9-2-2014 12:00 AM

Cell Death Regulates Injury and Inflammation During Renal Allograft Transplantation

Arthur Lau
The University of Western Ontario

Supervisor
Anthony M. Jevnikar
The University of Western Ontario Joint Supervisor
Zhuxu Zhang
The University of Western Ontario

Graduate Program in Pathology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

Cell Death Regulates Injury and Inflammation During Renal Allograft Transplantation

(Thesis format: Integrated Article)

by

Arthur Lau

Graduate Program in the Department of Pathology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

Renal transplantation invariably results in tissue injury resulting from ischemia reperfusion injury (IRI), inflammation, drug toxicity, and rejection. Tubular epithelial cells (TEC) comprise the majority of renal parenchyma and are susceptible to cell death and injury during diverse forms of inflammation, which has direct and indirect effects on long term allograft function. Renal TEC have the unique ability to attenuate inflammation and alloimmune injury through the expression of various mediators of cell death and inflammatory molecules. Inhibition of cell death pathways in renal allografts may influence outcomes of alloimmune responses and graft survival. In this body of investigation, alteration of apoptosis and necroptosis forms of TEC death *in vitro*, were tested for their ability to extend allograft survival *in vivo*. Apoptotic death induced by cytotoxic cells during allograft rejection was inhibited by TEC expression of Granzyme B inhibiting serine protease inhibitor-6 (SPI-6) and prolonged graft survival and function. Apoptosis death of TEC can also be initiated during renal IRI and with rejection by pro-inflammatory cytokines through surface death receptors. However, inhibition of TNFα-induced apoptosis in TEC through caspase-8 upregulated the receptor interacting protein kinase 1 and 3 (RIPK1/3)-mediated necroptosis pathway to limit graft survival. However, inhibition of RIPK1/3 necroptotic death during renal IRI and transplantation was able to preserve renal function and promote long term graft survival. Augmented pro-inflammatory effects following necrotic cell death were related to an increased release of high mobility group box 1 (HMGB1). Use of the HMGB1 inhibitor glycyrrhizic acid (GZA) inhibited inflammatory responses *in vitro* and was able to ameliorate renal IRI. Collectively these studies highlight the importance of endogenous donor kidney factors in regulating inflammatory cell death and subsequently the severity and outcomes of allograft rejection. Regulators of parenchymal cell death in kidney and other solid organs may provide entirely new therapeutic targets for transplantation which will promote long term allograft survival.

Keywords

Kidney transplantation, ischemia reperfusion injury (IRI), cell death, apoptosis, serine protease inhibitor-6 (SPI-6), necroptosis, receptor interacting protein kinase 1 (RIPK1), receptor interacting protein kinase 3 (RIPK3), high mobility group box-1 (HMGB1), glycyrrhizic acid (GZA)

Co-Authorship Statement

The following co-authors contributed to the publications listed in this thesis:

SPI-6 (Serine Protease Inhibitor-6) inhibits granzyme B mediated injury of renal tubular cells and promotes renal allograft survival

Karim Khan – research design

Alex Pavlosky – image analysis

Ziqin Yin – animal care

Xuyan Huang – animal care

Aaron Haig – histological analysis

Weihua Liu – immunostaining

Bhagi Singh – research design, contribution of reagents/equipment

Zhu-Xu Zhang – research design, writing of manuscript, data analysis

Anthony M. Jevnikar - research design, writing of manuscript, data analysis, contribution of reagents/equipment

RIPK3 mediated necroptosis promotes donor kidney inflammatory injury and reduces allograft survival

Shuang Wang – microsurgery

Jifu Jiang – microsurgery

Aaron Haig – histological analysis

Alexander Pavlosky – research design

Andreas Linkermann – research design

Zhu-Xu Zhang - research design, writing of manuscript, data analysis, contribution of reagents/equipment

Anthony M. Jevnikar - research design, writing of manuscript, data analysis, contribution of reagents/equipment

Glycyrrhizic acid (GZA) ameliorates HMGB1-mediated cell death and inflammation after renal ischemia reperfusion injury

Shuang Wang – microsurgery

Weihua Liu – immunostaining

Aaron Haig – histological analysis

Zhu-Xu Zhang - research design, writing of manuscript, data analysis, contribution of reagents/equipment

Anthony M. Jevnikar - research design, writing of manuscript, data analysis, contribution of reagents/equipment

Acknowledgments

I would like to thank my supervisors Dr. Zhuxu Zhang and Dr. Anthony M. Jevnikar for their mentorship and guidance throughout my Ph.D. candidacy. I would not have been able to complete this thesis nor publish my research projects without their significant support and patience. Their passion for transplantation research convinced me to join their laboratory as a graduate student and that same passion has motivated me to continue my journey to becoming a better researcher. I hope that I have met their expectations during the time that I have spent as their student and I will always grateful to them for the opportunities that they have provided for me.

I would also like to thank Dr. James Koropatnick and Dr. Weiping Min for serving on my advisory committee throughout my thesis. Their insightful advice and assistance has improved the quality of my research for which I am very grateful.

I would like to thank all past and present members of the Zhang/Jevnikar lab at the Matthew Mailing Centre including Kelvin Shek, Shuang Wang, Alex Pavlosky, Jifu Jiang, Winnie Liu, Xuyan Huang, Ziqin Yin, and Pamela Gardner. Any techniques that I have learned in the lab are a result of their patient guidance and support and I am greatly appreciative of the time and energy they have spent assisting me in my research endeavors. I also thank them for their kindness and friendship over the years that I have spent at Robarts/Matthew Mailing Centre as part of their ‘lab family’.

I would like to thank the Department of Pathology including past and present graduate students, faculty, and staff. I believe that our graduate department excels at fostering an environment conducive to the development of young scientists and I consider myself lucky to have benefitted from that. The opportunities that have been provided by the department have been instrumental to the progression and completion of my degree for which I am grateful for.

I am thankful for all the wonderful friendships that I have made with the people at Western University and in the community of London, Ontario. Their continued support and great company have made my time here an incredible and unforgettable experience. I will always

cherish their kindness and generosity and thank them for making my experience at Western full of enjoyment and laughter.

Finally I thank my family and girlfriend, Christina, for their love and support throughout my graduate school career. Without them, I would not have been able to make it through the difficult times in both school and in life. Most importantly, I thank them for reminding me to pursue the things in life that make me and the people I love happy with absolute dedication and passion. They have taught me the value of being a kind and loving person above all else and for that I will be forever grateful.

Table of Contents

List of Figures

1 Introduction

2 SPI-6 (Serine Protease Inhibitor-6) inhibits granzyme B mediated injury of renal tubular cells and promotes renal allograft survival

3 RIPK3 mediated necroptosis promotes donor kidney inflammatory injury and reduces allograft survival

List of Appendices

Chapter 1

1 Introduction

1.1 Current challenges in kidney transplantation

End stage renal disease (ESRD) arising from a variety of chronic kidney diseases (CKD) such as diabetes, high blood pressure, glomerulonephritis, and polycystic kidney disease affects millions of people worldwide. CKD often results in irreversible kidney which requires dialysis or kidney transplantation for survival. While dialysis is a viable treatment for ESRD, long term survival rates (>5 years) in patients receiving dialysis can vary from 90% to as low as 25% depending on the age and primary cause of ESRD making this a temporary solution for many patients[1]. In addition, the prevalence of ESRD has been increasing annually which not only applies financial burden on patients but also to the healthcare system as lifelong dialysis is costly and not every patient can receive a kidney graft. In Canada, approximately 55% of patients with ESRD are on some form of dialysis while the remainder are living with functioning kidney transplants[1]. Unfortunately as of 2013, approximately 3400 patients were still waiting for kidney transplants and only 1400 kidney transplants were performed that year[1]. As kidney transplantation is currently the preferred long term option for treatment of ESRD, it is crucial that the limited organs that are available for transplant are functional for as long as possible in transplant recipients.

While the first successful kidney transplantation was performed in 1950, it was not until 1953 when the first successful long term transplants were performed in Boston in identical twins. It has since become the most common form of solid organ transplantation. Due to differences in the genetic backgrounds of donors and recipients (with the exception of identical twins), all donor organs are allografts, and are thus recognized as foreign bodies by recipient immune systems. In the absence of clinical intervention, this results in immune-mediated responses against the allograft, which result in transplant rejection and possibly leading to graft loss and failure. Through the development of donor-recipient matching to ensure greater histocompability as well as improvements in immunosuppression therapies, the incidence of acute transplant rejection has been significantly reduced over the last few

decades[1]. Currently, short term survival rates for adult kidney recipients are excellent with 1 year survival rates at over 95% and 5 year survival rates at over 80%[2]. However, the ultimate goal of transplantation is to achieve long term graft survival with continuing good graft function. When kidney recipients are followed long term, graft survival rates are significantly reduced to as low as 20% with increased morbidity and early mortality[2]. Therefore, chronic loss of function from immune and non-immune mechanisms of injury remains a major challenge for kidney transplantation despite our advances in diagnostics and therapeutics.

There are many factors that contribute to chronic kidney rejection. Inherent to the procedure is ischemia reperfusion injury (IRI) that occurs early during the transplantation following the removal of organ from the donor and the clamping of blood vessels in the recipient. Following vascular anastomoses, the allograft must then contend with constant attack from the recipient immune system initially triggered by differences in cell surface molecules collectively known as the major histocompatibility complex (MHC) which defines the recognition of all tissue as 'self' or foreign[3,4]. Use of potent immunosuppressive drugs can prevent or atteuate acute rejection but recipients risk becoming immunodeficient and then can have increased susceptibility to infectious diseases and cancer. The challenge is to balance anti-rejection therapy and the need of recipients to maintain immune responses against pathogens and other dangers in order to enhance long term kidney allograft survival. While there has been a major focus on attacking immune responses of the recipient, the graft itself through injury and death of cells may influence those immune responses. Modifying the graft rather than the recipient immune response represents a departure from the current paradigm of transplant therapy. In this thesis, new mechanisms of cell death, graft injury, and inflammation will be presented to provide a more contemporary understanding of chronic graft rejection with the potential to discover and apply new therapeutic strategies and targets.

1.2 Renal ischemia reperfusion injury (IRI)

IRI invariably occurs as a result of organ transplantation and has effects on both acute kidney injury (AKI) as well as long term graft survival[5,6]. Ischemia occurs when blood supply is removed from tissue causing a hypoxic and nutrient deficient environment as well as the accumulation of metabolic byproducts. When blood is restored to the tissue, reperfusion injury occurs due to the reintroduction of oxygen and formation of reactive oxygen species (ROS), which causes oxidative stress. Collectively, IRI leads to the injury of tissue, recruitment of immune cells, and inflammation in the kidney, causing AKI and impaired kidney function. Renal parenchymal cells, particularly in the nephron, are particularly susceptible to damage by inflammation during IRI due to their high oxygen demands and important functional role. Tubular epithelial cells (TEC) comprise the majority of kidney cells and are sensitive to damage due to IRI, especially those located in the proximal tubules found in the cortical region[7–11]. Proximal TEC are specialized cells responsible for removing water and other solutes back into the blood. Therefore, their loss or dysfunction leads to organ dysfunction and with death that is not matched by replacement, there is fibrosis. TEC are thus critically involved in the progression of tubulointerstitial injury following IRI.

Inflammation as a result of renal IRI is a complex interaction between cellular and molecular components of both the immune system as well as renal parenchyma. After IRI, an influx of various immune cells begins to infiltrate the kidney tissue and migrate to sites of inflammation[7,12–15]. TEC are capable of producing a variety of pro-inflammatory cytokines such as interferon gamma (IFNγ), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1) that can both attract and activate immune cells[13,16–18]. Cells associated with the innate immune response such as neutrophils and macrophages are one of the earliest responders and are pathological hallmarks of IRI[13]. These cells can also release various inflammatory cytokines that in turn attrach more immune cells leading to the propagation of inflammation. Interestingly, traditional members of adaptive immune responses are also found prominently during IRI including T cells, B cells, and NK cells[7,13].

In addition to the production of pro-inflammatory cytokines, immune cells can also possess direct cytotoxic functions that if activated during inflammation, can kill TEC (Figure 1.1). Cytotoxic lymphocytes (CTL) can kill target cells directly via cell-to-cell interactions by

various mechanisms including Fas-Fas-ligand (FasL) and perforin/granzyme pathways[7,19,20]. Interestingly, TEC exposed to inflammatory cytokines can also participate in cell mediated death through fratricide by Fas-FasL interactions and TEC death can be independent of direct cytotoxic cell contact[8,21]. TEC can also be killed indirectly by other receptor mediated death pathways via tumor necrosis factor alpha (TNFα) or other death inducing molecules secreted by immune cells or neighbouring parenchymal cells[9,22–25]. Acute tubular necrosis (ATN) as a result of TEC destruction is a pathological hallmark of AKI and leads to the development of renal fibrosis which affects short term and long term kidney function[7,9,22,23,26,27].

The extent and severity of IRI is highly correlated with poor outcomes in renal transplantation in large due to its impact on TEC as well as endothelial cells. Currently, donor kidneys are divided into several categories based on the nature of the cause of death including following circulatory arrest or cessation of neurological activity or perfusion. As well, there are differences in donor grafts obtained from living or deceased patients which affects the outcome of transplantation[2,6,28]. Organs from living donors have better outcomes in recipients after kidney transplantation as surgeries can be planned to optimize the condition of the recipient and IRI can be limited by having much shorter storage times. In addition, the health status and pre-operative condition of the donor could also minimize the effects of IRI in live donations[29]. Ideally, all transplants would occur with grafts from living donors or from deceased donors in which IRI can be prevented. Donation after cardiac death (DCD) with the loss of heart function exposes kidneys to ischemic injury resulting in high delayed graft function rates. It is clear that the optimal storage of organs of deceased donors is crucial as the viability of the organ must be maintained before transplantation[30]. For this reason, the use of normothermic storage rather than hypothermic storage has recently been tested along with pulsatile perfusion rather than static perfusion. It has been well documented that IRI occurring during the preservation process affects graft function and survival and thus, further research into the prevention of IRI will allow for the use of deceased donor organs to greater beneficial effect in recipients[5,6,29,31,32].

