold storage (35 minutes) were transplanted into syngeneic WT recipients. (A) KIM-1 protein expression was confirmed in the kidney grafts of WT donor kidney recipients using Western blots. GAPDH was used as a loading control. (B) Average densitometric ratio of Western blots showing KIM-1 expression (n=3). (C) Serum creatinine was measured as a marker of renal function at days 1 and 3 post-transplantation (*$p < 0.05$). (D) Survival of the renal transplant recipients. Recipients of the KIM-1 deficient donor kidneys are denoted by

dotted line (---), and recipients of WT donor kidneys are denoted by straight bold line (*p=0.0105, log- rank, n=5-12/group).

Given the essential role of KIM-1 in the phagocytic clearance of apoptotic [27] and necrotic cells in the kidney [19], we expected that the poor transplant outcomes associated with donor KIM-1 deficiency would be associated with increased tubular cell apoptosis and necrosis (secondary necrosis of uncleared apoptotic cells [27]) early after transplantation. Mice transplanted with KIM-1 deficient donor kidneys exhibited increased numbers of both apoptotic (16.67 ± 3.333 vs. 3.75 ± 2.394, p= 0.0018; Figure 2-2A) and necrotic cells (11.5 ± 1.041 vs. 0.5333 ± 0.1202, p= 0.0005; Figure 2-2B) compared to mice transplanted with wild-type donor kidneys. In addition, we looked at acute tubular necrosis (ATN) to determine the degree of graft injury. As shown in Figure 2-2C, KIM-1 deficient donor grafts revealed significantly greater ATN scores compared to the wild-type grafts on day 3 post-transplantation (4 vs. 0.5, p=0.0286; Figure 2-2C). We also observed persistent tubular obstruction, which contributes to kidney dysfunction [11], more so in the KIM-$1^{-/-}$ grafts compared to the KIM-$1^{+/+}$ grafts (3.5 vs. 0.5, p=0.0286; Figure 2-2D). A lack of any difference at day 1 here is likely explained by the fact that KIM-1 does not affect PTECs death due to cIRI [20, 21], but rather the removal of debris after acute injury.

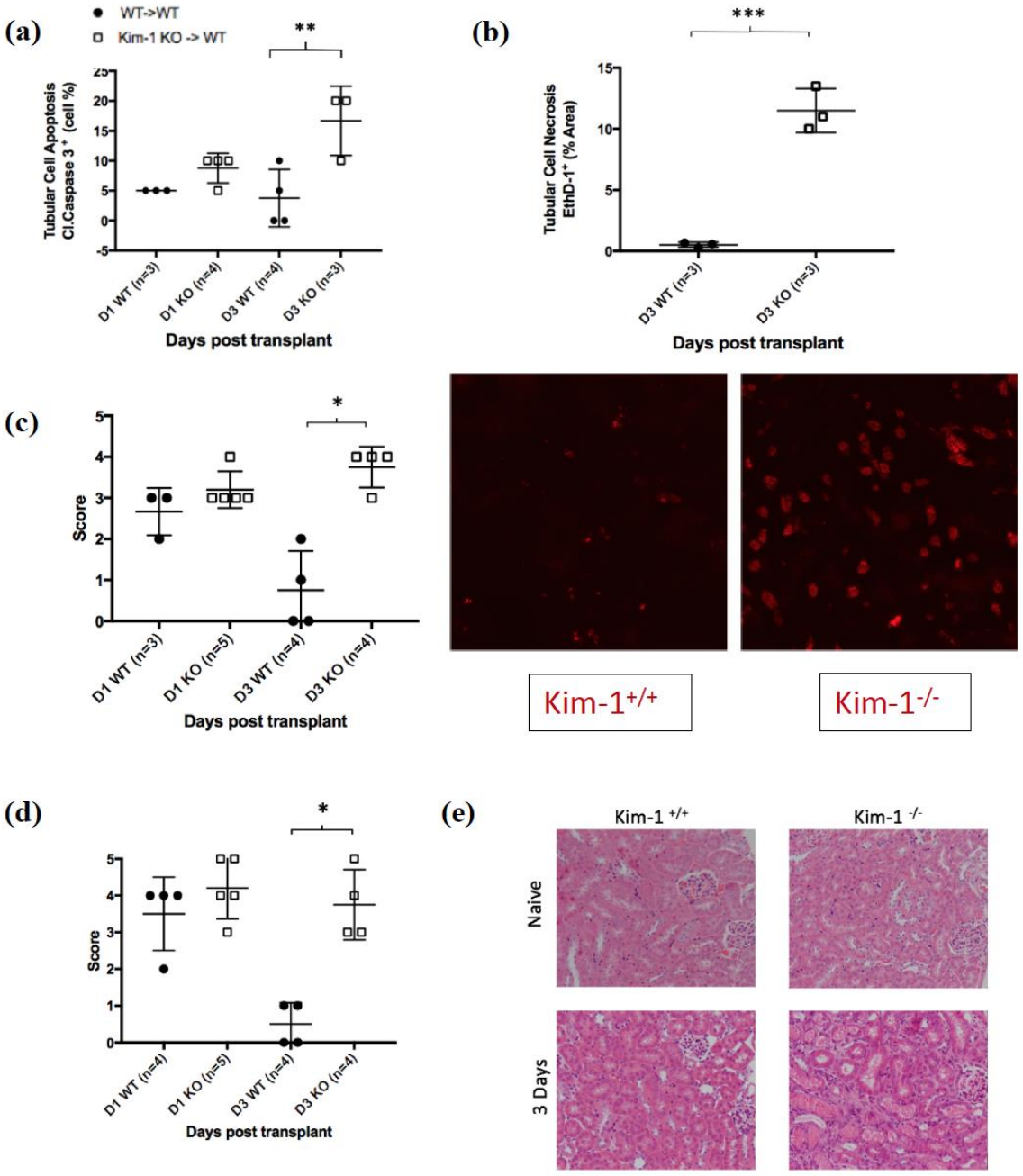
(a)
WT->WT
Kim-1 KO -> WT
**
Tubular Cell Apoptosis
Cl.Caspase 3+ (cell %)
D1 WT (n=3)
D1 KO (n=4)
D3 WT (n=4)
D3 KO (n=3)
Days post transplant
(b)
***
Tubular Cell Necrosis
EthD-1+ (% Area)
D3 WT (n=3)
D3 KO (n=3)
Days post transplant
(c)
*
Score
D1 WT (n=3)
D1 KO (n=5)
D3 WT (n=4)
D3 KO (n=4)
Days post transplant
Kim-1+/+
Kim-1-/-
(d)
*
Score
D1 WT (n=4)
D1 KO (n=5)
D3 WT (n=4)
D3 KO (n=4)
Days post transplant
(e)
Kim-1 +/+
Kim-1 -/-
Naive
3 Days

Figure 2-2. Renal tubular epithelial cell death and graft injury between the two donors following syngeneic renal transplantation.

(A) Apoptotic tubular cells were quantified using cleaved caspase-3 staining for early apoptotic cells at days 1 and 3 post-transplantation. Apoptotic cells were counted and averaged from 5 different fields at 400x magnification for each section (**$p < 0.01$). (B; Top) Necrotic tubular cells were quantified using EHD. (B; Bottom) Immunofluorescence images were taken at 400x magnification at day 3 post-transplantation (***$p < 0.001$). (C-E) Formalin fixed tissue sections were stained with H&E and were scored by a renal pathologist blinded to their genotype. Slides were based on a scoring system ranging from 0-5. 0= none, 1= < 10%, 2=11-25%, 3=26-45%, 4=46-75%, and 5= >75%. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n=3-5/group). (C) ATN score (D) Tubular obstruction score (E) Images of H&E stained tissue sections at 3 days post-transplantation.

An important, yet unanswered, question that remains is whether endogenous clearance of apoptotic and necrotic cells, and reduction in the burden of secondary necrotic or necrotic cells [28], would lead to a decreased release of DAMPs [27] and tissue inflammation. Furthermore, while mice carrying global defects in efferocytosis pathways are known to exhibit systemic autoimmunity [29, 30], systemic inflammation has not been attributed to such defects during acute tissue injury. When we analyzed the grafts for markers of inflammation, we noted that the absence of KIM-1 exacerbated the intra-graft inflammatory response. There was significantly more granulocyte infiltration at both days 3 (1.2 ± 0.7348 vs. 0 ± 0, $p=0.0060$) and 7 (2.333 ± 2.333 vs,0 ± 0, $p=0.0156$; Figure 2-3A). Similarly, there was significantly higher macrophage infiltration at day 7 post-transplantation (10 ± 1.225 vs. 4.75 ± 0.4787, $p=0.0072$; Figure 2-3B) in KIM-$1^{-/-}$ vs. *wild-type* grafts. In parallel, KIM-$1^{-/-}$ grafts also exhibited significantly greater expression of IL-6 (45.08 ± 13.07 vs. 11.32 ± 0.8026, $p=0.0419$) and MIP-2α (108.6 ± 22.5 vs. 29.48 ± 13.22, $p=0.0231$; Figure 2-3C) compared to mice transplanted with *wild-type* grafts at day 3 following transplantation.

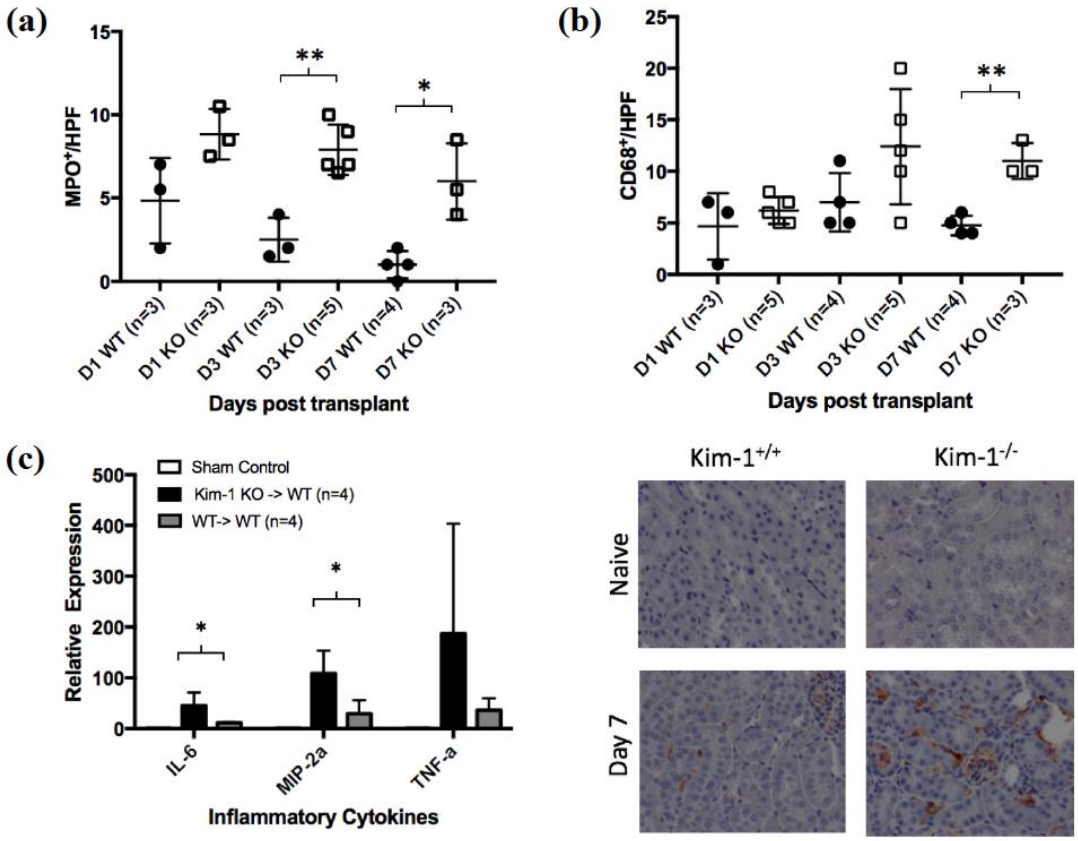
(a)
MPO+/HPF
15
10
5
0
**
*
D1 WT (n=3)
D1 KO (n=3)
D3 WT (n=3)
D3 KO (n=5)
D7 WT (n=4)
D7 KO (n=3)
Days post transplant
(b)
CD68+/HPF
25
20
15
10
5
0
**
D1 WT (n=3)
D1 KO (n=5)
D3 WT (n=4)
D3 KO (n=5)
D7 WT (n=4)
D7 KO (n=3)
Days post transplant
(c)
Sham Control
Kim-1 KO -> WT (n=4)
WT-> WT (n=4)
Relative Expression
500
400
300
200
100
0
*
*
IL-6
MIP-2a
TNF-a
Inflammatory Cytokines
Kim-1+/+
Kim-1-/-
Naive
Day 7

Figure 2-3. Absence of KIM-1 in the donor kidney exacerbates inflammation following syngeneic renal transplantation.

(A-B) Tissue sections were stained for MPO or CD68 to detect graft-infiltrating neutrophils and macrophages respectively, at days 1, 3 and 7 post-transplantation. The images were visualized at 400x magnification. (A) Number of MPO^+ neutrophils/HPF (*$p < 0.05$, **$p < 0.01$, n=3-5/group). (B; Top) Number of $CD68^+$ macrophages /HPF (**$p < 0.01$. n=3-5/group). (B; Bottom) Immunohistochemistry images of kidney graft sections staining CD68 at day 7 post-transplantation. (C) Measurement of pro-inflammatory cytokines (IL-6, MIP-2α, and TNF-α) on day 3 post-transplantation using quantitative RT-PCR. Data were normalized to GAPDH gene expression (*$p < 0.05$, n=4).

To formally test if the inability of PTECs to clear dying cells would lead to increased passive release of HMGB1, we conducted an *in vitro* study where either healthy, apoptotic (UV light-induced) [31] or necrotic (heat-killed) [19] cells were fed to primary PTECs isolated from the kidneys of either KIM-$1^{-/-}$ or KIM-$1^{+/+}$ mice, as previously shown [20], and analyzed the conditioned media for HMGB1 after 24h (Figure 2-4A). Virtually, none to very little HMGB1 was detected by Western blot when healthy cells were fed to PTECs. In contrast, the conditioned medium from KIM-$1^{-/-}$ PTECs fed with apoptotic cells (that underwent secondary necrosis after 24h) had significantly higher relative ratio level of HMGB1 compared to *wild-type* PTECs (3.568 ± 0.7507 vs. 1 ± 0, p= 0.0268; Figure 2-4B). As expected, when necrotic cells were fed to PTECs of either genotype, no significant difference in the level of HMGB1 was detected, since we previously showed that AIM (opsonin) is required for KIM-1-mediated clearance of necrotic cells [18]. Next, we determined if the phagocytic clearance of dying cells by KIM-1 would have any consequences on systemic HMGB1 levels. We performed an enzyme-linked immunosorbent assay to quantify serum HMGB1 *in vivo* post-transplant. Similar to our *in vitro* study, mice transplanted with the KIM-1 deficient kidneys exhibited significantly greater serum HMGB1 on days 1 (50.75 ± 3.473, vs.31.67 ± 3.844, p= 0.0146), 3 (41.33 ± 1.856, vs. 23 ± 3.512, p= 0.0099), and 7 (59.67 ± 9.171, vs. 27.33 ± 4.256, p=0.033; Figure 2-4C) compared to mice transplanted with *wild-type* kidneys.

During ischemic kidney injury, iNos-positive proinflammatory (M1) macrophages are recruited into the kidney in the first 1-2 days within injury, whereas arginase-1 and

mannose receptor-positive, (repair) noninflammatory (M2) macrophages predominate at later time points.

Given that previous work demonstrated that HMGB1 can facilitate M1 polarization during acute kidney injury [32], we therefore investigated whether the elevated HMGB1 levels in the KIM-$1^{-/-}$ mice correlated with increased M1 polarization in respective kidney grafts. Interestingly, KIM-1 deficient donor grafts had significantly greater M1: M2 (CD 80: CD 206) ratio compared to the *wild-type* donor grafts on both days 3 (712.1 ± 166.2 vs. 220.7 ± 58.66, p=0.0494) and 7 (260.3 ± 27.42, vs. 158.3 ± 21.43, p=0.0428; Figure 2-4D) following transplantation.

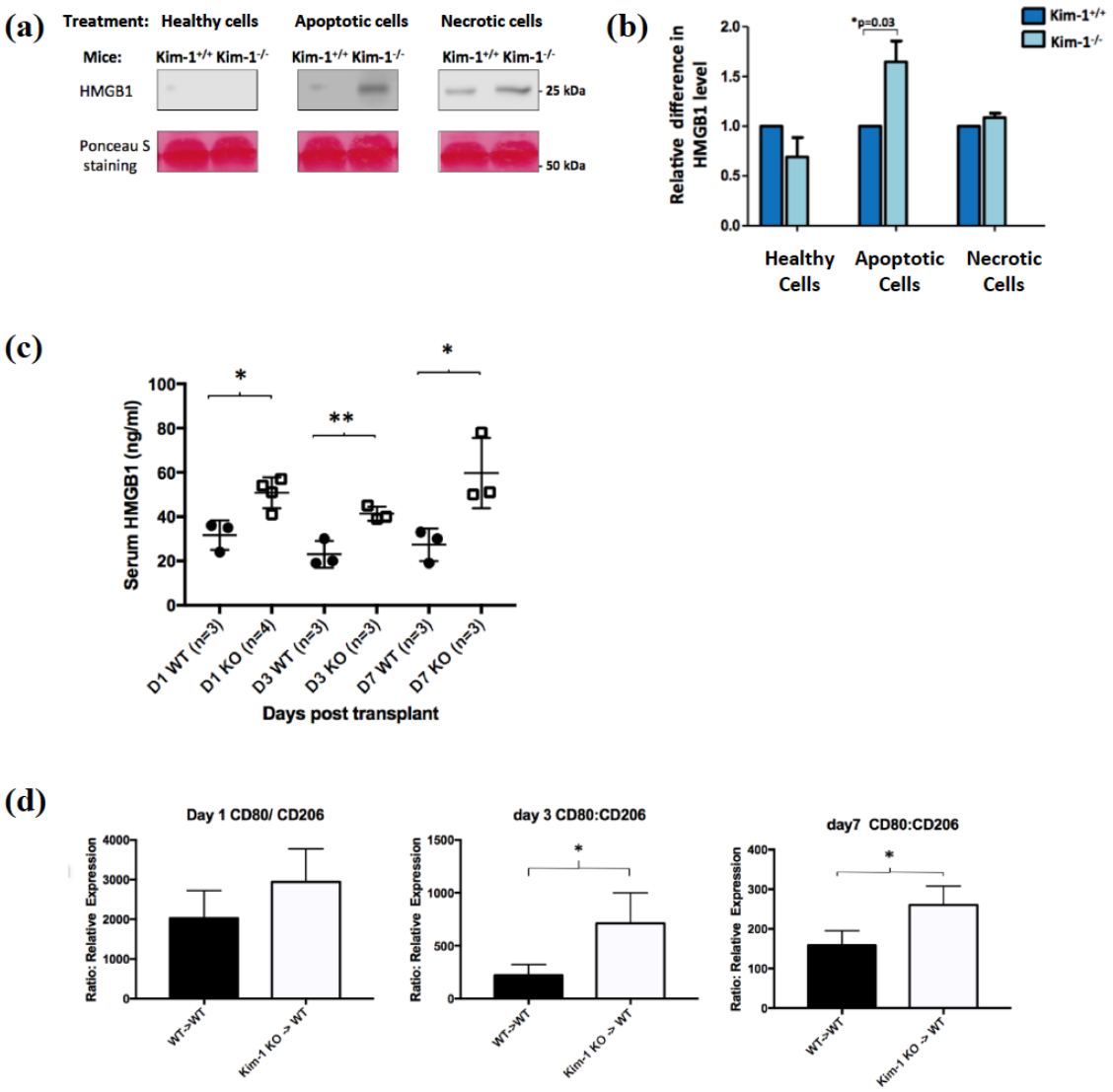
(a)
Treatment: Healthy cells
Apoptotic cells
Necrotic cells
Mice: Kim-1+/+ Kim-1-/-
HMGB1
Ponceau S staining
25 kDa
50 kDa
(b)
Kim-1+/+
Kim-1-/-
*p=0.03
Relative difference in HMGB1 level
Healthy Cells
Apoptotic Cells
Necrotic Cells
(c)
Serum HMGB1 (ng/ml)
D1 WT (n=3)
D1 KO (n=4)
D3 WT (n=3)
D3 KO (n=3)
D7 WT (n=3)
D7 KO (n=3)
Days post transplant
(d)
Day 1 CD80/ CD206
day 3 CD80:CD206
day7 CD80:CD206
Ratio: Relative Expression
WT->WT
Kim-1 KO -> WT

Figure 2-4. Absence of KIM-1 in the donor kidney leads to increased release of HMGB1.

(A-B) PTECs isolated from either KIM-1 deficient or WT mice were fed either thymocytes that were healthy, apoptotic, or necrotic. After 24 hours, conditioned media was collected and HMGB1 release was measured. (A) HMGB1 release was confirmed in the conditioned media using Western blots. (B) Relative quantification of Western blots (*$p < 0.05$, n=3). (C) In vivo serum HMGB1 was quantified using an ELISA kit at days 1, 3 and 7 days post-transplantation (*$p < 0.05$, **$p < 0.01$, n=3-4). (D) M1: M2 macrophage ratio using markers CD80 and CD206 respectively, on days 1, 3 and 7 post-transplantation (*$p < 0.05$, **$p < 0.001$, n=3).

Renal replacement therapy is absolutely needed for patients with end-stage renal disease, and renal transplantation is considered the best treatment [1]. However, the lifespan of the kidney grafts is limited as they are subjected to persistent inflammation and fibrosis due to repeated injury [1]. Graft inflammation can impair the induction of transplant tolerance and enhances acute and chronic rejection [12]. Emerging data suggest that necroinflammation triggered by cIRI may sustain graft inflammation via activation of innate and adaptive (and alloimmune) immune pathways [33, 34]. DAMPs, specifically HMGB1, have been shown to be a key mediator of this process [9, 14, 35].

HMGB1, an endogenous nuclear factor normally contained within the nucleus but released when a cell undergoes necrosis or secondary necrosis, has been implicated in the pathogenesis of IRI by stimulating TLR4 signaling. Through TLR4 signaling, HMGB1 has been reported to have a proinflammatory role during IRI, leading to organ damage including the kidneys [9].

Kidney Injury Molecule-1 (KIM-1) is a type 1 transmembrane glycoprotein that is specifically upregulated on the apical membrane of renal proximal tubular epithelial cells (PTECs) following AKI [16]. Given our previous work demonstrated that KIM-1 upregulation on PTECs is required for clearing apoptotic or necrotic cells during acute injury [18, 19], this study has uncovered a fundamental role for donor KIM-1-mediated efferocytosis in inhibiting local and systemic necroinflammation.

In summary, we were the first to elucidate the protective role of donor KIM-1 in mitigating tissue damage and cell death in post-renal transplant in a life-saving syngeneic murine model. Since delayed graft function is a risk factor for rejection and poor long-

term graft survival [3, 36], we provide the first evidence to support the use of therapeutic strategies to enhance the clearance of apoptotic and necrotic cells [18], or HMGB1 signaling [35], during renal transplantation to improve overall graft survival.

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Chapter 3

3 Recombinant apoptosis inhibitor of macrophage protein reduces delayed graft function in a murine model of kidney transplantation

This chapter is currently under review:

Lee JY, Lian D, Haig AR, Suri RS, Miyazaki T and Gunaratnam L. Recombinant apoptosis inhibitor of macrophage protein reduces delayed graft function in a murine model of kidney transplantation. Submitted to PLOS ONE. Manuscript #: PONE-D-20-27992

3.1 Introduction

Renal ischemia reperfusion injury (IRI) is an unavoidable consequence of renal transplantation. While most grafts will recover from IRI, prolonged cold and warm ischemia may lead to delayed recovery and impaired graft function, with clinically important consequences. Delayed graft function (DGF), defined as need for dialysis within 7 days of renal transplantation, affects 20-50% of deceased donor grafts (DCD) [1]. DGF not only results in acute morbidity and increased cost, but is increasingly recognized as a risk factor for rejection and long-term graft loss [2, 3]. As prolonged ischemia times are often unavoidable, especially with donation after cardiac death, a better understanding of the mechanisms leading to tissue damage during IRI is needed in order to develop effective therapeutic agents.

During renal IRI, ATP depletion and reactive oxygen species production leads to apoptosis and/or, more predominantly, necrosis of the renal tubular epithelial cells (TECs) [4-7]. To add insult to injury, dying cells lose membrane integrity, releasing intracellular damage associated molecular patterns (DAMPs) into the extracellular milieu, which trigger inflammation and promote further cell death. This positive-feedback loop of inflammation and cell death – or necroinflammation – exacerbates tissue damage [8] and potentiates both alloimmune - and non-alloimmune injury [7-9]. Although kidneys have marked regenerative capacity, such unregulated inflammation and/or severe injury can lead to irreversible allograft fibrosis [10, 11]. Thus, positing therapies that target enhancing clearance of dying cells which would dampen the release of danger signals

would break the feedback loop and may lead to protection against both graft damage from IRI and alloimmune injury.

Kidney Injury Molecule-1 (KIM-1) is an endogenous transmembrane glycoprotein transiently upregulated on proximal TECs during renal injury [12]. KIM-1 upregulation on renal TECs during injury transforms them into semi-professional phagocytes capable of clearing apoptotic cells (efferocytosis) [13], via direct binding of KIM-1 to phosphatidylserine on apoptotic cells. Importantly, efferocytosis by proximal TECs is solely dependent on KIM-1 [14]. We have previously reported that compared to *wild-type* mice, KIM-1 deficient or KIM-1 mutant (KIM-1 Δ mucin) mice exhibited greater renal tissue damage, inflammation and mortality when their native kidneys were subjected to warm IRI [14, 15]. In a different study, when we exposed donor kidneys from KIM-1 deficient to both warm and cold ischemia and then transplanted them into *wild-type* mice. We observed greater renal and systemic inflammation as well as increased susceptibility to renal dysfunction and tissue damage, compared to transplanted kidneys from *wild-type* donors [16]. Taken together, these studies suggest that KIM-1 plays a protective role in mitigating tissue damage by inhibiting necroinflammation [16].

While KIM-1 expression confers TECs with the capacity for direct phagocytosis of apoptotic cells, clearance of necrotic cells by KIM-1 expressing TECs is enhanced through the opsonization by the serum protein, apoptosis inhibitor of macrophage (AIM), also known as CD5L [17]. AIM is produced by macrophages to support their survival and circulates in the blood at high concentrations [18, 19]. Previous studies have delineated the role of AIM in several conditions [18, 20]. At steady state, AIM is bound to the much larger circulating IgM pentamer complexes, effectively preventing

trafficking into the renal tissue architecture [17, 19-21]. However, during acute kidney injury, AIM undergoes cleavage and dissociates from IgM pentamers through an unknown mechanism [17, 19]. It is then filtered by the glomerulus and accumulates on necrotic debris within renal tubules in both humans and in mice [17, 19, 21]. We have previously demonstrated that, during moderate warm IRI of native kidneys, filtered AIM accelerated renal recovery and improved mice survival in a KIM-1 dependent fashion [17]. However, the anti-inflammatory and therapeutic effects of rAIM in renal transplantation has not been studied.

Although KIM-1 expression is essential in the protection of sustained kidney damage and function following renal transplant IRI, early kidney pathology and dysfunction was still evident (Chapter 2) [16]. To this end, we tested whether administration of recombinant AIM can be used as a potential therapeutic agent to further boost the protective effect of KIM-1 and ultimately improve renal recovery after transplantation of kidneys exposed to severe warm and cold ischemia. If found to be beneficial, exogenous AIM administration could potentially be used to reduce incidence of DGF after transplantation of donor kidneys which have inevitably been exposed to prolonged ischemia, thus improving long-term graft outcomes in humans. We hypothesized that administration of exogenous recombinant AIM would augment KIM-1-mediated clearance of necrotic cells, mitigating necroinflammation, tissue damage, and renal dysfunction after transplantation of kidneys exposed to severe IRI.

3.2 Materials and Methods

3.2.1 Renal transplantation/ AIM administration

We performed single kidney transplants of male C57BL/6 (B6) mice kidneys into male B6 recipients following bilateral nephrectomy [22, 23]. Donor kidneys were exposed to approximately 60 min of warm ischemia and 35 min of cold ischemia during each procedure. Following kidney transplantation, recipients were administered a single 200 μl dose of either mouse rAIM (2mg/ml), or an equal volume of PBS intravenously via the tail vein. Dosage of rAIM was previously determined [17]. Murine rAIM was generated as previously described and provided by Dr. Miyazaki [17, 21]. All recipient mice were viable at 2 days post-transplantation at which point they were euthanized, and graft tissue and serum were collected. The primary outcome was renal function at 2 days and was quantified by serum creatinine as previously described [16]. We assessed tissue damage and local inflammation by histology; pro-inflammatory cytokine (IL-6, MIP-2α, IL-1α) expression in the graft; and systemic inflammation by serum HMGBI.

B6 mice were obtained from the Charles Rivers Laboratory. All animal procedures were pre-approved by Western University animal use subcommittee in accordance with the regulations of the Canadian Council on Animal Care.

3.2.2 Histology/ immunohistochemistry

A renal pathologist, blinded to the treatment groups, scored the degree of tubular necrosis and tubular obstruction on tissue H&E sections using a previously described semi-

quantitative method as follows: 0 = 0%, 1 = <10%, 2 = 11-25%, 3 = 26-45%, 4 = 46-75%, 5 = >75% [14]. Immunohistochemistry was performed on sections to visualize apoptotic cells using anti-cleaved caspase-3, infiltration of neutrophils using anti-myeloperoxidase antibody, and macrophages using anti-CD68 antibody. All antibodies were obtained from Abcam, Cambridge, MA. Quantification was done by counting and averaging from 5 different fields at 400x magnification for each section.

3.2.3 Inflammatory markers

Tissue sections were collected in TriPure isolation reagent (Roche Diagnostic, Basel, Switzerland) and total RNA extracted. qSCRIPT cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) was used to generate cDNA. Real-time polymerase chain reaction (RT-PCR) was completed using StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA) and SYBR Green (Thermo Fisher Scientific, Rockford, IL) detection was used to quantify relative expression. Primers (Integrated DNA Technologies, Coralville, IA) used were: IL-6: F-5'-TACTCCTTCCTACCCCA ATTTCC-3' R- 5'- TTGGTCCTTAGCCACTCCTTC-3'; MIP-2α: F-5'-CAAAGGCA AGGCTAACTGACC-3' R- 5'ACATCAGGTACGATC CAGG C-3'; and IL-1β: F-5'-ACCTAGCTGTCAACGTGTGG -3' R-5' TCAAAGCAATGTGCTGGTGC-3'. We used GAPDH: F- 5' TCAGCATCTCTAAGCGTGGT-3' R-5'-ATGTTGTCTTC AGCTCGGGA-3'as an internal control.

Serum HMGB1 was quantitatively determined using Sandwich-enzyme immunoassay kit in accordance with its protocol (Shino-Test Corporation, Tokyo, Japan). Multiskan GO (Thermo Fisher Scientific, Rockford, IL) was used for quantification.

3.2.4 Cell cultures

Human Embryonic Kidney 293 (HEK 293) cells and mouse kidney (Renca) cells were obtained from America Type Culture Collection (ATCC, Manassas, VA) and cultured at 37°C in 5% (vol/vol) CO_2 incubator. HEK 293 cells stably expressing human KIM-1 pcDNA (HEK293 -pcDNA) were generated by transfecting with plasmid construct encoding human KIM-1 using Lipofectamine® 2000 (Life technologies. Thermo Fisher Scientific, Rockford, IL). Stable cell lines were maintained with geneticin (G418) sulfate (Santa Cruz Biotechnology, Santa Cruz, CA) supplemented in DMEM (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen, Carlsbad, CA), and 1% Penicillin-streptomycin (Invitrogen, Carlsbad, CA). Renca cells stably expressing mouse KIM-1 were generated by transducing with Lenti ORF particles, Havcr1 (Myc-DDK-tagged) or LentiORF control particles (OriGene Technologies, Rockville, MD). These stable cell lines were maintained with puromycin dihydrochloride (Sigma-Aldrich) and cultured in RPMI-1640 medium containing 10% FBS, 5% PS, 0.1mM non-essential amino acids (ThermoFisher Scientific, Waltham, MA), 1 mM sodium pyruvate (ThermoFisher Scientific), and 2 mM L-glutamine (ThermoFisher Scientific). Hallmark appearance of these cells was confirmed by visual analysis.

3.2.5 Phagocytosis assay/ Flow cytometry

We collected thymocytes from B6 mice and heat-killed them by incubating the cells at 65°C for 20 minutes to induce necrosis. Necrotic thymocytes were verified by flow cytometry analysis showing double positive staining for propidium iodide (Biolegend,

San Diego, CA) and annexin V (Biolegend, San Diego, CA). Necrotic thymocytes were labeled with human rAIM (100 μg/ml) (R&D systems, Minneapolis, MN) at 37°C for 1 hour, or nothing. We then performed phagocytosis assay as previously described [17]. Briefly, approximately $1x10^6$ HEK 293 cells were plated and fed $3x10^6$ necrotic thymocytes with or without human rAIM, or no thymocytes (control). After incubation for 90 minutes at 37°C in 5% CO_2 incubator, cells were placed on ice to reduce non-specific binding for 30 minutes, washed 3 times with ice-cold PBS and harvested. The % phagocytosis, which represents the number of tubular cells that have phagocytosed the necrotic cells, was analyzed using BD LSR II flow cytometer (BD Biosciences, San Jose, CA). Similar procedure was performed using mouse Renca cells and mouse rAIM.