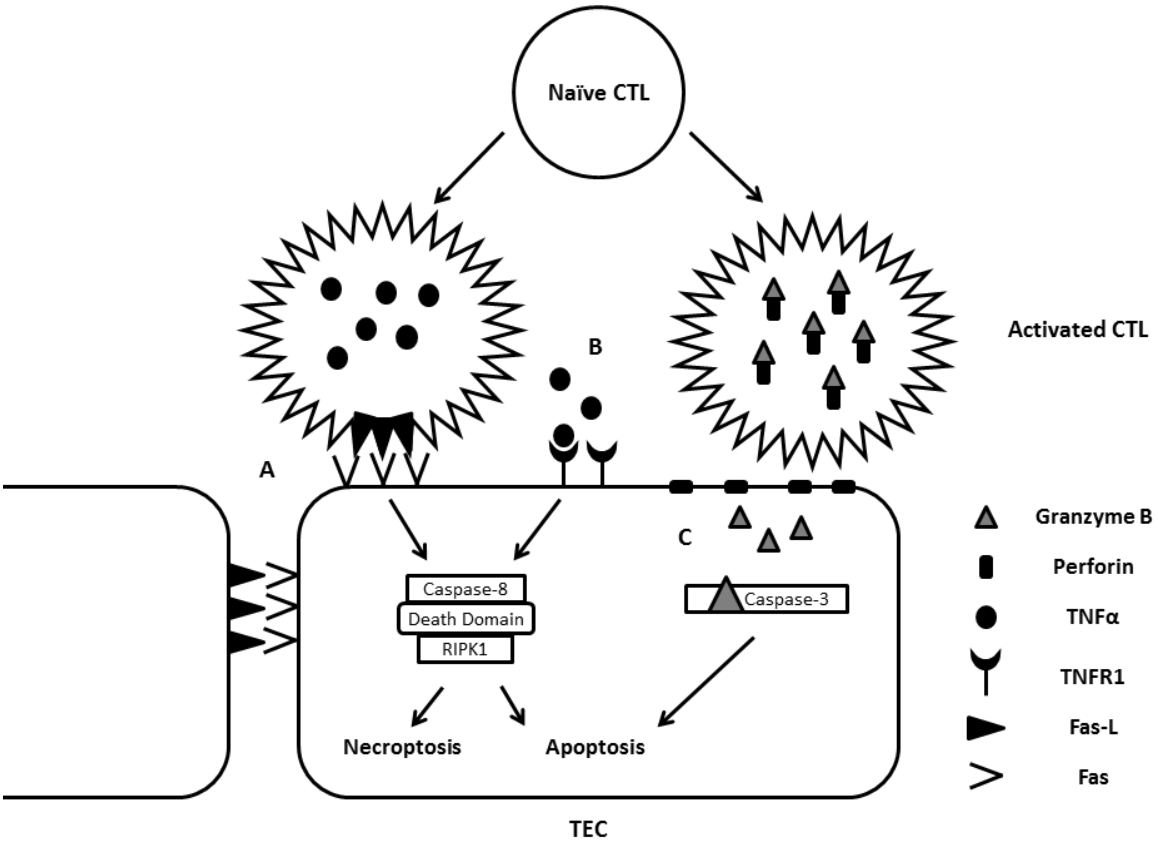

Naïve CTL
Activated CTL
A
B
C
Caspase-8
Death Domain
RIPK1
Caspase-3
Necroptosis
Apoptosis
TEC
Granzyme B
Perforin
TNFα
TNFR1
Fas-L
Fas

Figure 1.1 Mechanisms of cell death in renal IRI and transplantation.

Cell death is activated via various pathways in TEC by pro-inflammatory cytokines and cytotoxic lymphocytes (CTL). A) When CTL become activated and reach the target TEC, surface expression of Fas-L interacts with Fas receptors on TEC. This activates both apoptotic and necroptotic pathways in the TEC depending on the status of caspase-8 within the cell. B) When soluble TNFα is released from activated CTL, it can activate tumor necrosis factor receptor-1 (TNFR1) on TEC. This initiates both apoptotic and necroptotic pathways as well, similar to Fas signaling. C) Activated CTL upregulate production of granzyme B within granules which complex with perforin. Upon interaction with the target TEC, perforin forms pores in the surface of the TEC allowing for internalization of granzyme B. Intracellular granzyme B then activates caspase-3 and induces apoptosis in the target cell.

1.3 Allograft rejection

Allograft rejection occurs as a result of the recipient immune responses which arise from the recognition of the transplanted organ as a foreign body. This leads to an immune-mediated attack on the foreign tissue resulting in graft dysfunction or loss. Rejection is typically determined by clinical measures: 1) symptoms, 2) change in function as measured by serum creatinine and 3) histological analysis of biopsy tissue[32,33]. As the ability of the kidney to filter solutes, maintain acid-base pH, recover needed ions, minerals, and amino acids, and remove waste diminishes, the level of function decreases. Thus, the creatinine levels in the blood will accumulate and elevated levels of serum creatinine reflect a loss of renal function. Biopsies of recipients are routinely performed post-transplant to monitor any histological changes in the graft and are typically read by a transplant pathologist. The Banff score has been used to grade the level and type of rejection that is occurring and uses various pathological categories such as interstitial infiltration, fibrosis, tubular atrophy, and complement deposition[33]. Based on the results of these diagnostic tests, the clinician can then recommend an appropriate course of treatment depending on the type and severity of rejection.

Hyperacute/Acute rejection

Hyperacute rejection is defined by lack of function and loss of the allograft in a very short time span, usually hours to days after transplantation and can be life threatening. In the early days of transplantation, this was a common occurrence in solid organ transplantation as there were no methodologies to properly assess pre-existing antibodies to match donor organs with recipients and minimal immunosuppression therapies. As well, early transplants had the donor and recipient share little or any human leukocyte antigens (HLA), although this continues to be standard despite sophisticated methods of testing. HLA are the human version of genes which encode for MHC antigens presented on the surface of all cells. HLA mismatching between graft and recipient typically can increase cell mediated rejection and be the basis of antibody mediated rejection if alloantigen exposure in the past has resulted in pre-sensitization. Cellular mediated rejection can also be due to mismatched MHC class 1 and class II antigens although current drugs have been very successful in preventing acute rejection. Current strategies in HLA compatibility involve the use of both serology and

molecular methods such as PCR to identify the HLA profiles of both donor and recipient by crossmatching as many HLA groups as possible, as well as testing for and avoiding pre-existing allo-antibodies that could target the graft[34]. In the kidney, hyperacute rejection is antibody mediated as pre-existing antibodies against the donor antigen, potentially due to a prior blood transfusion, transplant, or pregnancy, will cause rapid loss of the graft unless antibodies are removed[35]. Current clinical practices involve use plasmapheresis, intravenous immunoglobulin (IVIG), as well as immunosuppressive drugs that target B cells such as anti-CD20 depleting antibody (rituximab), cyclosporine, rapamycin, and anti-thymocyte globulin (ATG) to prevent hyperacute rejection. They are not always successful and graft loss can be rapid and devastating.

Acute rejection can have a time course similar to hyperacute rejection (i.e. days) however the process may occur months to years after transplantation. Acute rejection can be suspected clinically and detected through the use of biopsies and functional assays such as serum creatinine. Episodes of acute rejection are treated with aggressive immunosuppressive therapies and are variably responsive[36]. The formation of acute rejection requires components of cellular-mediated rejection in which antigen presenting cells such as dendritic cells present donor antigen to T cells. This leads to effector immune responses that can result in CTL mediated or even antibody mediated destruction of the graft[35,37]. CTL recognize the foreign tissue via T cell receptor interaction with HLA and killing is through the use of various cytotoxic mechanisms, including perforin/granzyme and Fas-FasL. These cause death of target cells leading to graft dysfunction and loss[38]. In addition, infiltrating immune cells release pro-inflammatory cytokines which can either activate CTL or indirectly induce cell death through receptor mediated cell death in target cells. Current immunosuppression therapies are directed to deal with cellular-mediated and antibody-mediated rejection which has marginally increased 10 year survival rates of transplant recipients over the last decade[1,2]. However, repeated episodes of acute rejection may lead to the development of chronic rejection.

Chronic rejection

Chronic rejection occurs later, typically 5-10 years after transplantation and leads to slow loss of graft function. Loss of kidney graft function at 10 years post-transplant is invariably

associated with the development of fibrosis following rejection, drug toxicity, or recurrence of the original disease. Chronic rejection viewed as premature graft failure remains a challenge as up to 50% of kidney grafts are lost due to this[2]. Hallmarks of chronic rejection include tubular atrophy and fibrosis most prominent in the vascular and tubular compartments[32,34]. When fibrosis occurs in blood vessels, it is known as allograft vasculopathy, which appears as a narrowing of the blood vessels[32,34]. This can cause a decrease in blood flow leading to localized ischemic and inflammatory injury. As with acute rejection, both antibody and cellular mediated rejection can both contribute to chronic kidney rejection although their individual contributions are difficult to assess[35]. Due to the underlying inflammation that occurs throughout chronic rejection, various immune cells and damaged tissue upregulate transforming growth factor-beta (TGFβ) which stimulates the growth of fibroblasts and promotes fibrosis[35,39]. Fibroblasts secrete numerous extracellular matrix proteins including collagen which leads to formation of scarring in the tissue. The mechanism in which fibrosis occurs in the kidney during chronic rejection is not well understood however its effects are evident due to the eventual loss of graft function. Unfortunately, there are currently no strategies to prevent chronic injury or fibrosis and while the process can be slowed, generally it is irreversible. Following graft loss, patients must go back on dialysis or receive another transplant.

1.4 Cell death and kidney transplant injury

During IRI and transplantation, tissue injury occurs resulting in the death of parenchymal cells in the kidney[7,8,10,11,22]. The proximal tubular epithelial cells are particularly sensitive to ischemic and inflammatory injury due to their high metabolic activity and cell surface membrane polarity. Typically, necrosis of TEC (ATN) is the histological hallmark associated with AKI and early transplant injury. Depending on the severity and extent of ATN, this can recover with the regeneration of TEC or lead to the progressive development of renal fibrosis and ultimately graft failure. Therefore, cell death in the kidney parenchyma has profound effects on the outcome of graft function and survival. TEC can undergo various forms of programmed and non-programmed cell death due to complex and variable number of factors that regulate injury and inflammation during renal transplantation.

Classification of cell death and nomenclature

Cell death can be categorized as either programmed or non-programmed. Programmed cell death (PCD) refers to a process in which a cell undergoes a stepwise regulated biochemical process in response to specific stimuli leading to the death of the cell. Forms of classical PCD include apoptosis, a death pathway regulated by proteolytic activation of caspases, and autophagy, a form of cell death that occurs during nutrient deprivation. Conversely, non-programmed cell death, referred to as necrosis, leads to cell swelling, nuclear dissolution, loss of plasma membrane integrity, and cell lysis. However, advancements in the definition of new cell death pathways have revealed that various forms of regulated necrosis can occur which are collectively referred to as programmed necrosis (PN)[40]. Different forms of programmed necrosis can be classified by either their regulators or triggers which can vary from pathogens, death inducing ligands, metabolic byproducts, or chemical stress. Pyroptosis, a pathogen induced necrosis, is regulated by caspase-1 and activated by the formation of the apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) complex which induces a pro-inflammatory response towards the microbial infection[41]. Glutamate toxicity can lead to ferroptosis, an iron dependent form of necrosis, which results in lethal accumulation of ROS and activation of poly(ADP-ribose) polymerase 1 (PARP1) leading to necrosis[42,43]. Programmed necrosis represents a new direction in the field of cell

death research as previously unreported forms of programmed necrosis are being identified in diseases which may have effects on propagation of tissue injury and inflammation.

Apoptosis, a form of physiological programmed cell death, occurs in the kidney as TEC renewal occurs frequently. During renal transplantation, the rate of renal parenchymal cell death, particularly in epithelial and endothelial cells, outpaces renewal due to AKI and inflammation leading to graft dysfunction and decreased survival. Study of cell death pathways in renal allografts may reveal a major regulator of transplant injury as parenchymal cell death occurs predominantly through via cytotoxic lymphocytes that use Fas-FasL and/or perforin/granzyme or death inducing cytokines such as TNFα. Previous studies in mouse renal IRI models have reported that inhibition of caspase dependent apoptosis via shRNA targeted towards Fas and caspase-8 effectively reduced kidney injury[9]. In addition, inhibition of TNFα and its receptor TNFR1 was able to attenuate kidney injury in a mouse model of renal IRI which suggests a role for receptor mediated cell death in kidney inflammatory injury[44]. Analysis of human renal allograft biopsies undergoing acute rejection confirm that TNFα, TNFR1, and TNFR2 are expressed on renal parenchymal cells[45]. Expression levels of perforin and granzyme B were also upregulated in renal allografts undergoing acute rejection compared to non-rejection controls[46]. Furthermore, a form of programmed necrosis, termed necroptosis, may also be a regulator of allograft injury as murine studies in renal IRI and nephrotoxicity have revealed that inhibition of necroptosis attenuated kidney injury[47,48]. Together these studies support the hypothesis that programmed cell death, particularly apoptosis and necroptosis, may propagate inflammatory mediated injury during renal transplantation.

Cell death and induction by cytotoxic lymphocytes

During renal IRI and transplantation, graft infiltrating immune cells mediate tissue injury and graft dysfunction through cytotoxic activity largely directed towards kidney parenchymal cells, and in particular TEC and endothelial cells. Cytotoxicity can be mediated by a variety of immune cells including $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and other mononuclear cells through cell-to-cell contact as well as by release of mediators such as TNFα that bind to cell death receptors[35,37]. Activated cytotoxic cells have been shown to upregulate the serine protease, granzyme B, which forms in granules and complexes with perforin[49,50]. Upon

contact with the target cell, activated CTL secrete perforin/granzyme B which can enter target cells through perforin formed pores to activate caspase-3, leading to apoptosis (Figure 1.1). To prevent self-directed apoptosis, cytotoxic cells and other cells express the serpin protease inhibitor-9 (PI-9) to block granzyme B action[50–52]. PI-9, and its murine homolog serine protease inhibitor-6 (SPI-6), inhibits granzyme B activity by binding to it and through protease cleavage, causes irreversible binding between PI-9 and granzyme B[53]. Recent studies have identified the expression of PI-9 in renal allografts with subclinical rejection suggesting that the kidney may potentially be capable of self-regulating tissue injury by inhibiting cytotoxic attack from infiltrating cells[54,55]. Upregulation of PI-9/SPI-6 in TEC may thus represent a potential therapeutic strategy for limiting cytotoxicity in renal transplantation and may improve long term graft function and survival.

Apoptosis

Apoptosis is a form of programmed cell death occurring in almost all cell types. Cells undergoing apoptosis will express key morphological features including condensation and fragmentation of DNA, cell shrinkage, and membrane blebbing resulting ultimately in the death of the cell[56]. This phenotype of death is triggered by molecular events within the cell through the activation of a family of catalytic proteases known as caspases. Caspases can be either initiator caspases (*e.g.* caspase-8, caspase-9), which activate other caspases, or effector caspases (*e.g.* caspase-3, caspase-6), which cleave substrates that activate the apoptotic pathway. This series of protease activations is known as the caspase cascade[56]. Apoptotic cells typically undergo the following morphological changes:

1) Cell shrinkage due to changes in cytoskeleton.
2) Chromatin condenses in a process known as pyknosis
3) Degradation of the nucleus resulting in DNA fragmentation known as karyorrhexis.
4) Cell membrane blebbing leads to breakdown of the cell into vesicles known as apoptotic bodies.