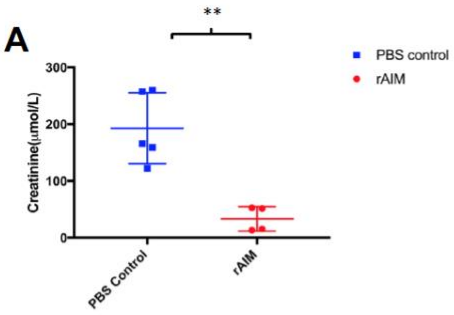
3.2.6 Statistics

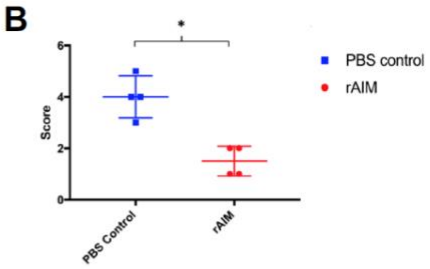
Continuous variables (creatinine, inflammatory markers, % phagocytosis) and tissue injury scores were compared between groups using one-way ANOVA/ Student's t- and Mann-Whitney U tests, respectively. All analyses were performed with GraphPad Prism (GraphPad Software Inc., La Jolla, CA). All data are presented as means ± SEM; p-values < 0.05 were considered statistically significant without adjustment for multiple comparisons.

3.3 Results

3.3.1 Renal Function and Tissue Damage

Recipient mice who received exogenous rAIM had significantly lower serum creatinine at 2 days following renal transplantation compared to those who received PBS controls (33.29 ± 10.83 vs. 192.7 ± 27.86 μmol/L, p=0.0019; Figure 3-1A). The rAIM treated mice also exhibited significantly less tubular necrosis (1.5 vs. 3.5, p=0.0286; Figure 3-1B), less tubular obstruction (1.5 vs. 3.5, p=0.0286; Figure 3-1C and 3-1D), and less apoptotic tubular cell death compared to PBS treated mice (2.5 ± 0.866 vs. 6 ± 0.7071, p=0.0203; Figure 3-1E and 3-1F).


A
**
PBS control
rAIM
Creatinine(μmol/L)
300
200
100
0
PBS Control
rAIM


B
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PBS control
rAIM
Score
6
4
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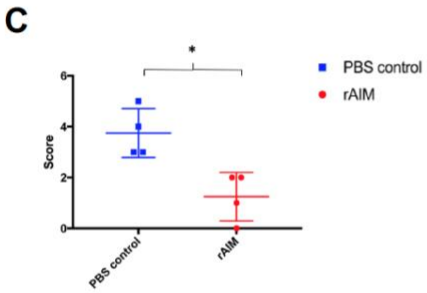
PBS Control
rAIM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rAIM
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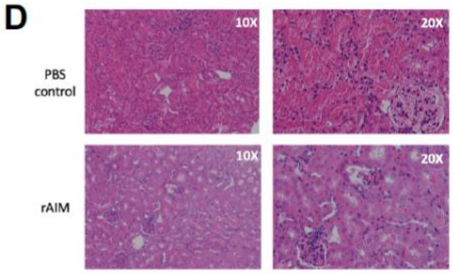
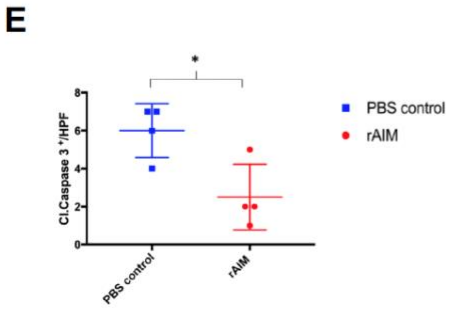
rAIM


D
PBS control
rAIM
10X
20X
10X
20X


E
*
PBS control
rAIM
Cl.Caspase 3 +/HPF
8
6
4
2
0
PBS control
rAIM

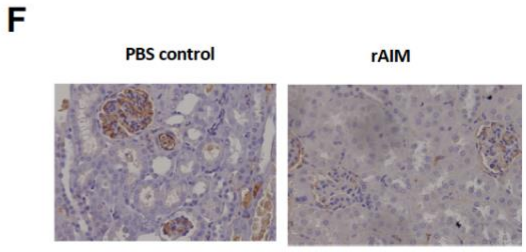

F
PBS control
rAIM

Figure 3-1. Administration of recombinant AIM improves graft function and mitigates graft damage.

Donor kidneys were transplanted into B6 wild-type recipients. Following transplantation, recipients were injected I.V. with either 200 μl of rAIM (2mg/ml) or PBS. On day 2 post-transplantation, mice were euthanized and (A) Serum creatinine was measured as a marker of renal function (B-D) Formalin-fixed tissue sections were stained with H&E and were scored in a blinded fashion. Scoring system: 0 = none, 1= <10%, 2 =11-25%, 3 =26-45%, 4 =46-75%, and 5 = >75%. (B) ATN score, (C) tubular obstruction score (D) H&E sections (E) early apoptotic tubular cells were quantified. Positive staining for apoptotic cells were counted and averaged from 5 different fields at 400x magnification for each section (F) Images of H&E -stained tissue sections. *$p<0.05$, **$<p<0.01$, n=4~5/group.

3.3.2 Local and Systemic Inflammation

Transplanted kidneys from mice treated with rAIM exhibited significantly less macrophage infiltration (2.25 ± 1.315 vs. 10.75 ± 1.493, p=0.0052; Figure 3-2A and 3-2B) and granulocyte infiltration (3.5 ± 1.041 vs. 12 ± 2.041, p=0.01; Figure 3-2C and 3-2D) compared to those from PBS treated mice. Transcript analysis of transplanted kidneys revealed that grafts from rAIM treated mice had significantly less expression of pro-inflammatory genes: IL-6 (1.478 ± 0.3042 vs. 25.61 ± 8.566, p=0.0306), MIP-2α (19.79 ± 4.45 vs. 158.8 ± 57.65, p=0.0352), and IL-1β compared to grafts from PBS treated controls (39.71 ± 7.822 vs. 142.1 ±34.11, p=0.0264; Figure 3-2E). We also observed significantly decreased serum levels of HMGB1 in rAIM-treated mice compared to PBS-treated mice (22.75 ± 6.098 vs. 40.48 ± 4.564 ng/ml, p=0.0450; Figure 3-2F).

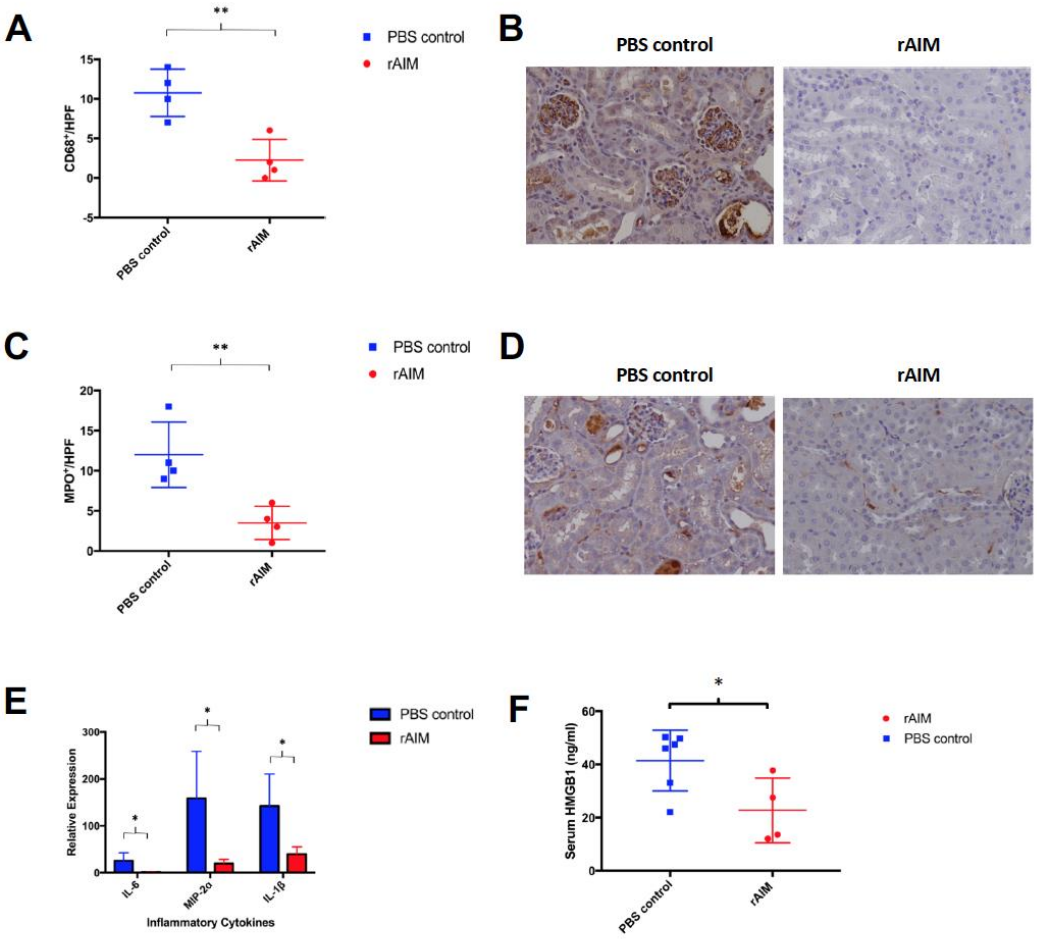

A
**
CD68+/HPF
15
10
5
0
-5
PBS control
rAIM
PBS control
rAIM
B
PBS control
rAIM
C
**
MPO+/HPF
20
15
10
5
0
PBS control
rAIM
PBS control
rAIM
D
PBS control
rAIM
E
*
*
*
Relative Expression
300
200
100
0
IL-6
MIP-2α
IL-1β
Inflammatory Cytokines
PBS control
rAIM
F
*
Serum HMGB1 (ng/ml)
60
40
20
0
PBS control
rAIM
rAIM
PBS control

Figure 3-2. Recombinant AIM administration alleviates local tissue and systemic inflammation following syngeneic renal transplantation.

(A-D) Kidney tissue sections were stained for CD68 or MPO to detect graft-infiltrating macrophages and granulocytes, respectively. (A) Number of $CD68^+$ macrophages/HPF. (B) Immunohistochemistry images of kidney graft sections staining CD68. (C) Number of MPO^+ granulocytes/HPF. (D) Immunohistochemistry images of kidney graft sections staining MPO. (E) Measurement of pro-inflammatory cytokines (IL-6, MIP-2α, and IL-1β) using quantitative RT-PCR. Data were normalized to GAPDH gene expression. *$p<0.05$, **$p<0.01$, n=4/group. (F) Serum HMGB1 levels were quantified using ELISA. $p= 0.1$, n=4-6/group.

3.3.3 *In vitro* Phagocytosis

Renca cells stably expressing murine KIM-1 were able to engulf necrotic cells significantly more in the presence of rAIM compared to without rAIM (17.7 ± 0.4726 vs. 12.9 ± 0.781%, p=0.0063; Figure 3-3A). Phagocytosis was found to be KIM-1 dependent as Renca cells transfected with empty vector failed to clear necrotic cells. Immunophenotyping by flow cytometry confirmed that the phagocytic target cells were indeed necrotic cells as they were either double positive for annexin V and PI or single positive for PI (Figure 3-3B). KIM-1 expression by the stably transfected Renca cells was verified using Western blot (Figure 3-3C).

Similarly, in the presence of rAIM, human kidney cells expressing KIM-1 exhibited significantly enhanced phagocytic activity of necrotic cells compared to controls (22.27 ± 0.4333 vs. 13.53 ± 0.7753%, p=0.0006; Figure 3-3D). Analogous to mouse Renca cells, rAIM-mediated phagocytosis of necrotic cells was found to be dependent on KIM-1 expression as non-KIM-1-expressing human kidney cells failed to exhibit increased clearance of necrotic cells in the presence of rAIM. KIM-1 expression on these cells was verified by Western blot (Figure 3-3E).

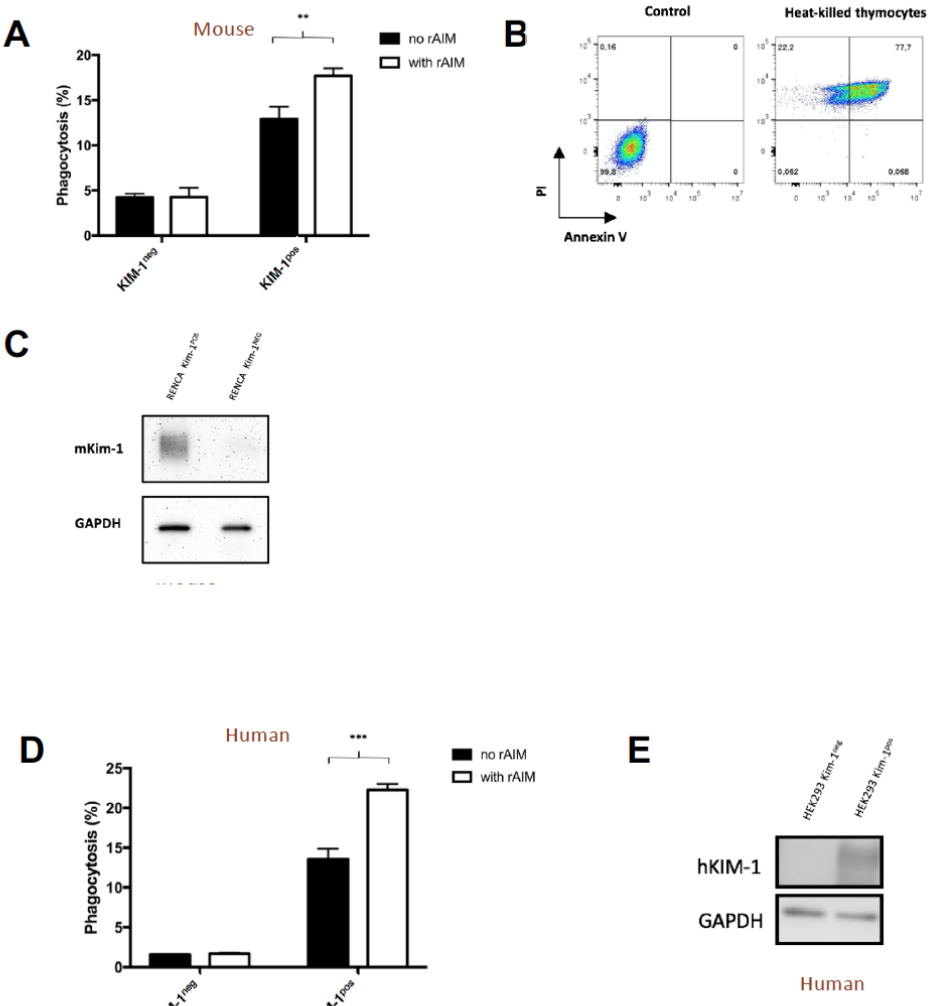

A
Mouse
**
no rAIM
with rAIM
Phagocytosis (%)
20
15
10
5
0
KIM-1neg
KIM-1pos
B
Control
Heat-killed thymocytes
0.16
0
99.8
0
22.2
77.7
0.062
0.068
PI
Annexin V
C
RENCA Kim-1pos
RENCA Kim-1neg
mKim-1
GAPDH
D
Human
***
no rAIM
with rAIM
Phagocytosis (%)
25
20
15
10
5
0
KIM-1neg
KIM-1pos
E
HEK293 Kim-1neg
HEK293 Kim-1pos
hKIM-1
GAPDH
Human

Figure 3-3. Recombinant AIM enhances the phagocytic uptake of necrotic cells by both KIM-1 expressing mouse and human kidney cells.

Renca cells (mouse) or HEK293 (human) expressing KIM-1 or without KIM-1 were fed necrotic thymocytes with or without rAIM (A-C). (A) Renca cells (mouse) after a 90-minute incubation, % phagocytosis was quantified using flow cytometry. (B) Immunotyping of necrotic cell populations. (C) Western Blots were performed confirming the expression of KIM-1 on Renca cells. (D) HEK 293 cells (human) after a 90-minute incubation, % phagocytosis was quantified using flow cytometry. (E) Western Blots were performed confirming the expression of KIM-1 on HEK293 cells. Histograms are representative results of at least 3 independent experiments. All flow cytometry experiments were analyzed using Flowjo X software. **$p<0.01$, ***$p<0.001$.

3.4 Discussion

AIM has been reported to be of therapeutic relevance in various conditions including obesity [18], autoimmune disease [24], and hepatocellular carcinoma [20]. Here, we demonstrate the therapeutic potential of rAIM against transplant-associated IRI using a clinically relevant model of kidney transplantation which incorporates severe forms of both warm and cold ischemia. We found that a single dose of rAIM given to transplanted mice almost completely normalized renal function at 48 h post-transplant and this was accompanied by markedly reduced tissue inflammation and damage compared to controls. Although AIM has been shown to protect against native renal warm IRI previously [17], our study is the first to elucidate the anti-inflammatory effect of AIM in a transplant IRI setting.