Induction of the apoptosis can occur through the intrinsic or extrinsic pathway as a result of cellular stress. The intrinisic apoptosis pathway is primarily controlled by mitochondrial membrane permeability through a family of apoptosis regulators known as Bcl-2. These

proteins control activation of mitochondrial permeability transition pores which allows for leakage of intra-mitochondrial proteins into the cytoplasm[48]. When mitochondria become dysfunctional and leak apoptosis inducing proteins such as cytochrome c and small mitochondria derived activator of caspases (SMAC), these proteins initiate apoptosis either by deactivating inhibitors of apoptosis proteins (IAP) allowing for caspase activation or they can directly induce apoptosis by binding to various such as apoptosis protease activating factor-1 (Apaf-1)[57]. Released cytochrome c complexes with Apaf-1 and ATP, resulting in the formation of the apoptosome, which in turn activates caspase-9 leading to apoptosis.

In contrast, extrinsic apoptosis occurs when an extracellular signal activates the programmed death pathway through a variety of cell surface receptors linked to intracellular adaptor proteins (Figure 1.2). These receptors are collectively known as death receptors and include members of the tumor necrosis factor receptor (TNFR) superfamily including TNFR1 which binds to TNFα and Fas (CD95) which binds to Fas-ligand (Fas-L, CD95L). Upon activation of the death receptor, adaptor proteins with death domains (DD) such as Fas associated death domain (FADD) forms a complex with caspase-8 collectively known as the death inducing signal complex (DISC) to initiate the caspase cascade leading to apoptosis[58]. Another method of caspase activation occurs when cytotoxic cells attach to target cells and granzyme B is released into the target cell which then activates caspase-8 and 3 to initiate apoptosis[49]. Caspase-8 activity is tightly regulated as its importance to cellular function is highlighted by embryonic lethality seen in caspase-$8^{-/-}$ mice[59]. One major regulator of apoptosis in TEC is cellular FADD-like IL-1β-converting enzyme (FLICE)-inhibitor protein (c-FLIP) which prevents catalytic activation of caspase-8 by binding to pro-caspase-8 and death domain complex[58].

Measurement of apoptotic death *in vitro* and *in vivo* has been well characterized with a variety of methodologies. Apoptotic cells *in vitro* or *in vivo* can be visually confirmed by microscopy using the aforementioned morphological features of apoptosis including cell shrinkage and DNA condensation. In addition, the inversion of the plasma membrane during apoptosis can also be detected via surface exposure of phosphatidylserine (PS) using Annexin-V labeling and analyses by flow cytometry[60]. Other molecular events such as the activation of caspases by protease activity can also be detected via immunoblotting to detect caspased related apoptotic death. However, as many modalities of cell death share similar

molecular characteristics, use of cell death inhibitors can be used for more definitive determinations of forms of cell death. Apoptosis can also be inhibited by genetic deletion embryonically or via caspase-targeted siRNA[9,61,62] as well as by small molecule caspase inhibitors such as carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (zVAD-fmk), a pan-caspase inhibitor[47,63], and Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone (zIETD-fmk), a caspase-8 inhibitor. *In vivo* studies of apoptosis in tissue and also in cells use terminal deoxynucletotidyl transferase dUTP nick end labeling (TUNEL) to detect DNA fragmentation. Immunostaining of tissue sections also allow for the detection of caspase activation *in vivo* to visualize apoptotic death.

Previous studies have revealed the importance of apoptosis in both IRI and transplant injury in the kidney. *In vitro* studies using caspase inhibition have demonstrated that TEC can undergo receptor mediated apoptosis by various ligands including TNFα and Fas-L[9,22,47]. Apoptotic TEC by TUNEL can be observed in cortical regions of the kidney after IRI and transplantation along with acute tubular necrosis[10,38,64,65]. Studies using siRNA as well as small molecule inhibitors targeted against caspase-3 and 8 in the kidney have been shown to be protective in renal IRI but additional studies are required to determine their efficacy in a transplant setting[9,61,62]. Therefore, inhibition of apoptosis in the kidney appears to protect against AKI in animal models and thus could potentially represent a novel target for the prevention of transplant injury and rejection.

Necroptosis

Recent studies into new cell death pathways have revealed a form of programmed necrosis known as necroptosis (Figure 1.2). Initial studies observed that some cells exposed to TNFα treated cells *in vitro* underwent a form of programmed cell death similar to necrosis rather than apoptosis when in the presence of caspase-8 inhibition[58,66–68]. Although apoptosis was inhibited in these cells, necrotic type death was clearly detected by flow cytometry using PI labeling and electron microscopy. This form of necrosis appeared to be negatively regulated by caspase-8 activity. Further studies revealed that two key checkpoint molecules were involved in the activation of the necroptotic pathway, namely receptor interacting protein kinase-1 and 3 (RIPK1/3). Inhibition of either protein prevented necroptosis as it is now referred to[22,58,66,69–71]. During receptor mediated cell death, caspase-8 activity usually allows

for apoptosis to occur. However, in the absence of caspase-8 activity, RIPK1 and RIPK3 are able to form a complex through RIPK homotypic interaction motifs (RHIM) and these serine kinases activate each other by phosphorylation which leads to necroptosis. Phenotypically and biochemically, cells undergoing necroptosis are identical to those undergoing spontaneous necrosis characterized by loss of cell membrane integrity and release of intracellular contents[72]. Preliminary studies have shown that the phenotype of death observed in necroptotic cells is mediated by a downstream regulator activated by RIPK3, termed mixed lineage kinase domain-like (MLKL). MLKL has been shown to form trimeric structures on the inner plasma membrane to induce Ca^{2+} influx and death. However, more research must be done to fully elucidate the mechanism[73–75].

Physiologically, the role of necroptosis appears to have evolved as a 'fail-safe' response during infection and immunity[76,77]. Upon infection by micro-organisms that attempt to silence caspase-8 and apoptosis, mammalian cells have adapted the ability to trigger necroptosis in order to elicit an even greater immune response to the infectious agent. The release of intracellular contents such as cellular death associated molecular pattern (CDAMP) molecules elicits much greater inflammation as compared to apoptosis which does not release CDAMP and results in minimal inflammatory responses[78]. This is supported by the evolution of M45 peptide expression, an inhibitor of RIPK3 and necroptosis, by cytomegalovirus (CMV) which allows them to evade host immune responses more efficiently[70,79]. Interestingly and keeping with the primary role of caspase-8 as a suppressor of necroptosis, caspase-$8^{-/-}$ mice, which are embryonic lethal, can be salvaged from lethality when crossed with RIPK$3^{-/-}$ mice. The resulting double knockout progeny are viable indicating that loss of caspase-8 regulation of RIPK3 mediated necroptosis but can be rescued by RIPK3 deletion[66]. Necroptosis was also observed *in vivo* in various inflammatory disease models in which caspase-8 activity was inhibited through genetic manipulation or chemical inhibition[67,80–84]. In this thesis, the roles of caspase-8 and RIPK1/3 have in propagating injury and inflammation in renal IRI and kidney transplantation is clarified.

Although necroptosis has only recently been characterized, several methods have been developed to distinguish this form of cell death *in vitro* and *in vivo*[72]. As necroptosis is a form of programmed necrosis, it shares a similar if not identical morphology to non-programmed necrosis, which can be observed using electron microscopy. The necroptotic

phenotype thus includes nuclear shrinkage and cell swelling. The loss of cell membrane integrity features prominently in necroptosis and can be identified through the use of intracellular stains and flow cytometry including propidium iodide (PI), an intercalating fluorescent molecule which can bind with DNA following the loss of cell membrane integrity[85]. Intracellular contents can leak out of necroptotic cells into the extracellular space allowing for the detection of lactate dehydrogenase (LDH) and CDAMPs including high mobility group box-1 (HMGB1) and various heat shock proteins (HSP)[72,78,85,86]. As RIPK1/3 are key regulators of necroptosis, detection of phophorylated RIPK1/3 by immunoblot also indicate activation of the necroptotic pathway. As often with the case with apoptosis, necroptosis can be most definitively identified through the use of specific inhibitors or by siRNA or genetic knockouts targeted towards RIPK1/3 or MLKL which have been shown to block necroptosis[66,73]. Necrostatins, a class of small molecule inhibitors targeted towards RIPK1, have also been effective in inhibiting necroptosis although this class of small molecules may bind to other targets and RIPK1 also has a role in apoptosis[82,87]. A novel *in vivo* technique for detection of necrosis in tissue which we have adapted to demonstrate necroptosis in this thesis[88]. Briefly, ethidium homodimer (ETH), a fluorescent intercalating agent, is perfused into the solid organ allowing for labeling of necrotic tissue in a similar fashion to PI labeling. This allows for the visualization and importantly for the quantification of tissue necrosis using fluorescent microscopy.

It is currently unknown what effect, if any, apoptosis and necroptosis inhibition have on clinical renal transplantation outcomes as there is complex biology that is affected by immunosuppression that was not used in the animal studies in this thesis. However, key studies in various acute and severe injury models including pancreatitis and toxic shock syndrome have demonstrated that necroptosis has a powerful capacity to propagate inflammatory injury and that this can be targeted[81,83]. As well, necroptosis has been identified to play a role in renal IRI as necrostatin-1 (Nec-1) was able to inhibit AKI in murine studies[47]. In summary, necroptosis is highly correlated with inflammatory injury and is involved in the progression of transplant injury, which is a chronic inflammatory mediated process. The availability of new therapeutics offers great potential benefit to transplant patients but must be based on biological insight and mechanistic understanding as targeted inhibition of specific components of cell death pathways may have unintentional

consequences. It may be that targeting this newly recognized form of programmed cell necrosis may be important in regulating renal graft function and improving long term survival.

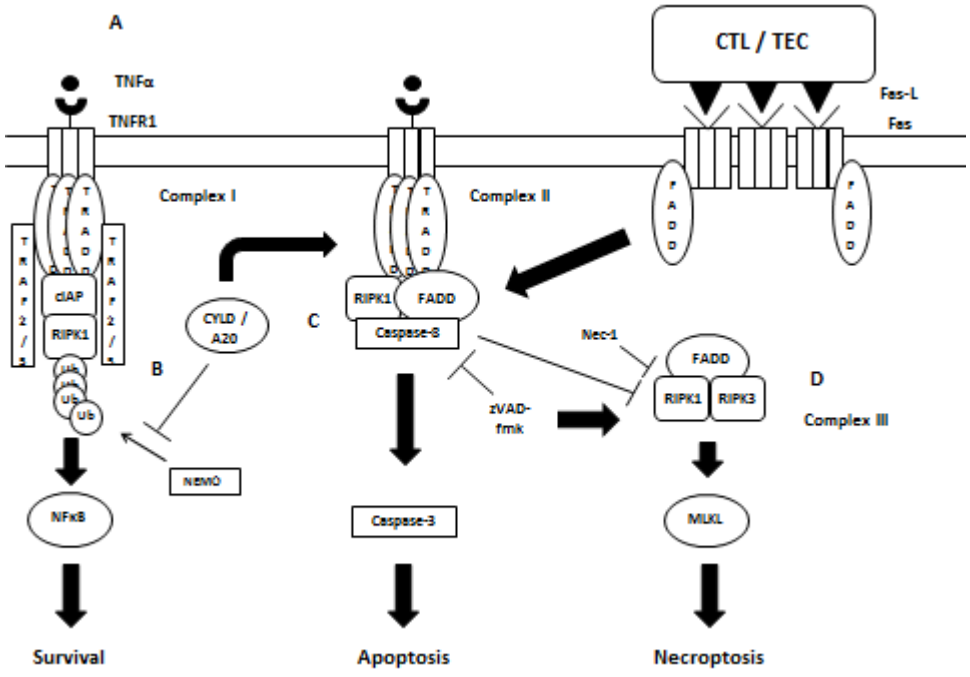

A
TNFα
TNFR1
Complex I
TRAF2/5
cIAP
RIPK1
Ub
Ub
Ub
Ub
TRADD
TRAF2/5
B
CYLD / A20
NEMO
NFκB
Survival
C
Complex II
TRADD
RIPK1
FADD
Caspase-8
Caspase-3
Apoptosis
zVAD-fmk
CTL / TEC
Fas-L
Fas
FADD
FADD
Nec-1
FADD
RIPK1
RIPK3
D
Complex III
MLKL
Necroptosis

Figure 1.2 Overview of receptor mediated apoptosis and necroptosis cell death pathways in TEC.

A) Extracellular death receptor ligands such as TNFα and Fas-L bind to their death receptors on the surface of TEC (TNFR1 and Fas resepectively) as a result of cytokine secretion or cell-to-cell activation. Activation of the death receptors results in recruitment of death domains (TRADD, FADD) allowing for the formation of death complexes. B) Formation of Complex I consisting of TRADD, TRAF2/5. cIAP, and ubiquitinated (Ub) RIPK1 results in activation of the NF-κB pathway and upregulation of pro-survival factors. Ubiquitination of RIPK1 is regulated by NF-κB essential modulator (NEMO) while de-ubiquitination is regulated by cylindromatosis (CYLD) and A20. When RIPK1 is de-ubiquitinated, it allows for the formation of Complex II and III, leading to cell death. C) RIPK1, FADD, and caspase-8 bind together upon TNFR1 signaling to form Complex II. This leads to activation of the caspase cascade leading to apoptosis. Caspase-8 activity can be inhibited by various regulators of cell death as well as small molecule inhibitors such as carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (zVAD-fmk). When caspase-8 is inhibited, Complex III can form leading to necroptosis. D) Inhibition of caspase-8 allows for FADD, RIPK1, and RIPK3 to complex, leading to the activation of mixed lineage kinase domain-like (MLKL). This results in activation of the necroptosis pathway which is phenotypically similar to necrosis leading to loss of cell membrane integrity and release of intracellular contents to the extracellular space. Inhibition of RIPK1 by necrostatin-1 (Nec-1) or RIPK3 prevents necroptosis.

1.5 Cell death results in the release of pro-inflammatory cellular death associated molecular patterns (CDAMPs)

The current understanding of infection and immunity has adopted the model of 'danger signaling' as the framework for immune surveillance and activation[89]. During infection, microorganisms can activate immune responses due to antigens present on their cell surfaces known as pathogen associated molecular pattern (PAMP) molecules. PAMPs are detected by various extracellular and intracellular receptors including Toll-like receptors (TLR) which are found on a variety of cell types including immune cells. Activation of TLR signaling by PAMPs results in the upregulation of pro-inflammatory responses in the form of cytokines which induce inflammatory responses towards the infectious agent in order to eradicate the pathogen. The principle of danger signaling is similar to that of PAMP signaling except that during necrotic cell death as a result of infection or injury, cellular death associated molecular pattern (CDAMP) molecules are released from cells[90]. CDAMPs also signal primarily through TLRs and can also upregulate the pro-inflammatory response during non-infectious injury leading to 'sterile' inflammation[17]. Necrosis thus acts as a 'beacon' for immune activation as unexpected or spontaneous death is typically due to infection or disease whereas apoptosis is a form of programmed death that is a part of normal physiological processes and therefore would not warrant an exaggerated immune response[91,92]. CDAMP molecules are typically proteins that reside ubiquitously in all cell types, where they carry out normal functions without immune responses, unless released into the extracellular space due to loss of plasma membrane integrity during necrotic cell death. Among the identified CDAMP molecules, high mobility group box-1 (HMGB1) and its role in immune activation and inflammation has been the most extensively studied.

High mobility group box-1 (HMGB1)

HMGB1 is a highly conserved protein found in all eukaryotic cells found primarily in the nucleus bound to chromatin and is involved in various DNA processes such as transcription[93]. The protein is comprised of Box A (amino acids (aa) 9-79), Box B (aa 88-162), and an acidic tail. Typically, HMGB1 is non-acetylated in order to maintain its presence in the nucleus however when acetylation occurs on the lysine residues located on the two nuclear localization signal (NLS) regions (aa 28-44, 179-185), HMGB1 disassociates

from DNA and translocates into the cytoplasm[94]. This post-translational modification can occur under pro-inflammatory conditions and can result in either secretion of HMGB1 or passive release due to necrosis[86]. HMGB1 can be secreted by certain activated immune cells such as macrophages[95] and dendritic cells[96] to act as a pro-inflammatory mediator in its extracellular form, while all cells can release HMGB1 when undergoing necrosis/necroptosis. Additional post-translational modifications can occur during cell death which affects the immunogenicity of HMGB1. For example, recent studies have demonstrated that oxidation of HMGB1 at cysteine resides (aa 23, 45, 106) can occur during pro-inflammatory conditions leading to cell death, likely due to the accumulation of reactive oxygen species[92]. The oxidized form of HMGB1 loses its cytokine-like activity and is non-immunogenic and in some cases, even promotes tolerance. In contrast, HMGB1 in a reduced state can be pro-inflammatory as either a chemoattractant or promoter of cytokine production[97].

HMGB1 is known to bind to various innate immune receptors including receptor for advanced glycosylation endproducts (AGE, RAGE), TLR2, and TLR4[98,99]. In some cases, HMGB1 in complexed with chromatin can also bind to TLR9 as well[100]. Specifically, the Box B region acts as the primary binding site for TLR4 and some studies have suggested that Cys-106 located in Box B may play a key role in HMGB1 and TLR4 interaction[97]. Many studies have highlighted the role of HMGB1 in various inflammatory diseases including renal IRI[101]. Neutralizing anti-HMGB1 antibodies during renal IRI in a murine model showed an inhibition of pro-inflammatory responses as well as improved kidney function although the amount of antibody needed and the degree of inhibition would suggest that this is not clinically feasible[101]. In addition, mouse chimera studies using $TLR4^{-/-}$ bone marrow transplants were resistant to renal IRI, supporting the importance of HMGB1/TLR4 interactions in AKI[16]. Interestingly, these studies showed that the greater benefit in IRI was with the loss of TLR4 in kidney cells rather than infiltrative cells. Thus, limiting extracellular HMGB1or limiting its interaction with kidney cells presents itself as an important target for the prevention of AKI and potentially renal transplant injury and rejection.

Other CDAMP molecules and signaling pathways

In addition to HMGB1, other CDAMP molecules have been associated with the innate immune response and inflammatory injury. Heat shock proteins (HSP) are protein folding

chaperones that are ubiquitously expressed and can be released upon necrotic cell death[102]. Of the HSP family of proteins, HSP60 and 70 are the best described as TLR signaling ligands, particularly TLR2 and TLR4[103,104]. In addition to its released pro-inflammatory stimulus form as a CDAMP, HSP70 can uniquely act as an antigen presenting molecule. Through interaction with antigen presenting cells (APC), HSP70 complexed with antigen through its chaperone function binds to CD91 resulting in immune activation[105]. Another potent CDAMP that is relevant to renal inflammatory injury is uric acid, a metabolic endproduct, due to the kidney's primary function of filtering out metabolic waste (and when dysfunctional can lead to accumulation of uric acid leading to kidney stones)[106]. When uric acid interacts with TLR2 or TLR4 on the surface of TEC, upregulation of various pro-inflammatory cytokines can be detected in TEC leading to inflammation[107]. In addition to released intracellular proteins, extracellular matrix proteins can also act as CDAMP molecules as well. During inflammatory injury, proteolytic damage to the extracellular matrix can occur caused by enzymes released by dying cells leading to the formation of protein fragments including hyaluronan, heparan sulfate, and biglycan[106]. These peptides can also bind to TLR2 and TLR4 leading to pro-inflammatory responses through Nfκb signaling. Emerging studies have suggested that other inflammatory pathways such as the NLRP3-mediated inflammasome pathway, which is typically activated when pathogens are detected, can be involved with CDAMP signaling when extracellular ATP released from necrotic is detected[108]. Further studies for delineating the mechanisms behind CDAMP-mediated inflammation are still required to identify the different biological functions of various CDAMP molecules and their contributions to inflammatory kidney injury. While the limitation of the effect of CDAMP within the kidneys seems attractive, there are currently no feasible therapeutic agents that are available that can do so effectively. It may be that the most effective approach in limiting inflammatory injury may include a combination of limiting apoptosis death significant enough to cause dysfunction, preventing necrotic cell death that release inflammatory CDAMP, and blocking the action of CDAMP that are released.

1.6 Aims and objectives

The overall aim of our research is to determine the impact of altering renal cell death on graft inflammation and its effects on overall kidney allograft function and survival. We hypothesize that endogenous factors in the kidney can regulate inflammatory cell death that will affect long term outcomes on graft function and survival by altering immune responses.

Aim 1 – Determine if renal allograft expression of SPI-6 can prolong graft function and survival.

Objective 1.1

Characterize the expression of SPI-6 in TEC *in vitro* under pro-inflammatory conditions and *in vivo* after renal transplantation.

Objective 1.2

Determine if SPI-6 expression in TEC provides resistance against cytotoxic lymphocytes.

Objective 1.3

Investigate the effects of SPI-6 expression in donor renal allograft on graft function and survival after kidney transplantation.

Aim 2 – Determine if inhibition of necroptosis in the kidney during renal IRI and transplantation is beneficial to renal function and graft survival.

Objective 2.1

Characterize expression of RIPK3 in TEC *in vitro* and kidney tissue *in vivo*.

Objective 2.2

Induce and measure necroptosis in TEC *in vitro* using small molecule inhibitors and genetically modified knockouts.

Objective 2.3

Determine if inhibition of necroptosis by loss of RIPK3 protects against renal IRI.

Objective 2.4

Determine if inhibition of necroptosis in the donor graft by loss of RIPK3 preserves graft function and prolongs survival after renal transplantation.

Aim 3 – Investigate the therapeutic potential of glycyrrhizic acid (GZA) on HMGB1 mediated inflammation and injury during renal IRI.

Objective 3.1

Characterize the release of HMGB1 after hypoxia induced TEC death.

Objective 3.2

Determine the effects of GZA on TEC death and production of pro-inflammatory molecules after hypoxia.

Objective 3.3

Investigate the effect of GZA therapy on renal function and tissue injury after renal IRI.

Chapter 2

2 SPI-6 (Serine Protease Inhibitor-6) inhibits granzyme B mediated injury of renal tubular cells and promotes renal allograft survival

Arthur Lau[1,2,3], Karim Khan[1,3], Alex Pavlosky[1,2,3], Ziqin Yin[1], Xuyan Huang[1], Aaron Haig[3], Weihua Liu[1,3], Bhagi Singh[1,4], Zhu-Xu Zhang[1,2,3*], Anthony M. Jevnikar[1,2,3*]

[1]Matthew Mailing Centre for Translational Transplantation Studies, London Health Sciences Centre; [2]Departments of Medicine, [3]Pathology, and [4]Microbiology & Immunology, Western University, London, Ontario, Canada.

Citation:

Lau A, Khan K, Pavlosky A, Yin Z, Huang X, Haig A, Liu W, Singh B, Zhang ZX, Jevnikar AM. SPI-6 (Serine Protease Inhibitor-6) inhibits granzyme B mediated injury of renal tublar cells and promotes renal allograft survival. *Transplantation*. 2014: Epub ahead of print. Wolters Kluwer Health Lippincott Williams & Wilkins©

2.1 Abstract

Background: Protease inhibitor 9 (PI-9) is an intracellular serpin that specifically inhibits granzyme B, a cytotoxic serine protease found in the cytosolic granules of cytotoxic T lymphocytes and natural killer cells. Enhanced cortical expression of PI-9 has been observed in kidney allografts with subclinical rejection, suggesting tubular epithelial cell (TEC) expression of this protein may have a protective role and attenuate overt allograft rejection.

Methods and Results: We demonstrate TEC express SPI-6 protein, the murine homolog of PI-9, basally with a modest increase following cytokine exposure. TEC expression of SPI-6 blocks granzyme B mediated death as TEC from SPI-6 null kidneys have increased susceptibility to cytotoxic CD8+ cells *in vitro*. The role of SPI-6 was tested in a mouse kidney transplant model using SPI-6 null or wild type donor kidneys ($H\text{-}2^b$) into nephrectomized recipients ($H\text{-}2^d$). SPI-6 null kidney recipients had reduced renal function at day 8 post-transplant compared to controls (creatinine: 113±23 vs. 28±3 μmol/L, n=5, $P<0.01$) consistent with observed tubular injury and extensive mononuclear cell infiltration. Loss of donor kidney SPI-6 shortened graft survival time (20±19 vs. 66±33 days, n= 8-10, $p<0.001$).

Conclusions: Our data shows for the first time that resistance of kidney TEC to cytotoxic T cell granzyme B induced death *in vitro* and *in vivo* is mediated by the expression of SPI-6. We suggest SPI-6 is an important endogenous mechanism to prevent rejection injury from perforin/granzyme B effectors and enhanced PI-9/SPI-6 expression by TEC may provide protection from diverse forms of inflammatory kidney injury and promote long term allograft survival.

2.2 Introduction

Protease inhibitor 9 (PI-9) is an intracellular serpin that specifically binds and inhibits granzyme B, a 29-kDa aspartic acid–directed protease, that is contained within the specialized secretory granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (1). PI-9 is expressed by CTL CD8+ T, CD4+ T, NK, Langerhan and dendritic cells (DC), as well as other mononuclear cells (2-6). Upon activation, CTL and NK cells synthesize granzyme B within granules which can “leak out” into cytoplasm, thus threatening ‘self’ viability through cleavage of caspases and initiation of apoptosis (2, 4-6). Activated effector cells proceed to induce apoptosis in target cells through release of perforin/granzyme B via surface pores allowing internalized granzyme B cleavage of intracellular caspases. As a mechanism of self-preservation, expression of PI-9 concurrently increases with granzyme B synthesis but is located prominently near granules where it can bind irreversibly to “leaked” granzyme B to protect cells from “misdirected” lethal granzyme B effects (2).

The role of PI-9 in renal transplantation has not been clearly defined. Kidney rejection is characterized histologically by CD4+ and CD8+ T, NK, B, and other mononuclear cell infiltrates. The invasion of these effectors into the tubular epithelium compartment (tubulitis) remains a central feature of cellular rejection (7), and intuitively contributes to progressive tubular injury and death. However, the relationship of infiltrate and severity of rejection is not entirely predictable due to the participation of multiple effector mechanisms utilized by different cell types (granzyme B, FasL, cytokines) as well as the expression of endogenous mechanisms of protection within TEC and other target cells. Thus, the presence of infiltrate in kidney allografts may not always represent clinically apparent rejection. Indeed, kidney allografts classified as having "subclinical rejection" have interstitial infiltration but lack a clinically apparent change in renal function (8). Interestingly in this regard, PI-9 has been observed to be more highly expressed in renal allografts undergoing subclinical rejection than those acutely rejecting grafts (8). As well, PI-9 mRNA expression in urinary cells of kidney transplant recipients undergoing acute rejection was higher compared to non-rejection controls (9). Therefore, kidney cells appear to have a capacity to express and regulate PI-9, which might afford protection against cytotoxic cell attack. Intriguingly, the progression from subclinical to clinically apparent rejection may be influenced both by the ability of

kidney cells to express PI-9 and the aggressiveness of infiltrating cytotoxic effector cells. This concept may provide insight into allograft rejection injury as there have been disparate results regarding the role of effector T cell expression of perforin/granzyme in mediating kidney allograft rejection. In previous studies, the absence of perforin/granzyme in recipient effector cells did not prevent tubular injury or rejection but these studies utilized 'non-life-supporting' murine renal transplant models and multiple effector mechanisms apart from perforin/granzyme provide redundant pathways of rejection *in vivo* (10, 11). Moreover, transcript levels of perforin and granzyme B were upregulated in grafts after transplantation which supports a potential role for perforin/granzyme mediated cytotoxicity (10).

Kidney tubular epithelial cells (TEC) represent the predominant parenchymal cell type within the renal cortex and as noted, are a primary target for cell mediated rejection. It has been previously observed that TEC exhibit resistance to effector cell mediated death, likely related to endogenous mechanisms of protection including regulated expression of mitochondrial proteins, c-FLIPs, IAPs, and TGF-β (12-16). As well the expression of members of the serpin family with TLR-4 and cytokine activation (17-22) suggests that the serpin PI-9 might similarly have a protective role during diverse forms of inflammatory renal injury, including ischemia reperfusion injury (IRI) and rejection (23-26). Although enhanced PI-9 expression has been observed in cortical areas of infiltrates in human kidney transplants undergoing rejection, it has not been unequivocally demonstrated that expression of PI-9 is restricted to infiltrating cells or to the TEC under effector attack. In this study, we demonstrate that renal TEC can express and upregulate SPI-6 in response to inflammation. Furthermore, the loss of SPI-6 in TEC increased susceptibility to granzyme B mediated death by cytotoxic cells *in vitro*. Most importantly the protective role of SPI-6 in the donor organ was clearly demonstrated during kidney transplantation with loss of SPI-6 resulting in increased tubular injury, reduced graft function, and shorter survival. These results may be of importance in considering therapeutic strategies in clinical renal transplantation.

2.3 Materials and Methods

Animals

B6, Balb/c, GranzymeA/B$^{-/-}$ (Grz$^{-/-}$) mice, and SPI-6$^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and maintained in the animal facility at Western University. Animal experiments were conducted in accordance with the Canadian Council on Animal Care guidelines under protocols approved by Western University.