As the name suggests, AIM was initially identified as an apoptosis inhibitor which ultimately supports the survival of macrophages [25]. Both humans and mice have relatively high levels of serum AIM (5μg/ml and 2μg/ml, respectively) and similar reported function [19]. With a half-life of 5 days for mice and 6 hours for humans, the majority of circulating AIM is bound to the Fc region of pentameric IgM (>500kDa), where one IgM pentamer houses one AIM. It is in this state where AIM is stabilized and not filtered, albeit inactive. It is likely that the interaction between AIM and IgM-Fc is due to the positively charged domain on the AIM protein being attracted to the negatively charged area in the IgM-Fc [21]. However, during kidney injury, AIM dissociates from IgM-Fc and is then filtered and accumulates on necrotic debris and interacts with KIM-1 thereby enhancing the phagocytic ability of KIM-1 to clear the necrotic debris [17]. The

mechanism as to how AIM dissociates from IgM-Fc is still unknown, but one speculation may be that during kidney injury, altered homeostasis may disturb the charge differences. However, this has yet to be investigated.

The main clinical manifestation of transplant IRI is DGF, resulting in increased morbidity, prolonged length of stay and increased resource utilization [1, 3]. Decreasing the incidence of DGF would thus be of major benefit. This is particularly true in the current era where the use of DCD kidney donors, which is associated with the highest rates of DGF, is approaching 20% in North America [26, 27]. To date, there are no effective therapies to prevent DGF. Thus, if rAIM were to be equally effective in humans as it was in mice, the therapeutic implications are likely to be of great importance.

In addition to the effect of rAIM on graft function, our data also highlighted the potent effect of rAIM administration on curtailing both graft and systemic inflammation (serum HMGB1) caused by excess necrosis in the graft. Based on our previous work [16, 17], as well as data presented in Figure 3-2, we conclude that the anti-inflammatory effect of rAIM is mediated by the enhanced phagocytic clearance of necrotic cell debris within the injured grafts. The ability to limit DAMP release from the damaged tissue may be particularly important in the setting of allogeneic transplantation where extracellular DAMPs have been shown to exacerbate alloimmunity [5, 16, 28]. Our syngeneic transplant model was well-suited to studying the effects of rAIM on transplant-associated IRI, but to test whether rAIM administration will mitigate alloimmune injury to the graft, which can be persistent unlike IRI, is an important question.

In addition to ameliorating inflammation, our work also demonstrated that rAIM treatment decreased dead cell debris within the tubular lumen, which also likely contributed to graft recovery given that tubular obstruction is a major mechanism contributing to decline in glomerular filtration rate during AKI [10]. Finally, our finding that rAIM augmented the phagocytic uptake of necrotic cells by human kidney cells expressing human KIM-1 supports the translation of our work to transplant patients.

In summary, our study is the first to delineate the therapeutic role of recombinant AIM in renal transplantation. Furthermore, our results demonstrate that KIM-1/AIM-mediated clearance of dying cells mitigates graft damage, and inflammation, while improving early graft function. Taken together, our findings suggest that the administration of recombinant AIM may be used as a therapeutic strategy to improve graft outcomes in kidney transplant patients.

3.5 References

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Chapter 4

4 Polymorphisms in *HAVCR1* alter KIM-1 mediated phagocytosis

4.1 Introduction

Renal ischemia reperfusion injury (IRI) occurs when blood flow to the kidney is significantly reduced for a period of time followed by reperfusion. IRI pathology has a two-pronged effect as ischemic conditions limits oxygen uptake resulting in ATP depletion and the build-up of toxic by-products, whereas the restoration of blood flow can produce harmful reactive oxygen species (ROS), leading to tissue damage [1, 2]. Notably, IRI is one of the most common mechanisms of kidney injury in many clinical conditions such as transplantation, sepsis and patients undergoing cardiac bypass surgery [3-5].

The destructive consequence of ATP depletion and ROS production during IRI cause renal tubular epithelial cells (TECs) to be more susceptible to cell death via apoptosis or necrosis [6, 7]. To add insult to injury, membrane integrity is typically compromised in necrotic cells leading to the release of their immunogenic intracellular contents, also known as danger, associated molecular patterns (DAMPs), into the extracellular milieu which further exacerbates inflammation [8, 9]. This in turn can trigger an auto-amplification loop of inflammation and subsequent cell death [10]. Thus, inducing rapid phagocytosis and clearance of these dying cells may suppress inflammation and mitigate kidney injury during IRI [11].

Kidney injury molecule -1 (KIM-1) is a cell-surface glycoprotein which transiently gets expressed on TECs following kidney injury [12]. As a transmembrane protein, KIM-1 has its extracellular portion consisting of Ig-like and mucin domains that binds apoptotic and necrotic cells allowing for the subsequent phagocytosis of neighbouring TECs [13]. KIM-1 binds directly to phosphatidylserine (PS) on apoptotic cells whereas phagocytosis

of necrotic cells requires KIM-1 to interact with another protein that acts as an opsonin, apoptosis inhibitor of macrophage (AIM) [14]. Using murine models, we have previously shown that compared to KIM-1 deficient or KIM-1 mutant (KIM-1 Δ mucin) mice, *wild-type* mice exhibited curtailed inflammation and limited tissue damage during renal transplant and native renal warm IRI [15-17]. In addition, we found that phagocytosis of PS expressing apoptotic cells was attenuated in KIM-1 Δ mucin mice [17]. Therefore, the alteration of the mucin domain or key residues required for PS binding may profoundly affect the biological function of KIM-1.

The KIM-1 gene (*HAVCR1*), which was initially identified as the Hepatitis A Virus Cellular Receptor 1 [18], is highly polymorphic and its variants are associated with differential susceptibility to severe hepatitis A infection, allergy and autoimmune disorders [18-20]. Considering the importance of the mucin domain to the functional capacity of KIM-1, we hypothesized that in human populations, certain high frequency *HAVCR1* variants specific to the coding regions of the mucin domain would be associated with altered phagocytic activity *in vitro*. This study provides novel insight into the potential prognostic risk factors associated with KIM-1 variants and renal transplantation.

4.2 Materials and Methods

4.2.1 Designing of the KIM-1 gene variants

Met158_Pro162del, Thr200del, and Thr207Ala mutations were created using pcDNA3 and the Quickchange lightning mutagenesis kit (Agilent Technologies) following the

manufacturer's directions. Primers were designed using Agilent's primer design program. Mutated plasmids were isolated from bacterial stock using a QIAprep spin miniprep kit following the manufacturer's directions (Qiagen, 27104). Mutations and deletions were confirmed by DNA sequencing at the London Regional Genomics Centre. Sequences were compared to Homo sapiens hepatitis A virus cellular receptor 1 (HAVCR1), transcript variant 1, mRNA NCBI Reference Sequence: NM_012206.3.

Primers used:

Met158_Pro162del	tgttccaacgacaacgactgttccaacgacaactgttc
Met158_Pro162del-antisense	gaacagttgtcgttggaacagtcgttgtcgttggaaca
Del200	cgacaacgagcattccaacaacaagtgttccagtg
Del200-antisense	cactggaacacttgttgttggaatgctcgttgtcg
Thr207Ala	gaacaaaggtagagacagctgttgtcactggaacact
Thr207Ala-antisense	agtgttccagtgacaacagctgtctctacctttgttc

4.2.2 Patients and Donor DNA sample collection

Total of 627 archived donor DNA samples from the histocompatibility laboratory at London Health Research Centre (London, ON, CA) for all consecutive recipients of deceased donor kidney transplants from 2008 to 2018 were collected. After verifying DNA quality, Sanger sequencing and fragment length analysis were performed for the

HAVCR1 variants, and genotype and allele frequencies were calculated by the members of the Hegele Lab. Donor DNA was extracted from peripheral blood mononuclear cells or splenocytes at the time of transplantation for HLA typing. Permission for use the leftover DNA samples for this study was granted by the research ethics board of the University of Western Ontario and Lawson Health Research Institute Research Ethics Committee of the UMC.

4.2.3 Transfection of KIM-1 variants/ Cell cultures

Human Embryonic Kidney 293 (HEK293) cells were obtained from America Type Culture Collection (ATCC, Manassas, VA) and cultured at 37°C in 5% (vol/vol) CO_2 incubator. HEK293 cells stably expressing *wild-type* human KIM-1 pcDNA (HEK293 - pcDNA) were generated by transfecting with plasmid construct encoding human KIM-1 using Lipofectamine® 2000 (Life technologies. Thermo Fisher Scientific, Rockford, IL), along with the 3 human KIM-1 variants described above. Stable cell lines were maintained with geneticin (G418) sulfate (Santa Cruz Biotechnology, Santa Cruz, CA) supplemented in DMEM (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen, Carlsbad, CA), and 1% Penicillin-streptomycin (Invitrogen, Carlsbad, CA). Hallmark appearance of these cells was confirmed by visual analysis.

4.2.4 KIM-1 surface staining/ Flow cytometry/ Immunofluorescence

Cells expressing human *wild-type* KIM-1 and the 3 variants were grown in media and then harvested and stained with florescent anti-human KIM-1 antibody (AKG α-hKIM-

1). They were then analyzed using BD LSR II flow cytometer (BD Biosciences, San Jose, CA) for mean florescent intensity (MFI) to detect KIM-1 cell surface staining. For immunofluorescence, cells were grown in normal media on cover slips then fixed with 2% paraformaldehyde. Cells were then incubated overnight with AKG α-hKIM-1 monoclonal primary antibody at 4°C followed by secondary incubation with fluorochrome-conjugated Alexa-Fluor 488® goat anti-mouse secondary antibody for an hour at room temperature. All images were analyzed using a FLUOVIEW X831 confocal microscopy (Olympus, Tokyo, Japan).

4.2.5 Phagocytosis assay/ Flow cytometry

Thymocytes were collected from 3-6-week-old C57BL/6 mice. Apoptosis was induced by exposure to UV light for 7 minutes followed by overnight incubation in DMEM media containing 1% Penicillin-streptomycin and 10% FBS at 37°C in 5% (vol/vol) CO_2. Apoptotic thymocytes were verified by flow cytometry analysis showing single positive staining for Annexin V (Biolegend, San Diego, CA) but negative for propidium iodide (Biolegend, San Diego, CA). Apoptotic thymocytes were labeled with pHrodo, and phagocytic activity was assessed as previously described [14]. Briefly, $1x10^6$ HEK293 cells, with differential KIM-1 variant expression, were in co-culture with $3x10^6$ pHrodo-labelled apoptotic thymocytes. After incubation for 90 minutes at 37°C at 5% CO_2, cells were placed on ice to reduce non-specific binding for 30 minutes and harvested. The % phagocytosis, which represents the number of tubular cells that have phagocytosed the apoptotic thymocytes, was analyzed using BD LSR II flow cytometer (BD Biosciences, San Jose, CA).

4.2.6 Western blot

KIM-1 variants' cell lysates were stored in 4% SDS. Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL) was used whereby isolated proteins from whole tissue sections were quantified. We incubated the blots with human KIM-1 (AKG) and mouse monoclonal GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Western blots were developed and quantified using the FluorChem M system (ProteinSimple, San Jose, CA).

4.2.7 Statistics

Percent phagocytosis and MFI (KIM-1 surface expression) were compared between groups using one-way ANOVA. All analyses were performed with GraphPad Prism (GraphPad Software Inc., La Jolla, CA). All data are presented as means ± SEM; p-values < 0.05 were considered statistically significant without adjustment for multiple comparisons.

4.3 Results

4.3.1 KIM-1 polymorphisms in the coding region have high allele frequencies

Polymorphisms occurring in the coding region of a gene can either change the amino acid sequence (nonsynonymous), or be silent (synonymous). Thus, we selected 3 known KIM-1 nonsynonymous variants that are of known to have high allele frequencies in a global population. Total of 627 donor DNA samples of recipients of deceased donor kidney transplants at London Health Research Centre was obtained. The genotype and allele frequencies of the 3 variants of our sample population were calculated which showed striking similarities to the global population frequencies reported in GeneCards (Table 4-1).

Table 4-1. Genotype and allele frequencies of 3 KIM-1 coding variants. Table of distribution of genotype and allele frequencies of KIM-1 variants from 627 patients.

Polymorphism rs number	Alleles	Genotype frequencies (% of absolute frequency)	Allele Frequencies	
			Ref allele	Alt allele
6149307	CATTGGAACAGTCGT/-	+/+: 15.789 +/-: 45.614 -/-: 38.596	0.386	0.614
45439103	TTG/-	+/+:64.753 +/-: 30.941 -/-: 4.306	0.802	0.198
12522248	T/C	+/+: 56.938 +/-: 32.855 -/-: 10.207	0.734	0.266

+/+: normal (no nucleotide changes/ deletion), +/-: WT/del or WT/nucleotide change (heterozygous), -/-: del/del or nucleotide change/nucleotide change (homozygous).

We then generated constructs for these coding variants through site-directed mutagenesis (Figure 4-1A and 4-1B). The 3 KIM-1 variants that we selected are as follows: KIM-1-1Met158-Pro162del (deletion of methionine to proline at positions 158-162); KIM-1-Thr200del (deletion of threonine at position 200); and KIM-1Thr207Ala (substitution of threonine with alanine at position 207) (Figure 4-1A). All of these polymorphisms are located in the extracellular mucin domain of KIM-1 which is known to have an important role in phagocytosis (Figure 4-1B).

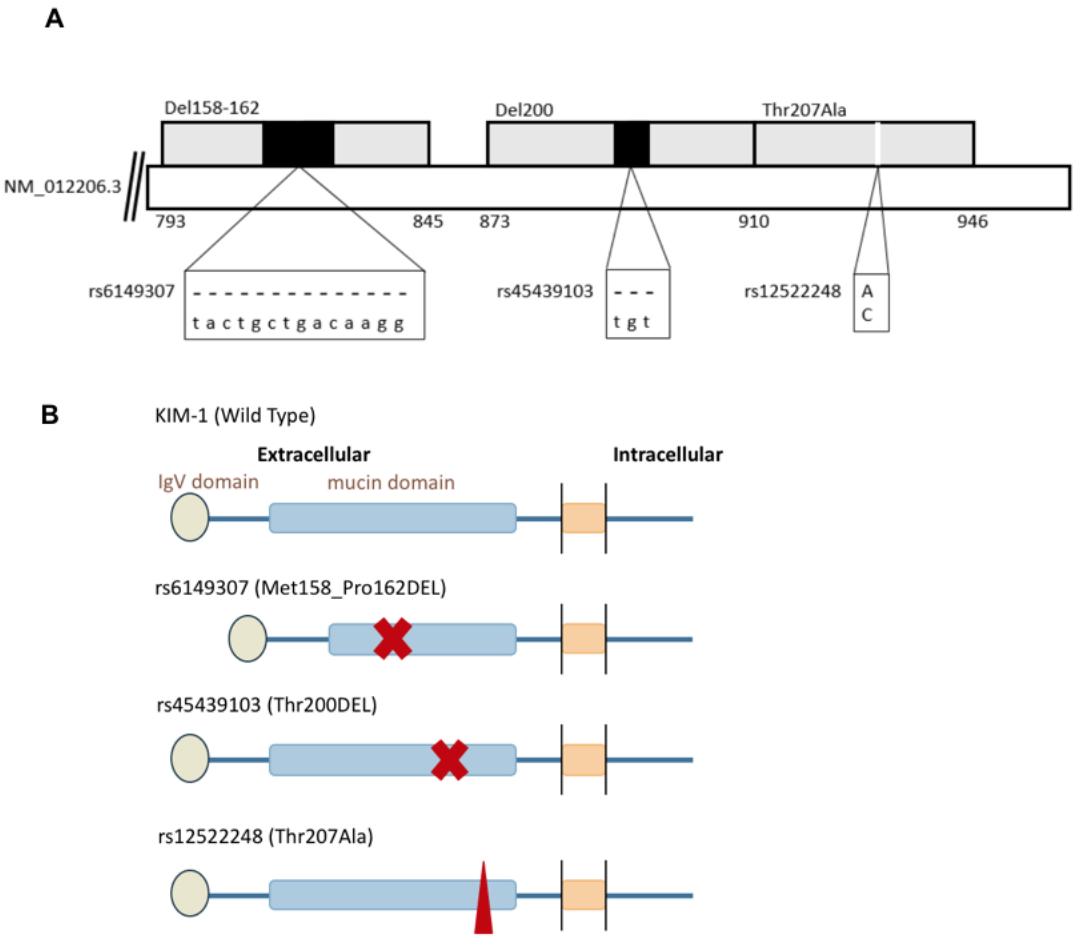
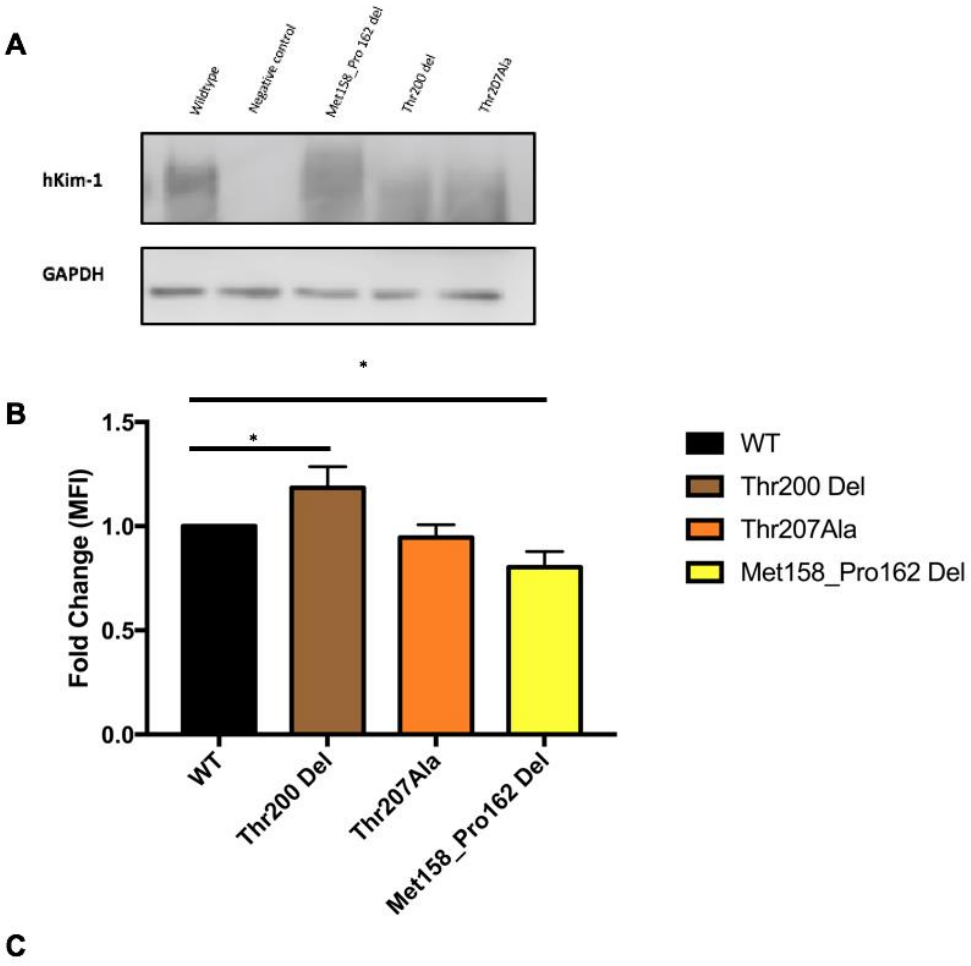