Tubular epithelial cell (TEC) culture

TEC were derived from B6 and SPI-6$^{-/-}$ mouse kidney cortex and characterized by typical cobblestone appearance of renal epithelial cells and expression of TEC markers (cytokeratin, CD13, CD26, and E-cadherin).

Real time PCR

cDNA was generated from isolated RNA from TEC and quantitative PCR was performed using the Brilliant SYBR Green QPCR Master Mix kit (Bio-Rad) and detected using the Mx4000 system (Stratagene). Primers for SPI-6: 5'-CCTCAGCAAGGTGGAAAACAATC-3' and 5'-TGAAGAAA AGG AAGGGGTGG TC-3'. □-actin was used as the endogenous control. The normalized delta threshold cycle value and relative expression levels ($2^{\Delta\Delta Ct}$) were calculated according to the manufacturer's protocol.

Immunoblotting

Total protein isolated from TEC or kidney tissue was probed with anti-SPI-6 (Abcam) or anti-β-actin (Sigma). Immunoblots were scanned and analyzed using GS 700 Imaging densitometry scanner (Bio-Rad).

Cytotoxicity assay

CD8+ T cells were isolated from wild type or Grz$^{-/-}$ mice. TEC (H-2^{b}) and CD8^{+} T cells (H-2^{d}) were activated overnight with IFNγ and IL-2 respectively. TEC were incubated with chromium^{51} (Perkin Elmer) and co-cultured with CD8^{+} T cells for 4 hours. Supernatant was collected and analyzed for gamma radiation by Wallac Wizard 1470 Automatic Gamma

Counter (GMI). Maximal TEC death was determined by the addition of 5% Triton X-100 to TEC and percentage of cell lysis was calculated as sample cpm / maximal death cpm.

Allogeneic (H-2^b to H-2^d) kidney transplantation

Male Balb/c recipient mice were bilaterally nephrectomized and transplanted with kidneys from male B6 or SPI-6$^{-/-}$ mice in a single procedure (13). Rejection was determined by clinical deterioration as defined by standard protocols in our institute including altered behavior, failure to feed, and weight loss of >15%. Mice were euthanized according to pre-defined animal care protocols. Serum creatinine was determined at time of sacrifice to establish graft dysfunction.

Histology and immunohistochemistry

Kidney sections were stained with H&E and scored in a double-blinded fashion by pathologist. Criteria for kidney injury included tubular necrosis, mononuclear cell infiltration, tubular casts, and glomerular necrosis. Kidney sections were labeled with either anti-SPI-6 (Abcam), anti-CD3 (Dako), or anti-Granzyme B antibody (Abcam) using a standard protocol. Quantification of positively labeled cells was scored in a blinded fashion by point-counting of five random fields. Results are presented as mean ± SEM. Cell death was determined by TUNEL assay (Calbiochem).

Statistical analysis

Data is presented as mean ± standard error of the mean (SEM). One-way ANOVA testing was used to compare between multiple groups of data and unpaired t-tests were used to test two group data. A Log rank test was used to test significant differences in recipient survival after kidney transplantation. Statistical significance was set at $p< 0.05$.

2.4 Results

SPI-6 expression in TEC is upregulated by pro-inflammatory cytokines

The expression of SPI-6 by TEC in response to pro-inflammatory cytokines was tested *in vitro* using TNFα and IFNγ. These cytokines are detected in renal IRI and may also play a role allograft injury (17-19). SPI-6 mRNA and protein levels in TECs were analyzed after 24 hours of treatment. While there was an increase in mRNA with TNFα and IFNγ (Figure 1A), SPI-6 protein was present in media controls and expression was modestly increased (Figure 1B, 1C)**.** The concentrations of cytokines used were selected to augment expression of proteins but minimize TEC death as we have previously reported. However incubation of TEC with increasing concentrations of IFNγ (10-250 ng/ml) induced greater expression of SPI-6 (Figure 1D). These data demonstrate for the first time that murine TEC express SPI-6 and are consistent with previous reports that demonstrated expression of PI-9 in the tubular cell compartment of human kidney transplant sections *in vivo* (8). Importantly, these data suggest that while TEC can upregulate SPI-6 under pro-inflammatory conditions, constitutive expression of SPI-6 suggests an important rapid function in the overall protection of TEC.

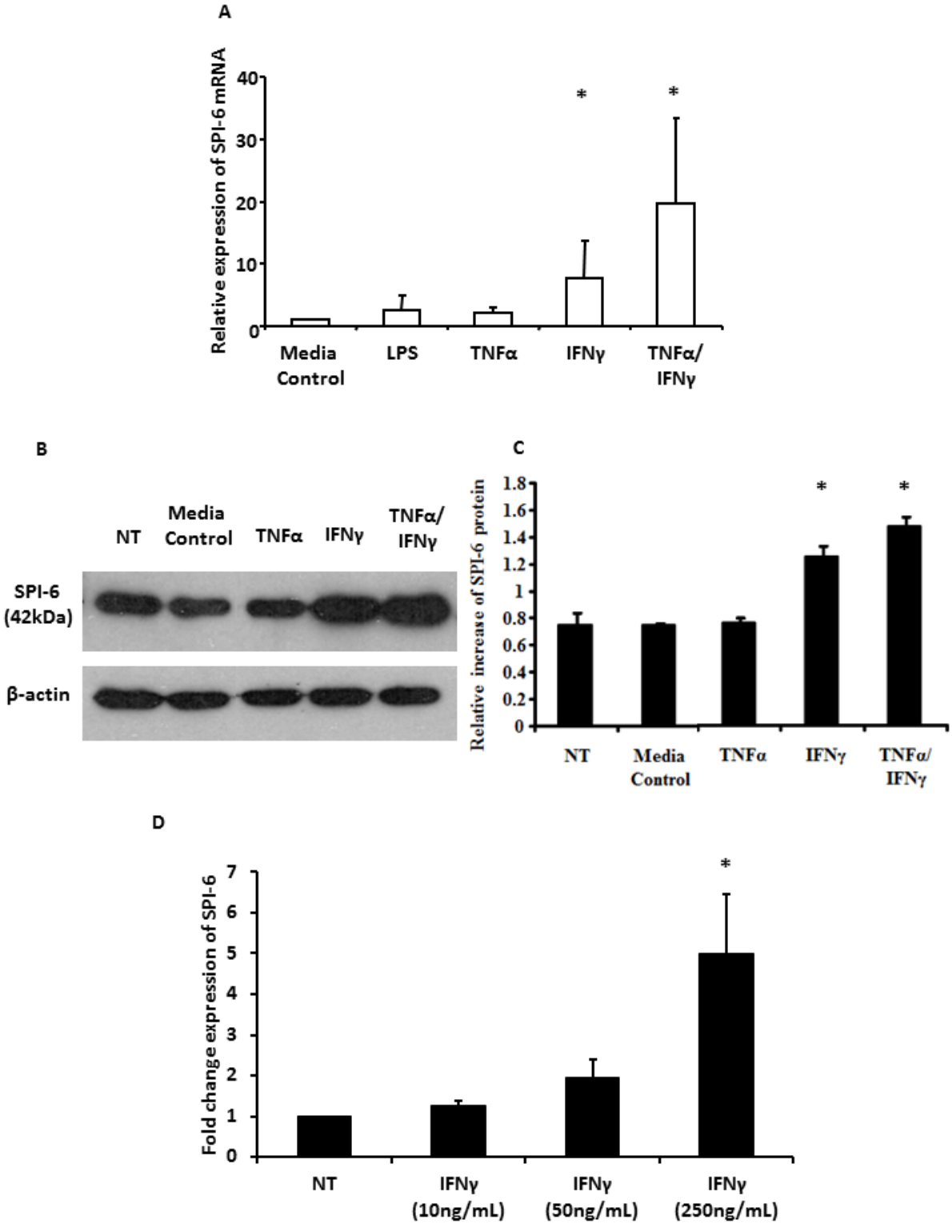

A
Relative expression of SPI-6 mRNA
40
30
20
10
0
*
*
Media Control
LPS
TNFα
IFNγ
TNFα/ IFNγ
B
NT
Media Control
TNFα
IFNγ
TNFα/ IFNγ
SPI-6 (42kDa)
β-actin
C
Relative increase of SPI-6 protein
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
NT
Media Control
TNFα
IFNγ
TNFα/ IFNγ
D
Fold change expression of SPI-6
7
6
5
4
3
2
1
0
*
NT
IFNγ (10ng/mL)
IFNγ (50ng/mL)
IFNγ (250ng/mL)

Figure 1. SPI-6 is upregulated in renal TEC in response to pro-inflammatory cytokines.

SPI-6 expression in renal TEC was analyzed 24 hours after treatment with IFN-γ (10ng/mL), TNF-α (10ng/mL), and LPS (1μg/ml). A) Total RNA was isolated from renal TEC after treatment and expression of SPI-6 mRNA was analyzed by Q-PCR. β-actin was used as an internal control. (*:$p<0.05$, n=3) B,C,D) Total cell lysate was isolated from renal TEC after treatment and expression of SPI-6 protein was detected by immunoblot. Bands were quantified by densitometry with β-actin as an internal control and compared to non- treated groups (NT). (*:$p<0.05$, n=3)

SPI-6 is an important mechanism to limit granzyme B mediated death

Previous studies have suggested that SPI-6 expression can protect CTL, NK cells, and DC from granzyme B mediated cell death during effector cell activation (2, 4-6). Therefore, we investigated whether SPI-6 expression by TEC can inhibit granzyme B mediated death**.** Balb/c CD8+ T cells were allo-activated by exposure to B6 splenocytes in IL-2 containing media and then co-cultured with either wild type or SPI-$6^{-/-}$ TEC. TEC were treated by IFN-γ (10ng/mL) to increase expression of SPI-6 and MHC without direct toxic effect on TEC. TEC death was measured used a chromium (Cr^{51}) release assay. Triton X-100 was added to TEC as a positive control for complete counts. CD8+ T cells were able to induce dose dependent (E:T) cell death in both wild type and SPI-$6^{-/-}$ TEC at 3:1 and 9:1 ratios of $CD8^+$ T cell: TEC (Figure 2). However, wild type TEC clearly showed greater resistant to CD8+ T cell mediated cytotoxicity compared to SPI$6^{-/-}$ TEC. This suggests that expression of SPI-6 is an important mechanism to limit granzyme B mediated death. However, loss of SPI-6 in TEC alone was not sufficient to provide complete protection from CD8+ mediated cytotoxicity as there are alternative mechanisms of cell death including granzyme A and Fas-FasL. Indeed the greatest reduction of TEC cytotoxicity was noted with loss of effector cell perforin/granzyme.

To confirm granzyme mediated cell death was a primary mechanism of TEC death in this assay, we utilized granzymeA/$B^{-/-}$ ($Grz^{-/-}$) CD8+ T cells with TEC in co-culture. $Grz^{-/-}$ CD8+ T cells had decreased cytotoxicity as compared to wild type CD8+ T cells supporting that 'granzyme' mediated cytotoxicity is a primary mechanism by which CD8+ T cells can kill TEC, at least *in vitro* (Figure 2). Collectively, these data demonstrate that expression of SPI-6 by TEC represents a potent protective response that allows TEC to resist granzyme B killing. Enhanced expression of SPI-6 by kidney parenchymal cells beyond its constitutive levels may be beneficial during inflammation to minimize injury and maintain kidney function.

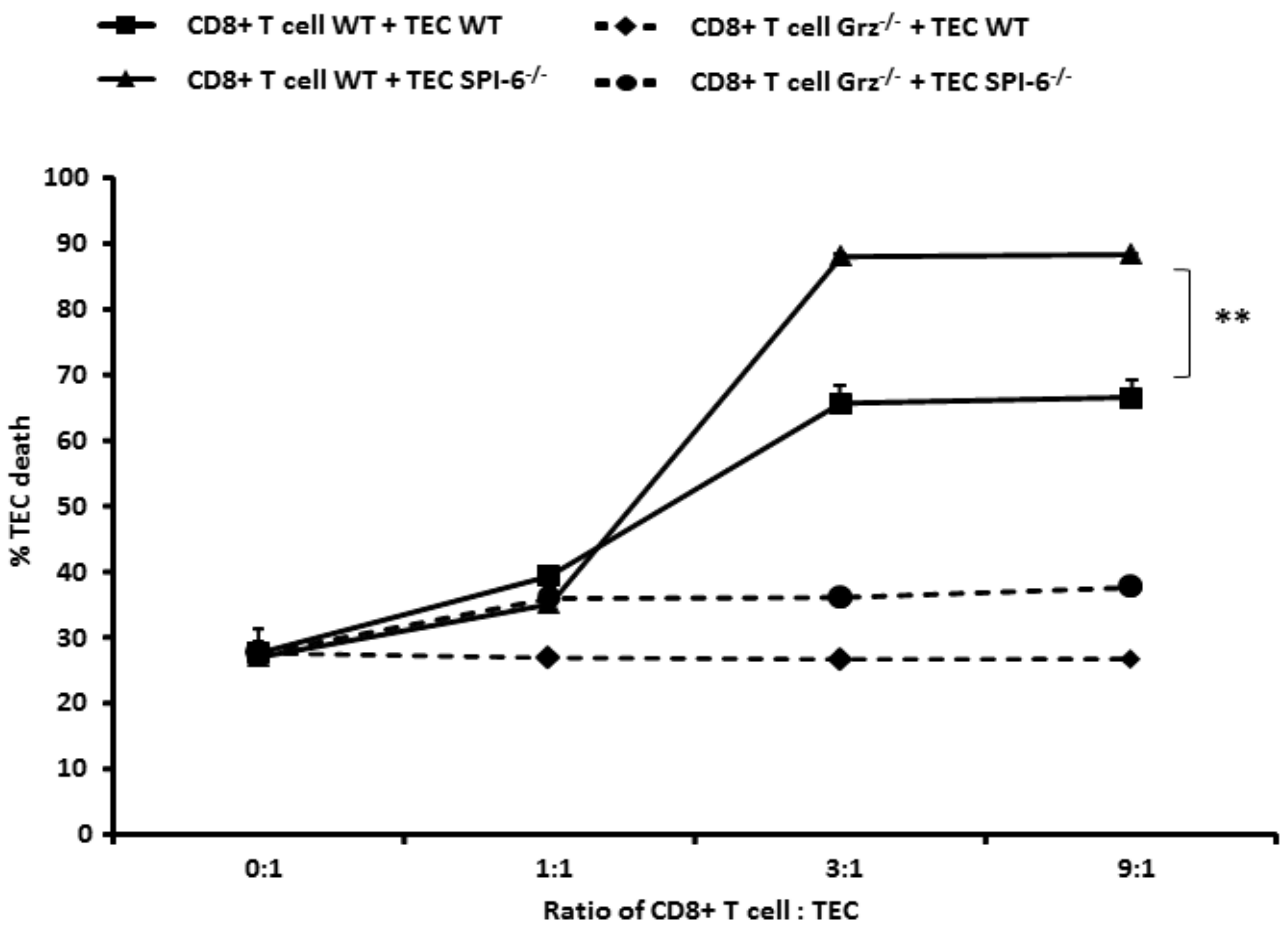

CD8+ T cell WT + TEC WT
CD8+ T cell Grz-/- + TEC WT
CD8+ T cell WT + TEC SPI-6-/-
CD8+ T cell Grz-/- + TEC SPI-6-/-
100
90
80
70
60
50
40
30
20
10
0
% TEC death
**
0:1
1:1
3:1
9:1
Ratio of CD8+ T cell : TEC

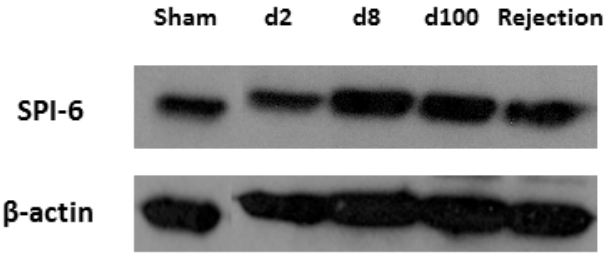
Figure 2. SPI-6 inhibits CD8+ T cell Granzyme B mediated cytotoxicity in TEC.