Figure 4-1. Generation of nonsynonymous KIM-1 variants.

(A) Diagram confirming the genotypes of the site-directed mutants corresponding to 3 nonsynonymous (coding) KIM-1- variants (KIM-1Met15_Pro162 del: deletion of methionine to proline at positions 158-162; KIM-1-Thr200 del: deletion of threonine at position 200; KIM-1-Thr207Ala: substitution of threonine with alanine at position 207). (B) Schematic showing the phenotypic changes of the 3 (coding) nonsynonymous KIM-1 variants relative to *wild-type*.

4.3.2 KIM-1 variants have altered cell surface KIM-1 expression

All 3 of the constructed KIM-1 variants expressed KIM-1 protein as efficiently compared to the *wild-type* (Figure 4-2A). However, the 3 variants showed variable cell surface KIM-1 expression *in vitro* (Figure 4-2B and 4-2C). Interestingly, HEK293 cells transfected with KIM-1-Thr200del and Met158_Pro162del exhibited significant upregulation and reduced expression of surface KIM-1, respectively, when compared to the *wild-type*, whereas KIM-1-Thr207Ala showed comparable levels of KIM-1 cell surface expression with the *wild-type* (Figure 4-2B). To further support our finding, fluorescent microscope images stained for KIM-1 also showed congruent results (Figure 4-2C) as our flow cytometry data.


A
Wildtype
Negative control
Met158_Pro 162 del
Thr200 del
Thr207Ala
hKim-1
GAPDH
B
*
*
Fold Change (MFI)
1.5
1.0
0.5
0.0
WT
Thr200 Del
Thr207Ala
Met158_Pro162 Del
WT
Thr200 Del
Thr207Ala
Met158_Pro162 Del

C

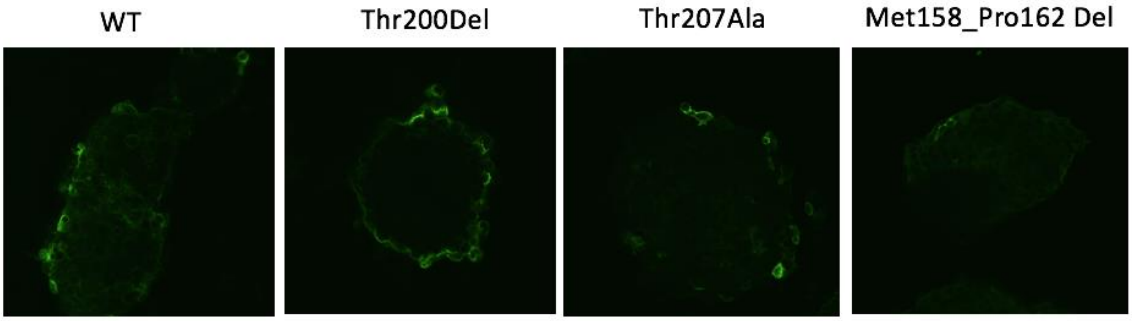

WT
Thr200Del
Thr207Ala
Met158_Pro162 Del

Figure 4-2. KIM-1 variants have altered cell surface KIM-1 expression.

Functional analysis nonsynonymous KIM-1 variants. KIM-1 *wild-type* (WT) and KIM-1 variants (Thr200 del, Thr207Ala and Met158_Pro162Del) were expressed by transfection in human embryonic kidney cells (HEK293). (A) Expression of KIM-1 protein in total cell lysates by Western blot. (B) Expression of KIM-1 protein at the cell-surface by fluorescently-conjugated human KIM-1 antibody via flow cytometry. Histograms are representative results of at least 3 independent experiments. All flow cytometry experiments were run on cytoFLEX cytometer and analyzed using Flowjo X software. *$p<0.05$ (C) Fluorescent microscopic images of HEK293 cells transfected with KIM-1 variants positively stained for KIM-1.

4.3.3 KIM-1 variants have lower phagocytic capability

The functional capacity of our KIM-1 variants was assessed. Interestingly, we found that all 3 coding variants exhibited significantly reduced phagocytic uptake of apoptotic cells compared to the *wild-type in vitro* (Figure 4-3). Specifically, HEK293 cells transfected with either Thr200del, Thr207Ala, or Met158_pro162del KIM-1 variants exhibited 25%, 57% and 10%, respectively, of the phagocytic ability of wild-type KIM-1. The corollary to this finding would suggest that these *HAVCR1* variants would significantly impair the protective function of KIM-1 in IRI.

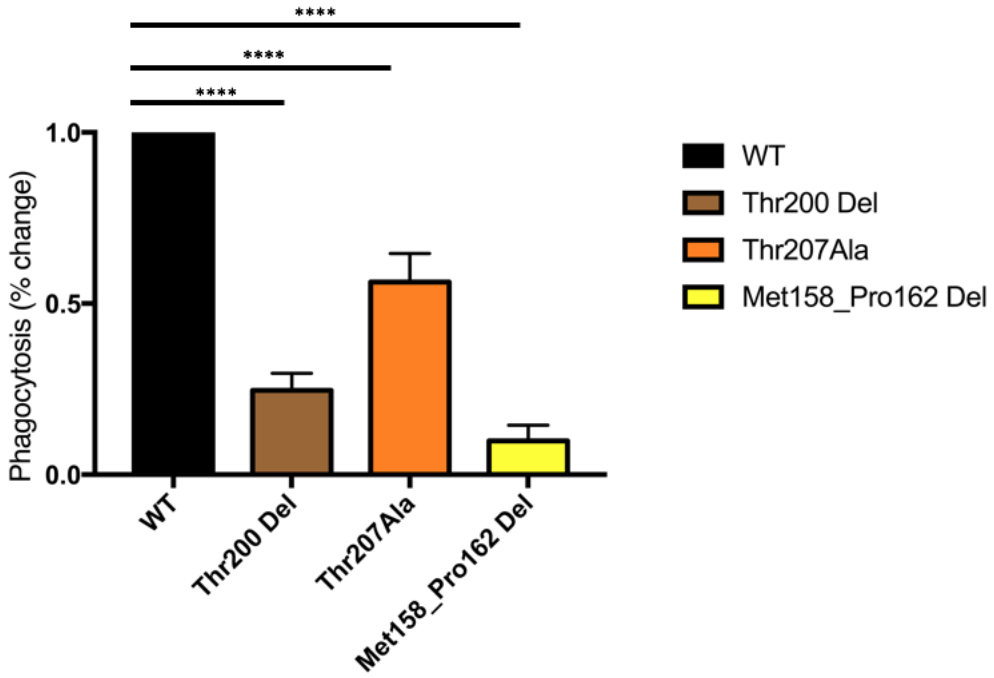


Figure 4-3. KIM-1 variants have lower phagocytic capability.

HEK293 cells transfected with nonsynonymous KIM-1 variants. KIM-1 *wild-type* (WT) and KIM-1 variants (Thr200 del, Thr207Ala and Met158_Pro162Del) were fed apoptotic thymocytes. After 90-minute incubation, % change in phagocytosis (relative phagocytosis of fluorescently labelled apoptotic cells) was quantified by flow cytometry. Histograms are representative results of 6 independent experiments. All flow cytometry experiments were run on cytoFLEX cytometer and analyzed using Flowjo X software. ****$p<0.0001$.

4.4 Discussion

Renal tubular epithelial cells upregulate the surface expression of KIM-1 upon injury which is then shed into the urine allowing for its detection as a biomarker for tissue damage [21]. Indeed, KIM-1 has been reported to be used as a biomarker for kidney injury and renal cell carcinoma [22, 23]. Additionally, KIM-1 is a receptor for phosphatidylserine which allows the phagocytosis of neighbouring dying cells by the KIM-1 expressing TECs which mitigates subsequent necroinflammation. Previous studies have shown the protective role of KIM-1 against renal ischemia reperfusion injury using various injury models [16, 17].

The KIM-1 gene (*HAVCR1*) is subject to high degrees of polymorphism, whereby *HAVCR1* variants have been linked with several clinical diseases including, hepatitis A infections, and a diverse range of autoimmune disorders [18-20, 24]. KIM-1 has also been shown to play an important role in renal transplantation, where its expression is readily upregulated in donor kidneys upon engraftment, which is indicative of injury [25, 26]. Ischemia reperfusion injury is one of the major clinical risk factors that affect the quality and longevity of donor kidneys [1, 27]. We have previously shown that KIM-1 expression in the donor kidney protects against renal transplant ischemia reperfusion injury using a mice model [15]. Thus, our question was whether mutations in the KIM-1 gene would alter its functional ability. Here we found that the 3 high frequency coding variants induced differential cell surface KIM-1 expression and universally decreased phagocytosis compared to the *wild-type*. Interestingly, only the Thr200Del variant exhibited increased cell surface KIM-1 expression, however its phagocytic ability was

still significantly reduced. It is possible that mutations in the mucin domain of KIM-1 allows for the binding of PS-expressing apoptotic cells but prevents the internalization and subsequent clearance. Future investigations into the specific mechanisms that would elucidate the diminished functional capacity of KIM-1 variants are warranted. Although our data highlights the association between genetic variation and phagocytosis *in vitro*, the role of these KIM-1 variants *in vivo* with respect to renal transplantation, graft damage, and signaling is unknown and requires further examination. Our findings have translatable implications, since all 3 variants were found to be of high frequency in human donor kidneys. It would be important to analyze whether the KIM-1 variant incidence would correlate with prognostic outcomes of kidney graft success.

In summary, our study is the first to delineate the functional role of KIM-1 gene variants *in vitro*. Furthermore, our findings show that the 3 distinct mutations in the coding region of *HAVCR1* have altered surface expression but universally lead to significantly reduced phagocytic capability. Taken together, our findings suggest that mutations in the KIM-1 gene may lead to increased risk of allograft rejection following renal transplantation.

4.5 References

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Chapter 5

5 Defective Kidney Injury Molecule-1 Polymorphisms and Risk of Delayed Graft Function in Humans

5.1 Introduction

Delayed graft function (DGF), defined as the need for dialysis with in the first 7 days post-transplant surgery, is a common complication faced by renal transplant recipients [1]. The incidence of DGF can vary from 20-30% in deceased donor kidney recipients, reaching >40% in recipients of donation after circulatory death (DCD) donors [2]. Patients with DGF have significantly poorer clinical outcomes, experiencing prolonged hospitalization, higher risk of developing acute rejection, and poor long-term graft survival [3-5]. Clinical risk factors of DGF include both donor and recipient characteristics but specific prediction tools are lacking. Some of these factors include duration of cold ischemia, DCD donor, duration of dialysis, terminal donor creatinine, donor and recipient sex, age, and body mass index (BMI), and recipients' race [5-7]. A growing body of experimental literature suggests that donor kidney-intrinsic genes also profoundly influence DGF risk [8, 9].

Ischemia reperfusion injury (IRI) is a largely unavoidable consequence of transplantation and is a major cause of DGF [9]. ATP depletion and the subsequent production of reactive oxygen species (ROS) during IRI induces vast amounts of tubular epithelial cells (TEC) to undergo sublethal injury or death via apoptosis or necrosis. Severe IRI leads to acute tubular necrosis whereby necrotic debris collects within the tubules and obstructs the flow of ultrafiltrate [10]. As a consequence, glomerular filtration rate declines, and may lead to delayed graft function [11, 12]. Secondarily, IRI can enhance the immunogenicity of the allograft by inducing the upregulation of HLA molecules and co-stimulatory on the surface of endothelial cells and antigen presenting cells (APCs)

(passenger leukocytes or infiltrating APCs), respectively which can engage the adaptive immune system to exacerbate alloimmune injury [13]. The underlying mechanism includes the release of danger signals such as High Mobility Group Box-1 (HMGB1), either passively from necrotic cells (e.g. TECs) or actively from injured and/or inflamed cells (e.g. macrophages), which then act on pattern recognition receptors on various parenchymal or innate immune cells within the injured graft to promote "necroinflammation" [14-16].

Kidney injury molecule -1 (KIM-1) is a glycoprotein whose expression is upregulated on the apical surface of renal proximal TECs following kidney injury [17]. The principle function of KIM-1 is to mediate the clearance of dying (apoptotic and necrotic) cells. The immunoglobulin variant (IgV) domain of KIM-1 directly recognizes and binds to phosphatidylserine (PS), which allows for the phagocytosis of the apoptotic cell bodies which display PS on their outer membrane for removal [18, 19]. In addition, KIM-1 expression enhances the uptake of necrotic cells through an opsonin, apoptosis inhibitor of macrophage (AIM) protein that is filtered by kidneys during IRI and binds to necrotic debris within the tubules [20]. This was evident in mice deficient for KIM-1, as they experienced significantly higher inflammation and tissue damage following native kidney warm IRI [19, 21]. Similarly, mice with a mutation in the mucin domain (KIM-1 Δ mucin) of KIM-1, rendering them incapable of binding to apoptotic cells and mediating their phagocytic uptake, are more susceptible to IRI compared to *wild-type* mice [22]. In the context of a mouse model of renal transplantation, KIM-1 deficiency in donor kidneys aggravated graft damage and resulted in severe renal function leading to lower overall survival [23]. Thus, these findings suggest that, not only the expression of KIM-1, but its

functional activity in TECs is essential in mitigating graft damage from IRI and improving graft function following renal transplantation.

The gene that encodes KIM-1 was initially identified as Hepatitis A virus cellular receptor-1 (*HAVCR1*), since KIM-1 was discovered as the cellular port of entry for Hepatitis A virus. KIM-1 is highly polymorphic with >5000 variants reported to date [24, 25]. A large number of the identified human *HAVCR1* polymorphisms are located within exon 4 which codes for the mucin domain. The degree of polymorphisms in exon 4 has may be driven by natural selection pressure driven by infection with Hepatitis A Virus [26]. Notably, a polymorphism resulting in a 6-amino-acid insertion in KIM-1/*HAVCR1* (157insMTTTVP) was associated with severe Hepatitis A Virus-induced liver disease [25]. The long allele of KIM-1 was shown to a more effective receptor for HAV than the short allele. Several other *HAVCR1* polymorphisms have been linked with increased susceptibility to hepatitis C infection, asthma, and eczema [25, 27-29]. Thus far, no studies have investigated the effect of *HAVCR1* polymorphisms on outcomes in acute kidney injury or transplant-associated IRI (e.g. DGF).