CD8+ T cells were isolated from Balb/c wild type (WT) and granzymeA/B$^{-/-}$ (Grz$^{-/-}$) mice and allogeneically primed with B6 splenocytes and IL-2 before TEC killing assay. TEC were isolated from B6 wild type and SPI6$^{-/-}$ mouse kidneys and activated with IFN-γ (10ng/mL) overnight before TEC killing assay. CD8+ T cells and TEC were co-cultured for 4 hours at various ratios and TEC death was measured using a chromium release assay. Cell death is shown as a percentage of total cell lysis (100%) as determined by the addition of Triton X-100 to TEC (**:$p<0.01$, n=3).

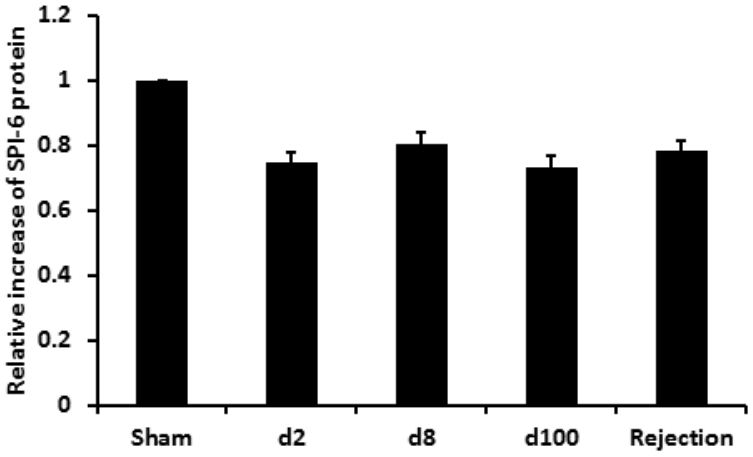
SPI-6 is expressed within the graft following renal transplantation

Our *in vitro* results demonstrate that SPI-6 is constitutively expressed by TEC and modestly upregulated under pro-inflammatory conditions. However, the expression and potential protective role of SPI-6 in renal allografts has to date not been tested. To determine the role of renal expression of SPI-6 in transplant injury, we used a B6 to Balb/c kidney transplantation model. Kidney grafts were assessed on day 0, 2, 8, 100 or on the day of rejection (clinically defined as in methods, prior to day 100 study end) and analyzed for SPI-6 expression. Immunoblot results of whole kidney lysates indicated that SPI-6 is constitutively expressed within kidneys and maintained over the course of transplantation from day 2 to day 100 (Figure 3A, 3B). While total allograft levels of SPI-6 appear to unchanged over the course of transplantation using whole tissue lysates, but this may not be representative of TEC expression of SPI-6 related to focal areas of inflammation. Immunohistochemistry analyses indicate that SPI-6 is upregulated post-transplantation, and persists to day 100 (Figure 3C). In addition, SPI-6 expression is not uniformly expressed within the cortex of kidney allografts and while not quantified appears to be predominantly expressed by TEC at day 8 in areas of inflammation and in infiltrating cells (Figure 3C). Expression was noted as well in day 100 post-transplantation (indicated by arrows) of long term survivors.

A


Sham
d2
d8
d100
Rejection
SPI-6
β-actin

B


Relative increase of SPI-6 protein
1.2
1
0.8
0.6
0.4
0.2
0
Sham
d2
d8
d100
Rejection

C

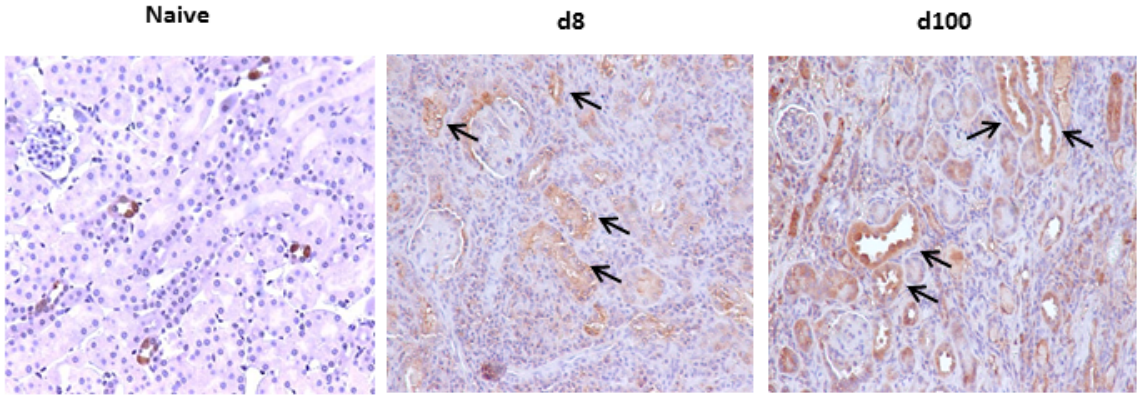

Naive
d8
d100

Figure 3. Donor kidney SPI-6 is expressed in TEC after kidney transplantation.

Single kidneys from B6 mice were transplanted into fully nephrectomised Balb/c recipients and followed for 100 days. Naïve kidneys and kidney allografts were analyzed at various time points post-transplantation for expression of SPI-6. A,B) Total protein was isolated from kidney allografts and analyzed for expression of SPI-6 by immunoblot at various time points. Bands were quantified by densitometry with β-actin as an internal control. (n=3) C) Naïve kidneys and day 8 and 100 post-transplantation allografts were analyzed by immunohistochemistry for expression of SPI-6. Tubules positive for SPI-6 labeling are indicated by arrows. Images were taken at 200X magnification.

SPI-6 deficiency enhances early tubular injury and accelerates rejection of kidney allografts

Kidney allografts persistently express SPI-6 after transplantation which suggests a potentially important homeostatic role for SPI-6 for kidney protection, particularly during inflammation. To test this hypothesis, we studied SPI-6 deficient mice in a 'survival model' using fully allogeneic mismatched B6 wild type or B6 SPI-$6^{-/-}$ to Balb/c kidney transplants. Kidney grafts and serum samples were collected by protocol on day 8 to assess tubular injury and graft function. As shown in Figure 4A, serum creatinine levels were higher in SPI-$6^{-/-}$ kidney recipients as compared to wild type recipients (113±23 vs. 28±3 μmol/L, n=5, $p<0.01$) consistent with greater graft dysfunction in SPI-$6^{-/-}$ kidneys. This was supported by histological analyses which demonstrated that SPI-$6^{-/-}$ grafts have greater tubular injury compared to wild type grafts (Figure 4B, 4C) (injury score: 8.8±2.2 vs 4.3±2.1, n=5/group, $p<0.01$). Further evidence in support of an important role for SPI-6 in inhibiting cell death was demonstrated by TUNEL staining. SPI-$6^{-/-}$ kidneys displayed much higher TUNEL positive (apoptotic) cell counts than wild type kidneys as shown in Figure 4B and 4D (17.2$\pm$6.4 vs 52.8$\pm$10.4, n=5, $p<0.001$). Collectively these data show that SPI-6 deficiency results in greater severity of early stage kidney injury and dysfunction.

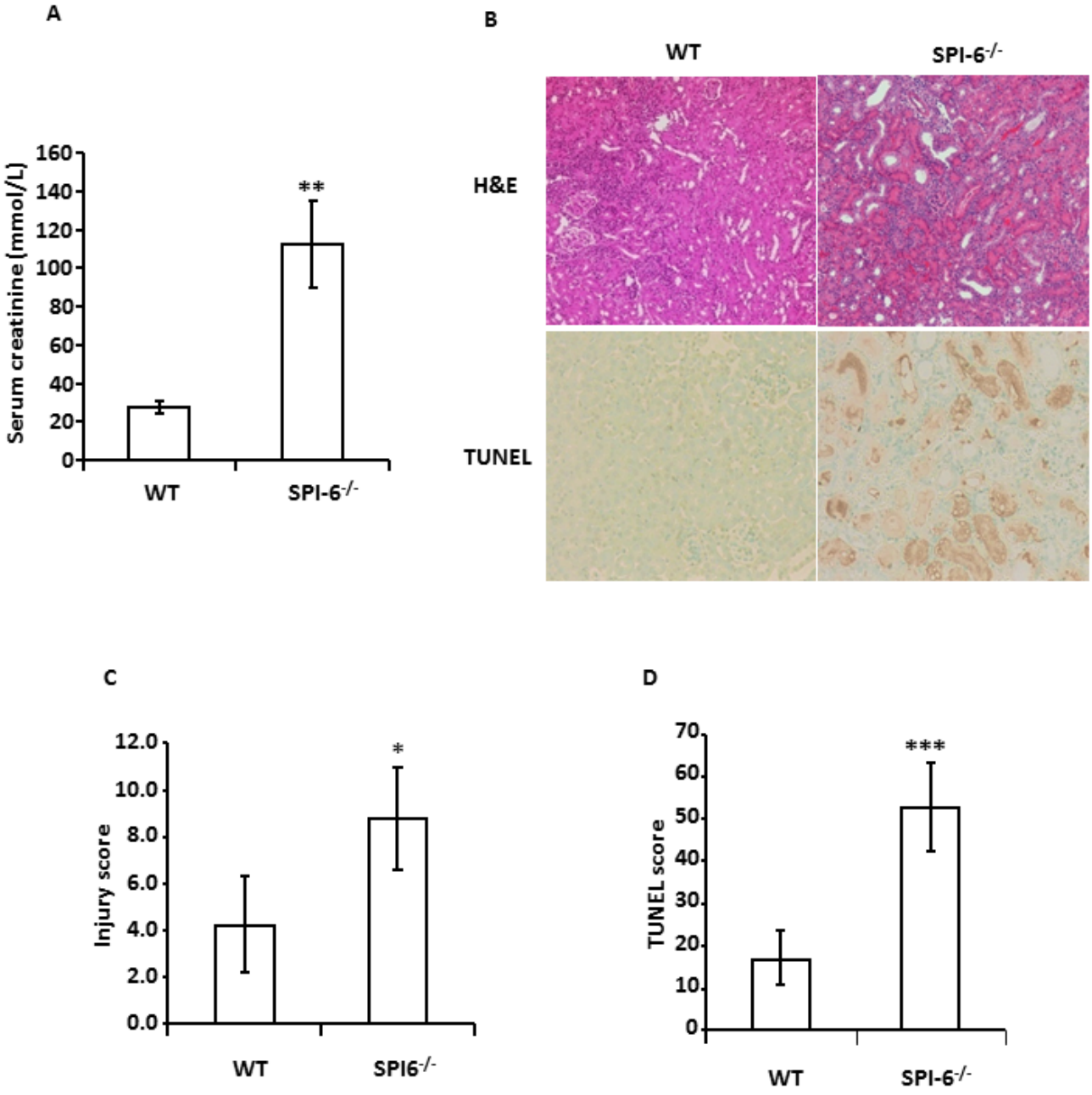

A
Serum creatinine (mmol/L)
160
140
120
100
80
60
40
20
0
**
WT
SPI-6-/-
B
WT
SPI-6-/-
H&E
TUNEL
C
Injury score
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
WT
SPI6-/-
D
TUNEL score
70
60
50
40
30
20
10
0
***
WT
SPI-6-/-

Figure 4. **SPI-6 in the renal allograft ameliorates injury after kidney transplantation.**

Single kidneys from B6 wild type (WT) and SPI-$6^{-/-}$ mice were transplanted into fully nephrectomised Balb/c recipients. A) Kidney function of recipients was measured by serum creatinine at day 8 post-transplantation. (**:$p<0.01$, n=5/group) B) Sections from day 8 renal allografts were stained with hematoxylin and eosin (H&E) or TUNEL and images were taken at 200X magnification. C) H&E sections were scored by blinded pathological analysis as described in Methods. (*:$p<0.05$, n=5/group) D) Cell death was measured by TUNEL staining and quantified by microscopy as described in Methods. TUNEL positive cells are indicated by brown color. (***:$p<0.001$, n=5/group)

We investigated the role of SPI-6 deficiency in renal allograft survival using B6 wild type or B6 SPI-$6^{-/-}$ to Balb/c kidney transplantation. As noted, kidney grafts were collected on day 8 by protocol for functional analyses and also analyzed for CD3+ T cell graft infiltration and granzyme B expression. As shown in Figure 5A, cell infiltrates are present in both wild type and SPI-$6^{-/-}$ renal allografts at day 8 post-transplantation. Quantification of CD3+ and granzyme B+ cells revealed prominent but equivalent numbers of infiltrating cells in both grafts (Figure 5B). Kidney graft recipient survival was monitored over 100 days (end point) and earlier clinical rejection was scored based on clinical deterioration as in Methods. As shown in Figure 5C, approximately 35% of wild type kidney recipients survived to day 100 while none of the recipients receiving SPI-$6^{-/-}$ kidney grafts survived to endpoint (Figure 5, $p<0.001$, n=8-10/group). SPI-$6^{-/-}$ kidney grafts were rejected more rapidly than wild type kidney grafts in Balb/c mice (20 ± 19 vs 66 ± 33 days, n=8-10/group, $P<0.001$). Importantly these data suggest that SPI-6 expression within the donor kidney contributes to allograft survival despite the presence of extensive infiltrating cytotoxic lymphocytes.

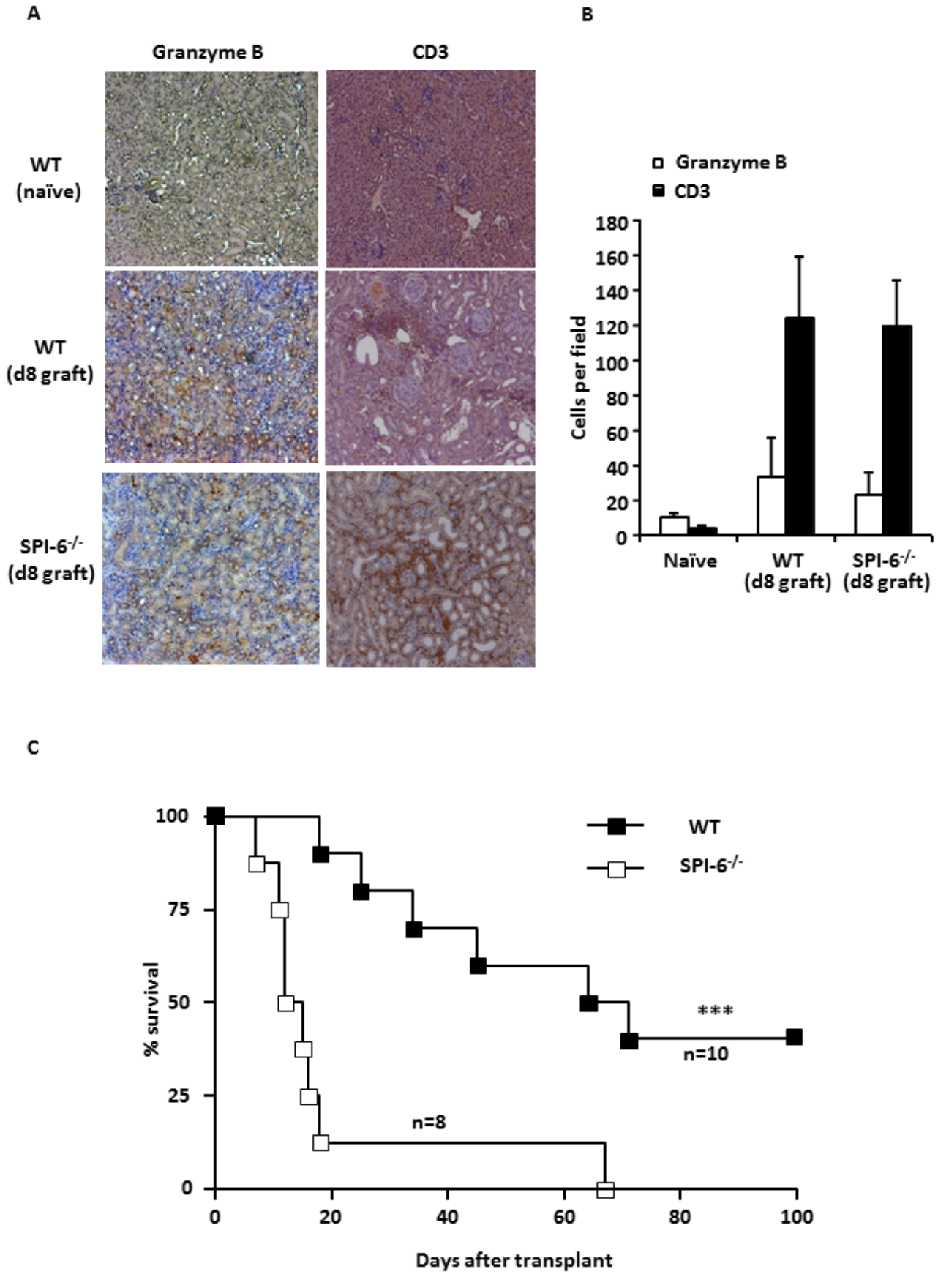

A
Granzyme B
CD3
WT
(naïve)
WT
(d8 graft)
SPI-6-/-
(d8 graft)
B
Granzyme B
CD3
Cells per field
180
160
140
120
100
80
60
40
20
0
Naïve
WT
(d8 graft)
SPI-6-/-
(d8 graft)
C
100
75
50
25
0
% survival
WT
SPI-6-/-
***
n=10
n=8
0
20
40
60
80
100
Days after transplant

Figure 5. SPI-6 in donor kidneys increased allograft survival in allogeneic kidney transplant recipients.

Bilaterally nephrectomized Balb/c recipients received a donor kidney from either B6 wild type (WT) or SPI-$6^{-/-}$ mice. Recipients were monitored as described in Methods. A, B) Naïve kidney and day 8 renal allograft sections were labelled with anti-CD3 and anti-granzyme B antibodies to identify infiltrating cytotoxic cells indicated by brown color. Positive cells were quantified as described in Methods. Images were taken at 200X magnification. (n=3) C) Kidney allograft receipients were followed for 100 days for survival. Recipients of wild type kidneys are denoted by black squares and recipients of SPI-$6^{-/-}$ kidneys are denoted by white squares. (***:$p<0.001$, n=8-10/group).

2.5 Discussion

With declining acute rejection rates, long term graft survival has become a major challenge in kidney transplantation. Although current strategies involving immunosuppression has improved incidences of acute rejection, there has been a disappointing lack in novel therapies that can prolong long term outcomes. As well, there has been a paucity of identified donor organ factors that might be targeted to improve long term graft survival. A pathological hallmark of cellular rejection is the presence of graft infiltrating immune cells which promote pro-inflammatory responses that can damage parenchymal cells within the graft. Cytotoxic effector cells that mediate rejection induce cell death through various mechanisms including Fas-FasL interaction, pro-inflammatory cytokines, and perforin/granzyme release by effector cells. Studies have shown that renal allograft recipients undergoing acute rejection have increased levels of urinary perforin and granzyme B mRNA (9). A potentially important role for perforin/granzyme in promoting kidney transplant rejection injury may be obscured by regulation of endogenous pathways of resistance to death in target cells. TEC can produce a variety of molecules that are capable of regulating inflammation such as IL-10, TNFα, Fas, Fas-L, TGF-β, IDO, and other cytokines as well as resistance to death including cFLIP and IAPs (12-14, 16, 17, 27, 28). To date, the expression and role of the serpin family member SPI-6 (PI-9) in attenuating TEC death by granzymes has not been studied.

Previous studies have demonstrated that viral infection in hepatocytes can induce expression of SPI-6 which increases resistance against CTL killing *in vivo* (29-31). This suggests that TEC expression of SPI-6/PI-9 may be a generalized pathway for epithelial cells to resist inflammatory cell death and may be a mechanism that regulates the aggressiveness of cell mediated rejection (32-35). In the current study, we found that SPI-6 was expressed in TEC and increased in response to pro-inflammatory stimuli (Figure 1), consistent with results in other cell types (29, 36). While SPI-6 protein increased modestly with cytokines, the high basal level in TEC is consistent with an important role in protecting these essential parenchymal cells from acute inflammatory injury. Also and consistent with our *in vitro* results and previous studies, SPI-6 expression in kidney grafts was prominently upregulated in TEC after allogeneic transplantation and persisted to day 100 (Figure 3). Interestingly, although some graft infiltrating lymphocytes were observed to express SPI-6, the

predominant expression appeared to be on surrounding TEC with unequal distribution *in vivo*. This likely reflected the patchy distribution of infiltrating cells during rejection. It may be that different phenotypes of TEC (i.e. proximal vs. distal) have differential capacity to express SPI-6. In contrast, increases in SPI-6 *in vitro* using cultures of primarily proximal TEC may have reflected more uniform and higher concentration exposure to cytokines than what occurs in vivo during rejection resulting in greater upregulation of SPI-6. Nonetheless, as several agents have been reported to augment SPI-6 expression (37), our results might provide a potential therapeutic strategy to limit the injurious effect of infiltrating cytotoxic cells during kidney inflammation and allograft rejection.

As noted, there are variable reports on the importance of granzyme B in inducing kidney injury (8, 9, 33-35). Clearly in our hands, $Grz^{-/-}$ CD8+ T cells were unable to induce cell death in TEC as effectively as wild type CD8+ T cells (Figure 2) and TEC death could be induced by CD8+ T cells despite the presence of SPI-6 in TEC. It is likely that other mechanisms of targeted cytotoxicity such as granzyme A in particular, and differences in models accounts for differences in previous studies (10, 11). Immunohistochemical studies of granzyme B in patients with acute renal graft rejection demonstrate that levels of granzyme B are higher (32) similar to that observed in a non-life supporting murine model of kidney transplantation (10). The effect of perforin and granzyme B deficiency in a non-life supporting kidney transplant model on tubular transcripts and histology rather than function and recipient survival in a life supporting models used here, may have additionally accounted for differences from results in our study. It is clear from the present data that a role for perforin/granzyme B exists in murine kidney rejection injury, and is consistent with clinical observations in human transplantation. The presence of tubular injury despite a loss of perforin/granzyme in recipient effector cells suggests that overt rejection leading to a loss of function and histological markers of injury are imprecisely correlated and might not be fully appreciated in a non-survival model of transplantation. The complexity of immune effector responses during rejection, our lack of detailed understanding of the various cytotoxic effectors, and the presence of endogenous mechanisms of ‘self-protection’ may collectively obscure the importance of granzyme B in kidney transplantation injury. As these pathways might be exploited to promote graft survival, a greater understanding of the regulation of the

granzyme B mediated injury pathway in TEC may generate new therapeutic strategies to protect grafts.

In this study, we have demonstrated that constitutive expression of SPI-6 in the donor kidney is associated with prolonged graft function and survival as compared to recipients receiving SPI-6 deficient kidneys (Figure 5). As this study has focused on TEC as functional parenchymal cells that are targeted during cellular rejection, we have not excluded the potential effect of SPI-6 on other donor kidney cells. Indeed the loss of SPI-6 in donor kidneys might promote the elimination of renal resident dendritic cells capable of either enhancing rejection (thus resulting in less rejection) or promoting tolerance through regulatory cell expansion (i.e. thus more rejection). As there were no significant differences in numbers of infiltrating CD3+ and granzyme B+ cells (Figure 5), it appears that the primary effect of loss of SPI-6 in donor kidney is on increased susceptibility to effector cell cytotoxicity. Future studies will be needed to clarify the effect on dendritic cell targeting. It was interesting to note that although allograft levels of SPI-6 protein was not globally increased within transplanted kidneys and was patchy, 35% of recipients still achieved long term survival (Figure 5) suggesting that basal expression of renal SPI-6 is sufficient to partially protect the graft from CTL mediated rejection. This may account for a high level of spontaneous acceptance of kidney allografts that has been noted in many previous studies in mice. While conventional therapeutics block immune pathways, augmentation of SPI-6/PI-9 above basal levels may provide a novel therapeutic target to limit rejection. Previous studies have demonstrated PI-9 upregulation induced by a constituent of soy (genistein) may be clinically relevant as this promoted the resistance of breast cancer cells to NK cell mediated killing (37) *in vitro*. Induction and maintenance of high levels of SPI-6 in the donor organ through perfusion solutions might be feasible to attenuate cytotoxicity by graft infiltrating cells, and thereby improving graft function and survival.

In conclusion, we show for the first time that kidney TEC express the granzyme B specific inhibitor SPI-6 *in vitro* and *in vivo* and it alters the resistance of these parenchymal cells to rejection injury by cytotoxic effector cells. These data suggest that the expression of SPI-6 may be renal protective during transplantation rejection and provide insights to generate new strategies that can prolong renal allograft survival.

2.6 Acknowledgements

The authors would like to thank Ms. Pamela Gardner for administration assistance. This study was supported by the Canadian Institutes of Health Research (XTW-90932, MOP-111180, MOP-115048), the Kidney Foundation of Canada (A.M.J., Z.X.Z), and the Program of Experimental Medicine (POEM) at Western University, London, Ontario, Canada.

2.7 Author Disclosure

The authors have no financial disclosures or conflict of interest in this study.

2.8 References

1. Shresta, S., C. T. Pham, D. A. Thomas, T. A. Graubert, and T. J. Ley. 1998. How do cytotoxic lymphocytes kill their targets? *Current opinion in immunology* 10: 581-587.

2. Laforge, M., N. Bidere, S. Carmona, A. Devocelle, B. Charpentier, and A. Senik. 2006. Apoptotic death concurrent with CD3 stimulation in primary human CD8+ T lymphocytes: a role for endogenous granzyme B. *J Immunol* 176: 3966-3977.

3. Bladergroen, B. A., M. C. Strik, N. Bovenschen, O. van Berkum, G. L. Scheffer, C. J. Meijer, C. E. Hack, and J. A. Kummer. 2001. The granzyme B inhibitor, protease inhibitor 9, is mainly expressed by dendritic cells and at immune-privileged sites. *J Immunol* 166: 3218-3225.

4. Hirst, C. E., M. S. Buzza, C. H. Bird, H. S. Warren, P. U. Cameron, M. Zhang, P. G. Ashton-Rickardt, and P. I. Bird. 2003. The intracellular granzyme B inhibitor, proteinase inhibitor 9, is up-regulated during accessory cell maturation and effector cell degranulation, and its overexpression enhances CTL potency. *J Immunol* 170: 805-815.

5. Zhang, M., S. M. Park, Y. Wang, R. Shah, N. Liu, A. E. Murmann, C. R. Wang, M. E. Peter, and P. G. Ashton-Rickardt. 2006. Serine protease inhibitor 6 protects cytotoxic T cells from self-inflicted injury by ensuring the integrity of cytotoxic granules. *Immunity* 24: 451-461.

6. Ida, H., T. Nakashima, N. L. Kedersha, S. Yamasaki, M. Huang, Y. Izumi, T. Miyashita, T. Origuchi, A. Kawakami, K. Migita, P. I. Bird, P. Anderson, and K. Eguchi. 2003. Granzyme B leakage-induced cell death: a new type of activation-induced natural killer cell death. *Eur J Immunol* 33: 3284-3292.

7. Colvin, R. B., A. H. Cohen, C. Saiontz, S. Bonsib, M. Buick, B. Burke, S. Carter, T. Cavallo, M. Haas, A. Lindblad, J. C. Manivel, C. C. Nast, D. Salomon, C. Weaver, and M. Weiss. 1997. Evaluation of pathologic criteria for acute renal allograft

rejection: reproducibility, sensitivity, and clinical correlation. *J Am Soc Nephrol* 8: 1930-1941.

8. Rowshani, A. T., S. Florquin, F. Bemelman, J. A. Kummer, C. E. Hack, and I. J. Ten Berge. 2004. Hyperexpression of the granzyme B inhibitor PI-9 in human renal allografts: a potential mechanism for stable renal function in patients with subclinical rejection. *Kidney international* 66: 1417-1422.

9. Muthukumar, T., R. Ding, D. Dadhania, M. Medeiros, B. Li, V. K. Sharma, C. Hartono, D. Serur, S. V. Seshan, H. D. Volk, P. Reinke, S. Kapur, and M. Suthanthiran. 2003. Serine proteinase inhibitor-9, an endogenous blocker of granzyme B/perforin lytic pathway, is hyperexpressed during acute rejection of renal allografts. *Transplantation* 75: 1565-1570.