It is increasingly recognized that donor kidney-intrinsic genetic factors influence the severity of DGF [8, 30]. Previous studies have examined whether certain donor- and/or recipient-derived polymorphic genes affect the outcome of transplanted kidneys [6, 31], but to date, few genetic determinants have been identified that predict the risk of DGF in patients [32]. Moreover, to our knowledge, no studies have associated genetic polymorphisms that encode for defects in proteins involved in the pathophysiology of acute kidney injury with altered clinical outcomes. Given that KIM-1-mediated phagocytic clearance of apoptotic and necrotic cells mitigates IRI and the absence of

KIM-1 in the donor kidney led to graft dysfunction and damage in a syngeneic mouse model of renal transplantation [18, 19, 22, 23], we investigated whether functional polymorphisms in affecting KIM-1 function in the donor kidney plays a role in DGF following deceased donor kidney transplantation. To this end, we have identified three coding variants of human *HAVCR1* encode for KIM-1 proteins that are significantly impaired in their phagocytic activity (Chapter 4 of this thesis). Based on these findings, we conducted a retrospective cohort study of patients who received a deceased donor kidney transplant to determine if those who received a kidney from donors with one or more KIM-1 variants are at increased risk of developing DGF.

5.2 Materials and Methods

5.2.1 KIM-1 variant selection

As correlational studies that examine the association between the phagocytic function of KIM-1 and clinical outcomes are lacking, we identified high-frequency allelic KIM-1 variants that have been previously reported to have an association in non-renal diseases [26, 33, 34]. KIM-1 variants with Minor Allele Frequencies (MAF) between 0.25-0.5 from North American populations previously reported from GeneCards were chosen. In total, 3 KIM-1 (non-synonymous) variants located in the coding region were selected. We chose to study these KIM-1 variants as a follow-up study to Chapter 4 based on the observed significant reduction on phagocytic function.

5.2.2 Population

The London Health Sciences Centre (LHSC, London, Ontario, Canada) Renal Transplantation Program has performed 49-126 renal transplant surgeries annually since 1995. Since 2008, DNA samples from kidney donors have been collected and are stored at the LHSC Histocompatibility Laboratory. For this study, we included all adults >18 years old who received a kidney transplant from a deceased donor between 2008 – 2018. Patients who had undergone combined transplantation with more than one organ (i.e. kidney/liver or kidney/pancreas), or who had missing, or unusable donor DNA samples were excluded. A total of 627 recipients met eligibility criteria. As this was a retrospective study with collection of de-identified data, patient consent was waived. The study was approved by Western University's (HSREB# 107181) and Lawson Health Research Institute's Research Ethics Boards (R-15-563).

5.2.3 Data collection

We reviewed recipient medical charts for baseline demographic information, duration of end-stage renal disease, type of dialysis, comorbidities, body mass index, and baseline laboratory values. We collected post-operative outcome data, including: immunosuppressive agents used, daily creatinine values, need for dialysis, and hospital length of stay, and graft rejection. All data was obtained from the patient's electronic medical record (PowerChart) for transplants performed since 2012. For those who received transplants prior to 2012, paper medical charts were retrieved and reviewed. Information on donor age and comorbidities, and warm and cold ischemia times were obtained. Each recipient donor pair was assigned a unique study ID number and all data

was entered into Microsoft Access spreadsheet which was later combined with the information on donor genotyping for further analysis.

5.2.4 Donor genotyping

Donor DNA were extracted using either the Bromide salts (dodecyltrimethylammonium bromide; DTAB/ cetyltrimethylammonium bromide; CTAB) method or using Roche magnetic glass particle kits and Roche MagNA Pure extractors (Roche Molecular Systems. Pleasanton, CA). Sanger sequencing and fragment length analysis were used to determine the genotypes for the following KIM-1 coding variants: rs6149307, rs45439103, and rs12522248. Genotyping was done by members of the Hegele Lab. Donor genotypes were linked to the corresponding recipients in our de-identified database.

5.2.5 Primary outcome definition

The primary outcome of the study was DGF, defined as requiring at least one session of dialysis within the first 7 days post-transplantation [1, 5]. To determine the risk of DGF with each genetic variant of KIM-1, the usage of relative risk was favored over odds ratio.

5.2.6 Statistical analysis

Significance of the deviation of variant genotype frequencies from Hardy-Weinberg equilibrium was assessed using chi-square analysis. Pairwise linkage disequilibrium between *HAVCR1* alleles was determined using Pearson correlation coefficients as

described [35]. Recipients were grouped into subjects and controls based on donor genotype. Throughout the study, the terms locus was used interchangeably with the corresponding KIM-1 variants (Table 5-1), the term variant was used to describe the defective KIM-1 allele, and *wild-type* was used to describe the non-defective reference allele of KIM-1. Subjects were defined as being homozygous for each *HAVCR1* variant (rs12522248, rs45439103, rs6149307), while controls were defined as being heterozygous for the respective variant or homozygous for the *wild-type HAVCR1*.

Baseline demographic and clinical variables were compared between subjects and controls using student's t-test for continuous variables and with Chi-squared test for categorical variables. Differences in the rate of the primary outcome of DGF between subjects and controls were assessed using univariate and multivariable logistic regression. For the multivariable models, we adjusted for the following potential confounders: (donor and recipient BMI, donor and recipient sex, history of hypertension, terminal creatinine, type of donor (expanded criteria or not) and cold ischemia time [2, 5, 36-38]. This exercise was repeated for each genotype comparison group. Results are expressed as relative risk with 95% confidence interval (CI); p-values were derived from likelihood ratio tests. All statistical analyses were performed using SAS version 9.4 (Cary, NC), with a nominal level of significance defined as $P < 0.05$ (two-sided).

5.3 Results

5.3.1 KIM-1 variants allele frequencies

We determined donor *HAVCR1* (KIM-1) genotypes in a cohort of 627 deceased donor kidney transplant recipients for three select functional (identified in Chapter 4) polymorphisms (Table 5-1). Table 5-2 reports the observed genotype and allele frequencies of the 3 variants in our population which were found to be in Hardy-Weinberg equilibrium corresponding to global population frequencies reported in GeneCards. For both rs12522248 and rs45439103, the polymorphism coding for impaired phagocytosis was less common. Surprisingly, for rs6149307, having the 5 amino acid deletion that impairs phagocytosis (see chapter 4) was more common (allele frequencies 0.614) (Table 5-2).

Table 5-1. Description of genotypes for the 3 KIM-1 variants studies.

KIM-1 Variant rs Number	rs12522248	rs45439103	rs6149307
Locus	1	2	3
KIM-1 variant aa changes	Thr207Ala	Thr200del	Met158_Pro162del
Variation in alleles	[T/C]	[TTG/-]	[CATTGGAACAGTCGT/-]
Homozygous wildtype (WW)	T/T	TTG/TTG	CATTGGAACAGTCGT/ CATTGGAACAGTCGT
Heterozygous Variant (WV)	T/C	TTG/-	CATTGGAACAGTCGT/-
Homozygous Variant (VV)	C/C	-/-	-/-

Abbreviations: aa= Amino acid, - = Deletion, WW = Homozygous *wild type*, WV = Heterozygous variant, VV= Homozygous Variant.

Table 5-2. Allele and Genotype frequencies of the 3 KIM-1 variants.

Locus	KIM-1 variant rs number	Observed Allele Frequencies		Observed Genotype Frequencies						Hardy Weinberg		
		Ref allele	Alt allele	WW		WV		VV		WW	WV	VV
				N	%	N	%	N	%			
1	rs12522248	0.734	0.266	357	56.938	206	32.855	64	10.207	0.538	0.391	0.071
2	rs45439103	0.802	0.198	406	64.753	194	30.941	27	4.306	0.644	0.317	0.039
3	rs6149307	0.386	0.614	99	15.789	286	45.614	242	38.596	0.149	0.474	0.377

Abbreviation: Ref allele= Reference allele, Alt allele= Alternative allele. WW = Homozygous *wild type*, WV = Heterozygous variant, VV= Homozygous Variant.

5.3.2 Baseline Characteristics

Baseline donor, recipient and transplant patient demographics influencing DGF were similar between subjects and controls for each KIM-1 variant, except for donor female sex, donation after cardiac death (DCD) donor, panel reactive antibody (PRA) and thymoglobulin induction therapy for subjects in the rs45439103 group (Table 5-3).

Table 5-3. Baseline demographic characteristics according to KIM-1 variant accession numbers.

KIM-1 variant rs number	rs12522248			rs45439103			rs6149307		
	Subjects (C/C)	Controls (T/T and T/C)	p-value	Subjects (D/D)	Controls (N/N and N/D)	P=value	Subjects (D/D)	Controls (N/N and N/D)	p-value
N	64	563		27	600		242	385	
Recipient Characteristics									
Age	53.45±14.58	53.61±13.63	NS (0.937)	55.70±14.24	53.50±13.70	NS (0.436)	54.00±13.71	53.33±13.73	NS (0.553)
Female sex, N (%)	25 (39.06)	206 (36.72)	NS (0.713)	10 (37.04)	221 (36.96)	NS (0.993)	84 (34.71)	147 (38.38)	NS (0.354)
BMI (kg/m^2)	27.96±5.04	27.47±5.19	NS (0.507)	27.10±5.20	27.54±5.17	NS (0.716)	27.89±4.65	27.28±5.48	NS (0.176)
Diabetes	27 (42.19)	220 (39.08)	NS (0.629)	12 (44.44)	235 (39.17)	NS (0.583)	96 (39.67)	151 (39.22)	NS (0.911)
PRA	27.57±37.42	22.19±33.27	NS (0.278)	9.30±18.43	23.35±34.15	0.000774	23.42±34.27	22.30±33.41	NS (0.688)
Preoperative Creatinine (µmol/L)	695.1±226.0	685.3±273.5	NS (0.749)	660.6±194.5	687.5±271.8	NS (0.496)	671.4±264.8	695.7±271.3	NS (0.269)
Donor Characterisitics									
Age	41.80±16.70	43.91±16.62	NS (0.339)	44.33±14.33	43.67±16.74	NS (0.817)	43.50±16.68	43.82±16.62	NS (0.817)
Female sex, N (%)	16 (25.0)	208 (36.9)	NS (0.059)	15 (55.56)	209 (34.83)	0.0279	82 (33.88)	142 (36.88)	NS (0.446)
BMI (kg/m^2)	27.87±8.53	26.31±5.61	NS (0.164)	28.07±4.96	26.39±6.00	NS (0.099)	26.74±6.70	26.29±5.46	NS (0.385)
HTN N (%)	14 (23.33)	137 (25.56)	NS (0.707)	7 (26.92)	144 (25.26)	NS (0.849)	67 (28.88)	84 (23.08)	NS (0.112)
Diabetes N (%)	5 (8.33)	39 (7.29)	NS (0.358)	2 (7.69)	42 (7.41)	NS (0.0957)	18 (7.89)	26 (7.12)	NS (0.727)
Terminal Creatinine (µmol/L)	85.02±70.63	75.39±49.44	NS (0.366)	80.80±77.51	76.13±50.85	NS (0.820)	74.16±50.38	77.59±52.56	NS (0.459)
Expanded Criteria Donor N (%)	10 (15.63)	136 (24.16)	NS (0.126)	7 (25.93)	139 (23.17)	NS (0.740)	58 (23.97)	88 (22.86)	NS (0.740)
Donation after Cardiac Death N (%)	23 (35.94)	191 (33.93)	NS (0.748)	4 (14.81)	210 (35.00)	0.0305	85 (35.12)	129 (33.51)	NS (0.678)
Transplant Characteristics									
Cold Ischemia Time (min)	735.7±338.6	717.8±343.3	NS (0.690)	667.9±420.2	720.0±338.9	NS (0.515)	735.4±349.3	709.8±338.4	NS (0.365)
Thymoglobulin Induction Therapy N (%)	46 (75.41)	396 (72.66)	NS (0.647)	15 (57.69)	427 (73.62)	0.074	181 (76.37)	261 (70.73)	NS (0.127)

Subjects = homozygous variant for each KIM-1 variant, Controls = heterozygous variant and homozygous *wild-type*. N indicates the number, % indicates the percentage of the study group exhibiting a particular characteristic. Means ± SD. Abbreviations: N/N = Normal/Normal, N/D= Normal/Deletion, D/D= Deletion/Deletion, BMI= Body mass index, PRA= Panel reactive antibody (peak), HTN= Hypertension, NS= Not significant.

5.3.3 Primary Outcome: Rate of DGF

Of 627 individuals in our cohort, 165 developed DGF (26.3%). In univariate analysis, DGF was not significantly different between subjects compared to controls (Table 5-4): the relative risk of developing DGF for patients that received donor kidneys that were homozygous for the polymorphisms at locus 1, locus 2 and locus 3 were 1.57 (95% CI = 0.97 – 2.53), 0.64 (0.25 – 1.65) and 1.01 (0.81 – 1.26), respectively. When we limited the control group to recipients of donor kidneys with at least one copy of the *wild-type* allele (at all 3 loci, N=203, see Table 5-7), the unadjusted relative risk did not change appreciably from the primary analysis. Multi-variable adjusted analysis yielded similar results (Table 5-4).

However, there was a trend to more DGF in patients who received donor kidneys homozygous for the rs12522248 substitution allele compared to controls, but this did not reach statistical significance (36% vs. 25.2%, unadjusted RR 1.57 (95% CI 0.97 – 2.53, p=0.062). Adjusted analysis revealed a similar trend but was not statistically significant (Table 5-4). Taken together, these findings suggest a potential risk of acquiring DGF associated with donor kidneys that are homozygous for the rs12522248 variant.

Table 5-4. Univariate and multivariate analysis for the deceased donors for the occurrence of delayed graft function.

Locus	KIM-1 variant rs number	Subjects		Controls							
		N	DGF N (%)	N	DGF N (%)	RR (unadjusted)	95% CI	p-value	RR (adjusted)*	95% CI	p-value
1	rs12522248	64	23 (36.0)	563	142 (25.2)	1.57	0.97-2.53	0.062	1.83	0.89-3.74	0.100
2	rs45439103	27	5 (18.2)	600	160 (26.7)	0.64	0.25-1.65	0.347	0.87	0.18-3.24	0.866
3	rs6149307	242	64 (26.5)	385	101 (26.2)	1.01	0.81-1.26	0.953	1.01	0.64-1.59	0.693

*Adjusted for: recipient age, recipient sex, recipient BMI (body mass index), and donor age, donor sex, donor hypertension, terminal creatinine, cold ischemia time, and DCD (donation after cardiac death)

Subjects = homozygous variant for each KIM-1 variant, Controls = heterozygous variant and homozygous *wild-type*.

Abbreviation: N=Number, DGF= Delayed Graft Function, CI= Confidence interval, RR= Relative risk.

5.3.4 KIM-1 variant haplotype groups and definition

Linkage disequilibrium is defined as nonrandom association between alleles at 2 or more loci [39]. Haplotypes under linkage disequilibrium have divergent frequencies compared to the expected values when looking at independent alleles. Linkage disequilibrium is said to be positive or negative, when 2 alleles occur together on the same haplotype beyond or below expected values, respectively [40]. Therefore, rather than a single variant being responsible for the association with an observed phenotype, it is possible that one or more inherited variants that are co-expressed within the haplotype may be contributing to the phenotype. Thus, to determine the cooperative consequence of KIM-1 haplotypes that get inherited together, we further dissected the prevalence of linkage disequilibrium within our sample population (Table 5-5). We found that rs6149307 and rs45439103 alleles are in close proximity and are in linkage disequilibrium with each other.

Table 5-5. Linkage disequilibrium D' values for KIM-1 variants.

	rs12522248	rs45439103	rs6149307
rs6149307	-0.386	0.626	
rs45439103	-0.068		
rs12522248			

Positive occurrence of linkage disequilibrium was determined by any D' values > 0.36.

Table 5-6 illustrates the haplotype groups with the presence or absence of mutation in each locus exhibiting the variants that exist in a single chromosome, and the corresponding frequencies observed in our cohorts. We then grouped the observed haplotypes according to chromosome pairs (Table 5-7). Strikingly, of the 64 patients that were homozygous variants for rs12522248 at locus 1 (Table 5-3), 56 patients expressed both the rs12522248 homozygous variant allele at locus 1 and homozygous variant rs6149307 allele at locus 3 on both chromosomes (Table 5-7).

Table 5-6. Definition and Frequencies of each Haplotype.

Haplotype Groups	Locus 1 (rs12522248)	Locus 2 (rs45439103)	Locus 3 (rs6149307)
1	N	N	N
2	N	N	D
3	N	D	N
4	C	N	N
5	C	N	D

Numbers 1-5 represent distinct haplotype groups for a chromosome where 1 is N/N/N; 2 is N/N/D; 3 is N/D/N; 4 is C/N/N; 5 is C/N/D for the 3 loci with rs125222248/rs45439103/rs6149307 respectively. N represents *wild-type* normal copy; D represents deletion, and C represents T to C substitution as described in Table 5-1.

Table 5-7. Haplotype Groups Definition in our Cohort and Frequencies.

Subject Groups	KIM-1 Haplotype Distribution	Haplotypes on both chromosomes Chr.1	Chr.2	Description	Frequency	%	Phenotype
Controls	1/1	N/N/N	N/N/N	Wild-type homozygous at all 3 loci	20	3.19	Controls: 2 normal copies of KIM-1
	1/4	N/N/N	C/N/N	heterozygote at Locus 1	2	0.32	Controls: One normal copy of KIM-1
	1/3	N/N/N	N/D/N	heterozygote at Locus 2	45	7.18	
	1/2	N/N/N	N/N/D	heterozygote at Locus 3	78	12.44	
	1/5	N/N/N	C/N/D	heterozygote for Locus 3 and Locus 1	58	9.25	
Group A: Compound Heterozygotes	2/3	N/N/D	N/D/N	heterozygote for Locus 2 and Locus 3	97	15.47	No normal copy of KIM-1 - BUT each variant limited to just one chromosome
	3/4	N/D/N	C/N/N	heterozygote for Locus 2 and Locus 1	4	0.64	
	2/4	N/N/D	C/N/N	*does not exist*	0	0	
	3/5	N/D/N	C/N/D	heterozygote at Locus 2 + Locus 3 + Locus 1	46	7.34	
Group B: Homozygous at Locus 1 only	4/4	C/N/N	C/N/N	homozygote at Locus 1	1	0.16	Locus 1 defective
	4/5	C/N/N	C/N/D	homozygote at Locus 1 and heterozygote for Locus 3	7	1.12	
Group C: Homozygous at Locus 2 only	3/3	N/D/N	N/D/N	homozygote at Locus 2	27	4.31	Locus 2 defective
Group D: Homozygous at Locus 3 only	2/2	N/N/D	N/N/D	homozygote at Locus 3	90	14.35	Locus 3 defective
	2/5	N/N/D	C/N/D	homozygote at Locus 3 + heterozygote at Locus 1	96	15.31	
Group E: Homozygous at TWO loci (1&3)	5/5	C/N/D	C/N/D	homozygote at Locus 3 + homozygote at Locus 1	56	8.93	Locus 1 and 3 completely defective - Double Mutant
TOTAL					627	100	

The sample population was grouped according to the KIM-1 haplotype distribution that exist in the human population. Numbers 1-5 represent distinct haplotype groups for a chromosome where 1 is N/N/N; 2 is N/N/D; 3 is N/D/N; 4 is C/N/N; 5 is C/N/D for the 3 loci with rs125222248/rs45439103/rs6149307 respectively (Table 5-6). N represents *wild-type* normal copy; D or C represents a mutational event described in Table 5-1.

5.4 Discussion

Kidney injury molecule-1 (KIM-1) is of particular interest in renal diseases due to its transient expression on the renal proximal TECs upon injury in both mice and in humans as well as its role in various renal diseases involving IRI [17, 19, 23, 41]. We have previously reported the protective effect of KIM-1 in renal transplantation in mouse models [23], however, whether our findings are translatable to patients was unknown and thus remained an important question that needed to be answered. The genetic variability of *HAVCR1* is linked with various degrees of susceptibility to diseases such as asthma and Hepatis A virus infection [25, 26, 28]. In Chapter 4, we identified 3 distinct genetic variants affecting the coding region of KIM-1. The ensuing amino acid change(s) resulting from the genetic variation significantly attenuated the phagocytic function of KIM-1 (Chapter 4). To that end, we selected these 3 coding variants for this study with the hypothesis that one or more of these variants that impair the function of KIM-1 would lead to an increased risk of DGF. This hypothesis was based on previous work done by the Bonventre laboratory [18, 22, 42] and our group [19, 21, 23] (including Chapters 2 and 3) suggesting that compromised clearance of dying cells would result in increased tubular obstruction and necroinflammation, which in turn would further aggravate graft damage and impair glomerular filtration, and ultimately lead to delayed graft function [16, 43]. Using a large cohort consisting of 627 deceased donor-recipient pairs, we tested whether one or more of the 3 donor coding variants of KIM-1 would predispose transplant recipients to an increased risk of DGF.

We report that compared to *wild-type* ("normal") KIM-1, none of the recipients of donor kidneys containing the KIM-1 variants were significantly associated with an increased risk of DGF following renal transplantation. Interestingly, the patients who inherited a kidney from a donor who was homozygous for rs12522248 exhibited 1.57 times greater risk of developing DGF compared to everyone else in the cohort, but this did not reach statistical significance. The DGF rate in this group was reached almost 36% compared to 25% in the controls group (Table 5-4). Notably, 56 of the 65 people who were homozygous for this allele in our cohort were also homozygous for the rs6149307 allele (Table 5-7), representing 9% of the population. It would be important to know if these two simultaneous changes to the coding sequence of KIM-1 would further impair phagocytic function when expressed in TECs compared cell expressing the rs6149307 variant (see Chapter 4). If so, it is plausible that having two phagocytic defects (i.e. homozygous at 2 loci) may confer an increased risk of DGF. However, our study was not powered to confirm this hypothesis, and further study is needed. Interestingly, males (donors) are more likely to have the rs12522248 allele compared to females.

Animal data presented in Chapter 2 clearly showed that donor KIM-1 expression is required for protection against graft dysfunction ("mouse DGF") in mice. Given the severity with which the rs6149307 affected KIM-1 function, if interpret the above data to mean that patients receiving a kidney from donors carrying two copies of this alleles did not experience any additional risk of DGF, this could imply that the phagocytic function of KIM-1 is not required (or is redundant) for protection against DGF. If so, the non-phagocytic functions of KIM-1 in DGF may need to be examined more carefully. Indeed, mutations in the IgV domain or the cytoplasmic domain can significantly alter KIM-1's

non-phagocytic function, but no variants that met our frequency criteria are known to exist (GeneCards). Our lab has previously reported that the intracellular domain of KIM-1 inhibits the harmful effects of activated Gα12 during IRI, ultimately enhancing kidney repair [19, 21]. Consequently, non-synonymous variations in KIM-1 intracellular domain that impede the interaction with Gα12 may lead to increased risk of DGF. The cytoplasmic and IgV domains of KIM-1 have been shown to interact with the ligand binding domain of pro-apoptotic nuclear receptor 77 (NUR77). This interaction did not require the mucin domain of KIM-1 where our three coding variants are located. Through this interaction, NUR77 is targeted for degradation, promoting epithelial cell survival [44]. Mutations in the cytoplasmic or IgV domains of KIM-1 may impede the interaction with NUR77 resulting in abrogation of NUR77 degradation and ultimately diminishing the survival of TECs. Taken together, KIM-1 may protect against DGF via mechanisms other than phagocytic clearance of apoptotic cells [22]. Identifying common non-synonymous variants that alter the non-phagocytic function of KIM-1 could enable us to test if they predispose to DGF development following renal transplantation.

In summary, in our cohort, we were not able to demonstrate that polymorphisms in any of the 3 coding KIM-1 loci (rs12522248, rs45439103 and rs6149307) are associated with increased risk of delayed graft function following renal transplantation. Our study was limited by a relatively small sample size: <10% of donor kidneys were homozygous for variant allele at rs12522248, and rs6149307. We did uncover that the variant allele for rs6149307 was more common than the reference allele in the population (Table 5-2) and that it is in linkage disequilibrium with the defective variant of rs12522248 making the double mutant relatively common (Table 5-7). However, whether inheriting double

mutations on KIM-1 on both alleles increases the risk of DGF requires further study. Alternatively, it may be that the phagocytic function of KIM-1 alone does not play a significant role in mitigating early allorecognition and rejection of the graft in humans, but most likely a combination of multiple pathways is involved in the development of DGF.

5.5 References

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Chapter 6

6 Discussion and Conclusion

6.1 Discussion

Renal transplantation is the optimal treatment for patients suffering from end-stage renal disease [1, 2], however, ischemia-reperfusion injury (IRI) is an inescapable consequence of transplantation that can limit the longevity of the kidney grafts [3-5]. The pathological process of IRI is two-fold; firstly, ischemia is initiated when adequate blood flow is restricted leading to decreased production of ATP, secondly reperfusion, which occurs when the flow of oxygenated blood is restored, can induce burst of ROS production [6-8]. Renal tubular epithelial cells (TECs), which are highly susceptible to IRI, can either overcome this insult leading to recovery or otherwise undergo various modes of cell death [9-11]. The accumulation of dying cells in the microenvironment due to inefficient clearance paves the way for secondary necrosis of apoptotic bodies. Although primary and secondary necrotic cells exhibit different characteristics and morphology - secondary necrotic cells are smaller in size and are depicted by the loss of chromatin, high levels of monosodium urate microcrystals, and low ATP - they both lead to the release of DAMPs (i.e. HMGB1; free, reduced form for primary and nucleosome bound and oxidized form for secondary necrotic cells), which ultimately further exacerbates inflammation [9-11]. To add insult to injury, inflammation is progressively amplified via an auto-amplification loop. Together, the culmination of this inflammatory cascade manifests as severe secondary tissue damage [12]. Thus, targeting the efficient removal of dying cells in IRI-impacted kidneys may be a promising therapy for mitigating graft damage. In this thesis, I outlined the protective mechanism of Kidney injury molecule -1 (KIM-1) against transplant related -IRI in a murine model of renal transplantation. Moreover, with the aim of translating our findings from the bench to the bedside, I also demonstrated how

recombinant AIM can be used to enhance KIM-1 function to ameliorate DGF/tissue damage and examined the effect of KIM-1 genetic variation in human patients in predicting DGF following deceased donor renal transplantation. To this end, I elucidated the importance of the phagocytic ability of KIM-1 in renal transplantation using in both mice and in humans.

6.1.1 Kidney injury molecule-1 expression in donor kidneys alleviate graft damage, renal dysfunction and graft failure through mitigating necroinflammation following renal transplantation

In Chapter 2, using a murine syngeneic renal transplant model, I determined the role of donor kidney KIM-1 expression in transplant-associated graft damage. In these studies, the donor kidneys were retrieved while the heart was still beating, therefore, our transplant model most closely resembles transplants where the donors were deemed to be neurological determination of death (NDD). I discovered that kidney KIM-1 expression in the donor kidney significantly improved early graft function and improved overall survival (Figure 2-1). In addition, KIM-1 expression mitigated graft damage and decreased local and systemic inflammation (Figure 2-2 and 2-3). Interestingly, serum levels of the danger signal, HMGB1, were elevated and increased M1 polarization of macrophages were observed in recipients that received kidneys from KIM-1 deficient mice compared to those who received kidneys from wild type mice (Figure 2-4 C-D). Collectively, these findings suggest that KIM-1 mediated clearance of dying cells during

syngeneic renal transplantation is required for limiting necroinflammation, ultimately conferring a survival advantage. Although previous studies have delineated the protective effect of KIM-1 in native kidney warm IRI [13, 14], my work is the first to define the role of KIM-1 in renal transplantation, where both warm and cold ischemia occur concurrently.

By using a syngeneic transplant model, we eliminated the potential confounding effect of allogenic immune response, and thus we were able to elucidate the pathology strictly stemming from IRI. The functional significance of KIM-1 in allogeneic transplantation, however, is an important and clinically relevant question since human transplants occur predominantly between HLA-mismatched donor and recipient pairs. Autotransplants are done in cases of loin pain hematuria or mechanical issues pertaining to the ureter or renal vasculature [15]. Notably, allogeneic transplants between HLA mismatched pairs exhibit greater rejection and poorer overall survival compared to transplants between HLA identical siblings [16]. Whether KIM-1 would also provide a protective benefit in the context of allogenic transplants warrants further investigation.

Regulatory T cells (T regs), an important population responsible for suppressing inflammation, has shown to be protective in kidney injury by participating in renal repair following IRI [17], and by preventing transplant rejection through maintaining immune tolerance [18-20]. Although danger signals are not strictly a prerequisite for graft rejection, they may block tolerance induction via the inhibition of Tregs [21, 22]. The inflammatory cytokine, IL-6, plays a key role in the blocking of Treg expansion [23, 24], whereas IL-6 neutralization has been shown to enhance the proliferation of Tregs [25]. The production of IL-6 has been purported to be sourced from injured tubular epithelial

cells following IRI and dendritic cells in response to DAMPs [22, 25]. In my model, I observed increased levels of DAMPs (Figure 2-4 C) and IL-6 production (Figure 2-3 C) in the recipients harboring KIM-1 deficient kidneys, which suggests a possible mechanism by which KIM-1 could confer protection from alloimmune injury via the promotion of Treg expansion.

Preconditioning is a protective clinical measure to render tolerance to severe IRI through the application of brief episodes of ischemia and reperfusion to the organ prior to transplantation [26]. A recent meta-analysis using experimental animal models reported that ischemic preconditioning exhibited protected effects by reducing serum creatinine, blood urea nitrogen, and histological damage following renal IRI compared to non-treated [27]. Similarly, Torras J *et al.* reported that a brief cycle of ischemic preconditioning attenuated damage from renal transplant associated IRI in Sprague-Dawley rats [28]. It would be interesting to investigate the potential link between KIM-1 expression and preconditioning. It is possible that KIM-1 expression may be upregulated during preconditioning and contribute to its protective phenotype.

The protective effects of transient KIM-1 expression during renal injury notwithstanding, chronic expression of KIM-1 has been associated with regions of fibrosis, chronic inflammation and progression of chronic kidney disease in human patients [29, 30]. Additionally, constitutive expression of KIM-1 in the absence of renal insult leads to spontaneous development of renal fibrosis, renal failure and death in mice [31]. Taken together, these findings highlight that a delicate balance limited KIM-1 expression is necessary for optimal renal function and that therapies aimed to enhance KIM-1 function must be cognizant of its proclivity towards pathogenicity.

Although this dissertation has focused on the protective role of KIM-1 in renal transplantation, it is true that transplant-associated pathologies can arise from diverse aetiologies involving numerous pathways. Therefore, it is likely the case that a combinational therapy that targets KIM-1 in addition to current clinical regimens, such as immunosuppressants, may be the most ideal approach going forward. In this chapter, I discovered that KIM-1 mitigates early graft inflammation and damage and increased overall survival following renal transplantation by dampening necroinflammation through phagocytosis of dying cells. My findings add to the body of work describing the role of KIM-1 in kidney injury and provides a "stepping-stone" in delineating KIM-1 function in long-term pathologies associated with allogeneic renal transplantation.

6.1.2 Recombinant apoptosis inhibitor of macrophage protein ameliorates graft damage and renal dysfunction via enhancement of KIM-1 mediated clearance of necrotic cells following renal transplantation

In chapter 3, I examined the therapeutic potential of recombinant AIM (rAIM) in mitigating graft damage following renal transplantation by enhancing the KIM-1 mediated removal of dying cells. Arai S *et al*. first described the protective role of AIM administration in native kidney IRI, where they reported that AIM opsonized necrotic cells were effectively cleared by KIM-1 [32]. This study suggested that the filtration of endogenous AIM in the body is not maximized during kidney injury, and that additional administration of exogenous AIM confers a therapeutic effect against kidney IRI. Here, I

have shown that administration of rAIM in transplant recipient mice resulted in markedly improved renal function and minimized graft damage (Figure 3-1). In addition, rAIM treated mice released significantly less DAMPs into the circulation and displayed decreased graft inflammation (Figure 3-2). Finally, we assessed whether rAIM would potentiate the phagocytic ability of KIM-1 by enhancing the clearance of cellular debris. As expected, I showed that the addition of rAIM to KIM-1 expressing human HEK 293 cells significantly increased the overall phagocytic activity *in vitro* (Figure 3-3D). Taken together, my findings show that rAIM potentiates the functional activity of KIM-1 in the clearance of necrotic cells which conferred a protective phenotype in the context of renal transplantation.

Alloimmune injury is a major component of graft injury during renal transplantation which is not addressed in syngeneic models [33]. Moreover, IRI has been found to increase sensitization to donor alloantigens [5] and promote alloreactive $CD4^+$ T cells responses [34, 35] in allogeneic models of transplantation. Indeed, allogeneic transplantation results in tissue damage to be incurred from both alloimmune responses and IRI. In addition, autoantibodies (e.g. against LG3) can also aggravate IRI during allotransplantation [36]. Thus, an important line of investigation is whether the rAIM administration would similarly confer protection in an allogeneic transplant model where alloimmune and autoimmune injuries significantly contribute to the overall pathology.

One limitation of this chapter is that it is unclear whether the protective effects attributed to the administration of rAIM is solely mediated by KIM-1 and/or through the enhancement of macrophage activity. Although Arai S *et al*. reported that in the absence of KIM-1, administration of rAIM did not exhibit any protective effect following native

renal warm IRI [32], whether rAIM will also be protective independent of KIM-1 in transplant IRI needs further investigation. Notably, the original function of AIM was identified to have pro-survival effects on macrophages [37]. Thus, how the presence of additional exogenous AIM could contribute the survival and the recruitment of macrophage to the kidney grafts and whether it exhibits additional protective effects independent of KIM-1 by regulating macrophages needs to be further studied in detail.

Numerous studies have targeted various key molecules involved in necroinflammation in attempt to block or abrogate the auto-amplification loop in order to mitigate kidney damage [38]. In particular, Kumer S *et al*. described another DAMP, extracellular histones, in the context of glomerulonephritis, exacerbated inflammation and kidney damage, however DAMP neutralization markedly improved severe glomerulonephritis in mice [39]. My own findings showed enhancing the clearance of dying cells via rAIM, mitigated the release of DAMPs. Future investigations should examine whether rAIM administration could stop the inflammatory feedback loop in an allogeneic model of renal transplantation, which would ostensibly reduce the immunogenicity of the kidney graft ultimately resulting in prolonged graft survival. Taken together with data from chapter 2, my findings highlight the therapeutic application of rAIM to potentiate the protective effects of KIM-1 in renal transplantation.

6.1.3 The effect of genetic variations in human Kidney injury molecule-1 in altering its phagocytic function and in predicting delayed graft function

In chapter 4, I uncovered that 3 HAVCR1 variants that altered the region encoding for the mucin domain of KIM-1 all significantly impaired its (apoptotic cell) phagocytic function compared to *wild-type* KIM-1 *in vitro*. Interestingly, the total protein levels of each variant when expressed in HEK 293 cells remained unaltered. However, there was some variability in the relative cell-surface expression of each variant compared to cells expressing wild-type KIM-1 (Figure 4-2). Furthermore, out of the 3 variants, rs6149307 exhibited greatest reduction in its phagocytic ability of apoptotic target cells compared to the *wild-type* (Figure 4-3).

Considering that KIM-1 has a critical role in ameliorating graft damage and renal dysfunction in kidney transplantation (Chapter 2) [40], coupled with the finding that variations in the coding region result in impaired phagocytic activity (Chapter 4), the next question was to assess whether donor kidneys that were positive for the select variants examined in Chapter 4 predisposes recipients to an increased risk of DGF. In Chapter 5, logistic regression analysis of a clinical database revealed that none of these variants were significantly correlated with increased risk of DGF (Table 5-4). However, our cohorts that inherited kidneys from donors homozygous for rs12522248 allele exhibited increased DGF rate and 1.664 times greater risk of developing DGF compared to the control groups (Table 5-4). Thus, taken together with the increased DGF rate observed in the 64 patients with homozygous mutation in rs12522248, and 56 patients of these

patients exhibiting 2 significant defects in loci on both chromosomes (double mutants), this group of cohorts therefore may be an interesting group and requires further study.

KIM-1 expression has been reported to be correlated with renal function and fibrosis following renal transplantation [41]. DGF, although an important indicator of transplantation prognosis, is only one of many clinical factors that may influence a transplant's success. It may also be of interest to examine the potential association of each KIM-1 variant with risk of long-term graft survival since KIM-1 expression is known to correlate with fibrosis and progression to CKD [31, 42, 43].

The contribution of necrotic cell death to IRI and DGF is another factor that could have confounded our multivariate analysis. Indeed, the predominant form of cellular injury during renal IRI is believed to be via necroptosis [44, 45]. Although, we showed that genetic polymorphisms in select KIM-1 variants resulted in defective clearance of apoptotic cells (Chapter 4), whether these variants also were defective in its ability to bind to AIM on opsonized necrotic cells was not tested. Therefore, it is possible that these select variants still retained the ability to clear necrotic cells which would render a negligible association with DGF.

Although Chapter 5 took sex into consideration when determining the risk of DGF with the three functional KIM-1 variants, Chapters 2 and 3 of my thesis only examined the role of KIM-1 in the donor kidneys of male mice. Numerous studies have implicated that males are more prone to IRI and consequently exhibit poorer transplant outcomes compared to females [46, 47]. In addition, when subjecting mice and rats to bilateral ischemia reperfusion injury, males developed significantly greater amount of kidney

deterioration, renal dysfunction and severe tubular necrosis compared to females [48, 49]. Administration of testosterone exacerbated damage due to renal IRI in females. Similarly, estrogen administered to castrated males provided significant protection [50]. Taken together, these results propose that testosterone is a key determinant for enhanced susceptibility to IRI. Therefore, examining the potential for sex differences in the role of KIM-1 in kidney transplants is an interesting question and needs further investigation.

Here we show for the first time that 3 select variations in the coding region of KIM-1 causes alteration in cell surface expression and significantly attenuates its phagocytic function. However, whether these select variants are predictive for the potential risk of developing DGF require additional studies. Although further analysis of the data is necessary in uncovering potential associations with clinical prognostic markers, these findings may provide novel insight in potentially linking the non-phagocytic function of KIM-1 with long-term transplant outcomes in human patients.

6.2 Conclusion

The overarching theme of this thesis is to examine the role of KIM-1 in mitigating graft damage in the hopes of providing evidence for potential therapies that would prolong the lifespan of the transplanted kidney. Ischemia reperfusion injury is a major element that limits the lifespan of the kidney grafts [3, 4, 6, 7, 51, 52] and delayed graft function is a major consequence of IRI which

in turn is associated with graft rejection and poor long-term survival [53, 54]. This thesis systematically describes how efficient clearance of dying cells could markedly improve transplant outcomes via regulating necroinflammation that occurs downstream of KIM-1 (Figure 6-1). First, I identified KIM-1 as a target protein in mitigating graft injury which consequently improved overall survival. Second, I validated a KIM-1 specific therapy, rAIM, as a potential treatment agent in transplantation. Finally, I assessed the clinical significance of various KIM-1 haplotypes in human kidney transplantations. There are still unanswered questions including: the role of KIM-1 in an allogeneic transplantation; whether chronic expression of KIM-1 following renal transplantation is pathogenic; and whether genetic variants of KIM-1 in donor kidneys predispose human recipients to chronic kidney disease and subsequent graft failure. Nevertheless, this thesis elucidates the protective role of KIM-1 and rAIM administration during renal transplantation, and highlights the effective targeting of KIM-1 to be an attractive therapeutic strategy that may prolong kidney transplant survival.

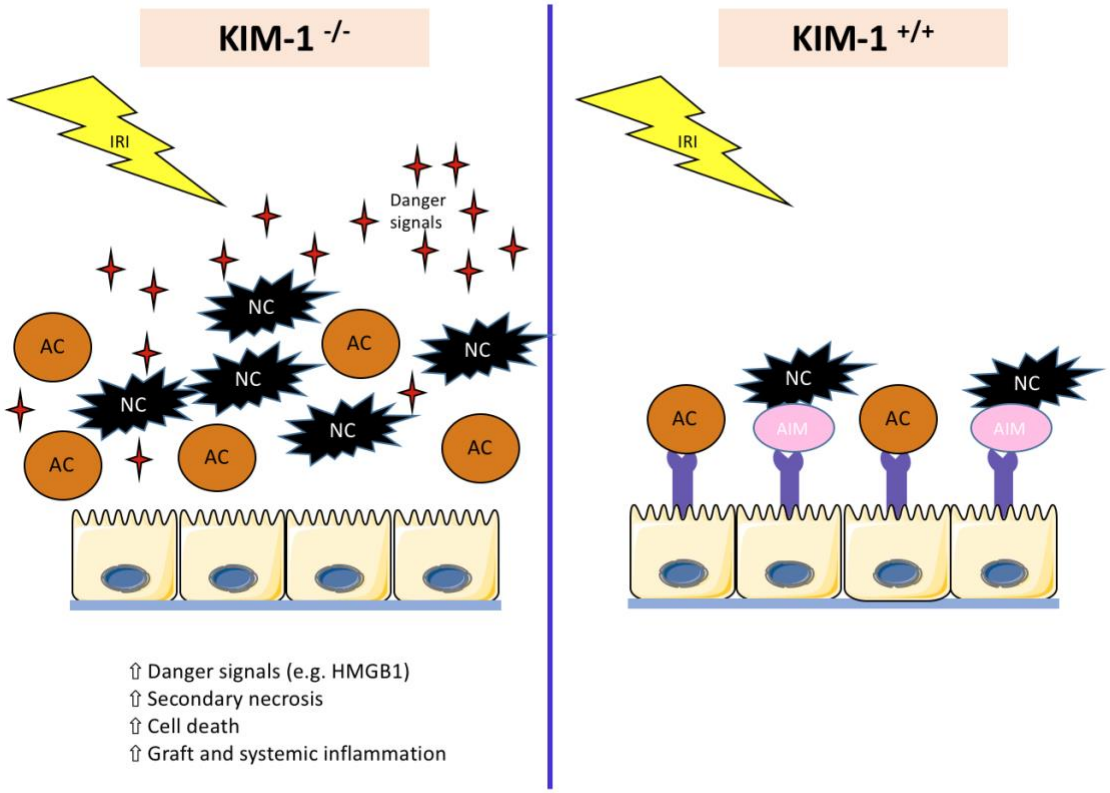

Figure 6-1. Expression of KIM-1 in the donor kidneys protects against transplant associated-IRI through regulation of necroinflammation via the clearance of dying cells.

Abbreviations: AC = Apoptotic cell, AIM= Apoptosis inhibitor of macrophage protein, IRI= Ischemia-reperfusion injury, NC= Necrotic cell.

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Appendices

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Curriculum Vitae

NAME: **Ji Yun Lee**

EDUCATION

2015-Present **Philosophy of Doctorate**

Department of Microbiology and Immunology, Western University

Thesis: Examining Kidney injury molecule-1 mediated phagocytosis and its therapeutic application in ameliorating renal transplant ischemia reperfusion injury.

Supervisor: Dr. Lakshman Gunaratnam

2011-2015 **Hon. Bachelor of Science**

Double Major in Biology and Medical Sciences, Western University

HONOURS and AWARDS

2019-2020 Ontario Graduate Scholarship

2019 Dr. Frederick W. Luney Graduate Travel Award in Microbiology & Immunology, Western University

2019 Canadian Society of Nephrology: 3rd place at platform presentation

2017 Canadian Society of Nephrology: 3rd place at platform presentation

2017 Dr. Frederick W. Luney Graduate Travel Award in Microbiology & Immunology, Western University

2015-2019 Western Graduate Research Scholarship, Western University

2013-2014 Science Award: Laurence Paterson Scholars, Western University

2013-2014 Dean's Honor List, Western University

PUBLICATIONS

Published

1) **Lee JY**, Ismail OZ, Zhang X, Haig A, Lian D, Gunaratnam L. 2018. Donor kidney injury molecule-1 promotes graft recovery by regulating systemic necroinflammation. *Am J Transplant* 18(8): 2021-2028.

Under Review

1) **Lee JY**, Lian D, Haig AR, Suri RS, Miyazaki T and Gunaratnam L. Recombinant apoptosis inhibitor of macrophage protein reduces delayed graft function in a murine model of kidney transplantation. Submitted to *PLOS ONE*. Manuscript ID: PONE-D-20-27992

2) Lee JC, Yotis D, **Lee JY**, Sarabusky M, Shrum B, Champagne A, Tutunea-Fatan E and Gunaratnam L. Inhibitory effect of KIM-1 on metastasis of renal cell carcinoma. Submitted to *Scientific Reports*. Manuscript ID: fff90d55-8e4e-4524-8f86-e43448397733

In Preparation

1) **Lee JY**, Shrum B, Lee SH, Ban MR, McIntyre AD, Hegele RA, Suri RS and Gunaratnam L. Defective Kidney injury molecule-1 polymorphisms and risk of delayed graft function in humans (Anticipated submission date: Feb 2021)

ORAL PRESENTATIONS

1) *Canadian Society of Transplantation (CST)*: "Recombinant Apoptosis Inhibitor of Macrophage Protein Ameliorates Transplant Ischemia-Reperfusion Injury." Oct 17th, 2019, Banff, CAN

2) *Canadian Society of Immunology (CSI):* "Recombinant apoptosis inhibitor of macrophage protein ameliorates transplant ischemia-reperfusion injury." June 14th 2019 Banff, CAN

3) *Canadian Society of Nephrology (CSN)*: "Recombinant apoptosis inhibitor of macrophage protein ameliorates transplant ischemia-reperfusion injury." May 3rd, 2019 Montreal, CAN

4) *Canadian Society of Nephrology (CSN)*: "Kidney injury molecule-1 mitigates tissue damage from transplant renal ischemia reperfusion injury." May 5th, 2017, Montreal, CAN

5) *American Transplant Congress (ATC):* "Kidney injury molecule-1 mitigates tissue damage from transplant renal ischemia reperfusion injury" May 1st, 2017, Chicago, USA.

PUBLISHED ABSTRACTS

1) **Lee JY**, Shrum B, Gunaratnam L. 2020. Polymorphisms in HAVCR1 alter KIM-1 mediated phagocytosis: American Society of Nephrology; Online.
2) **Lee JY**, Zhang X, Lian D, Haig A, Miyazaki, Gunaratnam L. 2019. Recombinant apoptosis inhibitor of macrophage protein ameliorates transplant ischemia-reperfusion injury. Canadian Society of Transplantation; Banff, CAN.
3) **Lee JY**, Zhang X, Lian D, Haig A, Miyazaki, Gunaratnam L. 2019. Recombinant apoptosis inhibitor of macrophage protein ameliorates transplant ischemia-reperfusion injury. London Health Research Day; London, Ontario, CAN.
4) **Lee JY**, Zhang X, Lian D, Haig A, Miyazaki, Gunaratnam L. 2019. Recombinant apoptosis inhibitor of macrophage protein ameliorates transplant ischemia-reperfusion injury. Canadian Society of Immunology; Banff, CAN.
5) **Lee JY**, Zhang X, Lian D, Haig A, Miyazaki, Gunaratnam L. 2019. Recombinant apoptosis inhibitor of macrophage protein ameliorates transplant ischemia-reperfusion injury. American Transplant Congress; Boston, USA.
6) **Lee JY**, Zhang X, Lian D, Haig A, Miyazaki, Gunaratnam L. 2019. Recombinant apoptosis inhibitor of macrophage protein ameliorates transplant ischemia-reperfusion injury. Canadian Society of Nephrology; Montreal, CAN.
7) **Lee JY**, Ismail OZ, Zhang X, Haig A, Lian D, Gunaratnam L. 2018. Donor kidney injury molecule-1 promotes graft recovery by regulating systemic necroinflammation. Department of Medicine Resident Research Day; London, Ontario, CAN.
8) **Lee JY**, Ismail OZ, Zhang X, Haig A, Lian D, Gunaratnam L. 2018. Donor kidney injury molecule-1 promotes graft recovery by regulating systemic necroinflammation. London Health Research Day; London, Ontario, CAN.

9) **Lee JY**, Ismail OZ, Zhang X, Haig A, Lian D, Gunaratnam L. 2017. Donor kidney injury molecule-1 promotes graft recovery by regulating systemic necroinflammation. Department of Medicine Resident Research Day; London, Ontario, CAN.
10) **Lee JY**, Ismail OZ, Zhang X, Haig A, Lian D, Gunaratnam L. 2017. Donor kidney injury molecule-1 promotes graft recovery by regulating systemic necroinflammation. London Health Research Day; London, Ontario, CAN.
11) **Lee JY**, Gu X, Gangireddy R, Zhang X, Haig A, Gunaratnam L. 2017. Persistent kidney injury molecule-1 expression promotes AKI to CKD transition after severe renal ischemia-reperfusion injury. Canadian Society of Nephrology; Montreal, CAN.
12) **Lee JY**, Ismail OZ, Zhang X, Haig A, Lian D, Gunaratnam L. 2017. Donor kidney injury molecule-1 promotes graft recovery by regulating systemic necroinflammation. Canadian Society of Nephrology; Montreal, CAN.
13) **Lee JY**, Ismail OZ, Zhang X, Haig A, Lian D, Gunaratnam L. 2017. Donor kidney injury molecule-1 promotes graft recovery by regulating systemic necroinflammation. American Transplant Congress; Chicago, USA.

RESEARCH EXPERERIENCE

2015-present **Ph.D. Candidate,** Schulich Medicine and Dentistry: Department of Microbiology and Immunology
- Investigating the role of Kidney Injury Molecule -1 in Renal Transplantation

2013-2015 **Research Assistant,** Western University: Department of Biology
- Looked at the effect of different biotic and abiotic conditions on flight performance of the army worm

TEACHING EXPERIENCE

2018-2019 **Teaching Assistant**, Western University

MICROIMM 3300B

2015-2017 **Exam Marker**, Western University Medical School

Infection and Immunity (1st year medical students)

SUPERVISORY EXPERIENCE

2017-2019	Undergraduate Student Michael Lee
2017-2018	Honours Students Ingrid Hon and Jerry Ding
2016-2018	SRTP Students Jamie Lee and Haider Abed
2016-2017	Honours Student Shivam Singh