10. Halloran, P. F., J. Urmson, V. Ramassar, A. Melk, L. F. Zhu, B. P. Halloran, and R. C. Bleackley. 2004. Lesions of T-cell-mediated kidney allograft rejection in mice do not require perforin or granzymes A and B. *Am J Transplant* 4: 705-712.

11. Einecke, G., T. Fairhead, L. G. Hidalgo, B. Sis, P. Turner, L. F. Zhu, R. C. Bleackley, G. A. Hadley, K. S. Famulski, and P. F. Halloran. 2006. Tubulitis and epithelial cell alterations in mouse kidney transplant rejection are independent of CD103, perforin or granzymes A/B. *Am J Transplant* 6: 2109-2120.

12. Yard, B. A., M. R. Daha, M. Kooymans-Couthino, J. A. Bruijn, M. E. Paape, E. Schrama, L. A. van Es, and F. J. van der Woude. 1992. IL-1 alpha stimulated TNF alpha production by cultured human proximal tubular epithelial cells. *Kidney International* 42: 383-389.

13. Porter, C. J., J. E. Ronan, and M. J. Cassidy. 2000. fas-fas-ligand antigen expression and its relationship to increased apoptosis in acute renal transplant rejection. *Transplantation* 69: 1091-1094.

14. Gupta, S., R. Bi, and S. Gollapudi. 2005. Central memory and effector memory subsets of human CD4(+) and CD8(+) T cells display differential sensitivity to TNF-

{alpha}-induced apoptosis. *Annals of the New York Academy of Sciences* 1050: 108-114.

15. Sanna, M. G., J. da Silva Correia, O. Ducrey, J. Lee, K. Nomoto, N. Schrantz, Q. L. Deveraux, and R. J. Ulevitch. 2002. IAP suppression of apoptosis involves distinct mechanisms: the TAK1/JNK1 signaling cascade and caspase inhibition. *Molecular and cellular biology* 22: 1754-1766.

16. Lemay, S., H. Rabb, G. Postler, and A. K. Singh. 2000. Prominent and sustained up-regulation of gp130-signaling cytokines and the chemokine MIP-2 in murine renal ischemia-reperfusion injury. *Transplantation* 69: 959-963.

17. Domanski, L., A. Pawlik, K. Safranow, K. Jakubowska, V. Dziedziejko, D. Chlubek, J. Rozanski, M. Myslak, M. Romanowski, T. Sulikowski, J. Sienko, M. Ostrowski, and K. Ciechanowski. 2007. Purine and cytokine concentrations in the renal vein of the allograft during reperfusion. *Transplant Proc* 39: 1319-1322.

18. Daemen, M. A., C. van't Veer, T. G. Wolfs, and W. A. Buurman. 1999. Ischemia/reperfusion-induced IFN-gamma up-regulation: involvement of IL-12 and IL-18. *J Immunol* 162: 5506-5510.

19. Li, L., L. Huang, S. S. Sung, P. I. Lobo, M. G. Brown, R. K. Gregg, V. H. Engelhard, and M. D. Okusa. 2007. NKT cell activation mediates neutrophil IFN-gamma production and renal ischemia-reperfusion injury. *J Immunol* 178: 5899-5911.

20. Daemen, M. A., M. W. van de Ven, E. Heineman, and W. A. Buurman. 1999. Involvement of endogenous interleukin-10 and tumor necrosis factor-alpha in renal ischemia-reperfusion injury. *Transplantation* 67: 792-800.

21. Halloran, P. F., P. Autenried, V. Ramassar, J. Urmson, and S. Cockfield. 1992. Local T cell responses induce widespread MHC expression. Evidence that IFN-gamma induces its own expression in remote sites. *J Immunol* 148: 3837-3846.

22. Kim, B. S., S. W. Lim, C. Li, J. S. Kim, B. K. Sun, K. O. Ahn, S. W. Han, J. Kim, and C. W. Yang. 2005. Ischemia-reperfusion injury activates innate immunity in rat kidneys. *Transplantation* 79: 1370-1377.

23. Chen, K., J. Huang, W. Gong, P. Iribarren, N. M. Dunlop, and J. M. Wang. 2007. Toll-like receptors in inflammation, infection and cancer. *International immunopharmacology* 7: 1271-1285.

24. Vabulas, R. M., P. Ahmad-Nejad, S. Ghose, C. J. Kirschning, R. D. Issels, and H. Wagner. 2002. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *The Journal of biological chemistry* 277: 15107-15112.

25. Yoh, K., M. Kobayashi, N. Yamaguchi, K. Hirayama, T. Ishizu, S. Kikuchi, S. Iwabuchi, K. Muro, S. Nagase, K. Aoyagi, M. Kondoh, K. Takemura, K. Yamagata, and A. Koyama. 2000. Cytokines and T-cell responses in superantigen-related glomerulonephritis following methicillin-resistant Staphylococcus aureus infection. *Nephrol Dial Transplant* 15: 1170-1174.

26. Du, C., Q. Guan, Z. Yin, R. Zhong, and A. M. Jevnikar. 2005. IL-2-mediated apoptosis of kidney tubular epithelial cells is regulated by the caspase-8 inhibitor c-FLIP. *Kidney International* 67: 1397-1409.

27. Goes, N., J. Urmson, V. Ramassar, and P. F. Halloran. 1995. Ischemic acute tubular necrosis induces an extensive local cytokine response. Evidence for induction of interferon-gamma, transforming growth factor-beta 1, granulocyte-macrophage colony-stimulating factor, interleukin-2, and interleukin-10. *Transplantation* 59: 565-572.

28. Barrie, M. B., H. W. Stout, M. S. Abougergi, B. C. Miller, and D. L. Thiele. 2004. Antiviral cytokines induce hepatic expression of the granzyme B inhibitors, proteinase inhibitor 9 and serine proteinase inhibitor 6. *J Immunol* 172: 6453-6459.

29. Stout-Delgado, H. W., Y. Getachew, T. E. Rogers, B. C. Miller, and D. L. Thiele. 2007. The role of serpinb9/serine protease inhibitor 6 in preventing granzyme B-dependent hepatotoxicity. *Hepatology (Baltimore, Md* 46: 1530-1540.

30. Stout-Delgado, H. W., Y. Getachew, B. C. Miller, and D. L. Thiele. 2007. Intrahepatic lymphocyte expression of dipeptidyl peptidase I-processed granzyme B and perforin induces hepatocyte expression of serine proteinase inhibitor 6 (Serpinb9/SPI-6). *J Immunol* 179: 6561-6567.

31. Wagrowska-Danilewicz, M., and M. Danilewicz. 2003. Immunoexpression of perforin and granzyme B on infiltrating lymphocytes in human renal acute allograft rejection. *Nefrologia* 23: 538-544.

32. Sharma, V. K., R. M. Bologa, B. Li, G. P. Xu, M. Lagman, W. Hiscock, J. Mouradian, J. Wang, D. Serur, V. K. Rao, and M. Suthanthiran. 1996. Molecular executors of cell death--differential intrarenal expression of Fas ligand, Fas, granzyme B, and perforin during acute and/or chronic rejection of human renal allografts. *Transplantation* 62: 1860-1866.

33. Mengel, M., I. Mueller, M. Behrend, R. von Wasielewski, J. Radermacher, A. Schwarz, H. Haller, and H. Kreipe. 2004. Prognostic value of cytotoxic T-lymphocytes and CD40 in biopsies with early renal allograft rejection. *Transpl Int* 17: 293-300.

34. Pascoe, M. D., S. E. Marshall, K. I. Welsh, L. M. Fulton, and D. A. Hughes. 2000. Increased accuracy of renal allograft rejection diagnosis using combined perforin, granzyme B, and Fas ligand fine-needle aspiration immunocytology. *Transplantation* 69: 2547-2553.

35. Bots, M., E. de Bruin, M. T. Rademaker-Koot, and J. P. Medema. 2007. Proteinase inhibitor-9 expression is induced by maturation in dendritic cells via p38 MAP kinase. *Human immunology* 68: 959-964.

36. Jiang, X., Patterson, N.M., Ling, Y., Xie, J., Helferich, W.G., Shapiro, D.J. 2008. Low concentrations of the soy phytoestrogen genistein induce proteinase inhibitor 9 and block killing of breast cancer cells by immune cells. *Endocrinology* (149)11: 5366-5373.

37. Du, C., Q. Guan, H. Diao, Z. Yin, and A. M. Jevnikar. 2006. Nitric oxide induces apoptosis in renal tubular epithelial cells through activation of caspase-8. *American Journal of physiology* 290: F1044-1054.

Chapter 3

3 RIPK3 mediated necroptosis promotes donor kidney inflammatory injury and reduces allograft survival

Arthur Lau[1,3], Shuang Wang[2,4], Jifu Jiang[1], Aaron Haig[3], Alexander Pavlosky[1,3], Andreas Linkermann[5], Zhu-Xu Zhang[1,2,3,4,*], Anthony M. Jevnikar[1,2,4,*]

[1]Matthew Mailing Centre for Translational Transplant Studies, Lawson Health Research Institute; Departments of [2]Medicine and [3]Pathology, London Health Sciences Centre and [4]Western University, London, Canada, and the [5]Division of Nephrology and Hypertension, Christian-Albrechts University, Kiel, Germany

Citation:

Lau A, Wang S, Jiang J, Haig A, Pavlosky A, Linkermann A, Zhang ZX, Jevnikar AM. RIPK3 mediated necroptosis promotes donor kidney inflammatory injury and reduces allograft survival. *American Journal of Transplantation*. 2013 (11): 2805-2818. John Wiley & Sons©

3.1 Abstract

Kidney transplant injury occurs with ischemia and alloimmunity. Members of the receptor interacting protein kinase family (RIPK1,3) are key regulators of 'necroptosis', a newly recognized, regulated form of necrosis. Necroptosis and apoptosis death appear to be counterbalanced as caspase-8 inhibition can divert death from apoptosis to necrosis. Inhibition of necroptosis in donor organs to limit injury has not been studied in transplant models. In this study, necroptosis was triggered in caspase inhibited tubular cells (TEC) exposed to TNF□ *in vitro*, while RIPK1 inhibition with Nec-1 or use of RIPK3$^{-/-}$ TEC, prevented necroptosis. *In vivo*, shRNA silencing of caspase-8 in donor B6 mouse kidneys increased necroptosis, enhanced HMGB1 release, reduced renal function and accelerated rejection when transplanted into BALB/c recipients. Using ethidium homodimer (EHD) perfusion to assess necrosis *in vivo*, necrosis was abrogated in RIPK3$^{-/-}$ kidneys post-ischemia. Following transplantation, recipients receiving RIPK3$^{-/-}$ kidneys had longer survival (p=0.002) and improved renal function, (p=0.03) when compared to controls. In summary, we show for the first time that RIPK3 mediated necroptosis in donor kidneys can promote inflammatory injury, and has a major impact on renal IRI and transplant survival. We suggest inhibition of necroptosis in donor organs may similarly provide a major clinical benefit.

3.2 Introduction

Kidney dysfunction following transplantation has multiple etiologies. Tubular epithelial cells (TEC) comprise more than 75% of renal parenchymal cells and are highly susceptible to death from ischemia-reperfusion injury (IRI), reactive oxidative species (ROS), nitric oxide (NO), pro-inflammatory cytokines, antibodies, and cytotoxic T and NK cells. Their viability, as well as other renal parenchymal cells, directs both short and long term kidney allograft survival (1-5). IRI enhances adaptive immune responses and pro-inflammatory cytokine expression that promote rejection (6-8) and the recruitment of T, NK and other cell effectors (9, 10). Collectively these mechanisms affect organ function and homeostasis by the elimination of parenchymal cells as well as promoting further inflammatory organ injury. It follows that prevention of TEC and inflammatory forms of parenchymal cell death would be expected to reduce delayed graft function and attenuate rejection responses in transplanted kidneys.

While targeting cell death might be useful as a therapeutic strategy in transplantation, a greater understanding of complex intra-cellular interactions that lead to various forms of cell death will be required for such strategies to be applied effectively. Caspase-dependent apoptosis or “programmed cell death” (PCD) has been regarded as the prototypic form of regulated cell death. While apoptosis induces minimal inflammation and may contribute to immune tolerance (12, 13), necrosis and caspase-independent cell death (CICD) are regarded as unregulated forms of cell death induced by severe nonspecific and non-physiological stress (14). Necrosis promotes inflammatory injury in kidneys (15, 16) as membrane rupture results in the release of pro-inflammatory endogenous molecules including heat-shock proteins (HSP), unprocessed high-mobility group box 1 (HMGB1), uric acid, fibronectin, IL-33, and others (17, 18). These cellular death associated molecular patterns (CCDAMPs) participate in IRI and allograft rejection through interaction with Toll Like Receptors (TLR) and other innate receptors (16, 19-21). Ligation of surface death receptors (TNFR1, Fas/CD95 and TRAIL-R) recruits adapter proteins, such as Fas-associated death domain (FADD), TNFR-associated factor with death domain (TRADD), RIPK1, and other proteins which allow formation of a complex which triggers the autocatalytic activation of caspase-8 homodimers and apoptosis. However recent studies have indicated that a primary function of

caspase-8/FLIP-long heterodimers is to prevent a ‘regulated’ form of necrosis (RN) termed necroptosis, which is mediated by RIPK1 and 3 proteins (22, 23). Necroptosis is morphologically and biochemically indistinguishable from most other forms of necrosis (23-31). RIPK1 and RIPK3 are serine/threonine kinase family members which interact through RIPK homotypic interaction motifs (RHIM) to permit necroptosis to take place (24, 32) in addition to mixed lineage kinase domain-like protein (MLKL). As necroptosis is a 'failsafe' mechanism to eliminate caspase-8 inhibiting virus infections (32, 33), inhibition of caspase-8 may be detrimental by triggering necroptosis. A recent study showing benefit in renal IRI by blocking necroptosis though RIPK1 (34) suggested necroptosis may play a role in the pathogenesis of diverse kidney injury including allograft rejection.

Our previous studies have demonstrated that tumor necrosis factor alpha (TNFα) can induce apoptosis in renal TEC and that TEC participate in cytokine enabled, Fas-FasL mediated fratricide (3). This has been recently confirmed in cisplatinum activated TEC (35). We and others have also shown that inhibition of caspase-8 (4), interleukin-2 (36, 37), and indolamine 2,3 dioxygenase (IDO) (38, 39) can attenuate various forms of TEC death and improve renal injury in short term IRI models. In the present study, we demonstrate the differential effects of inhibiting caspase-8 mediated apoptosis and necroptosis following IRI or kidney transplantation. While caspase-8 inhibition can improve IRI, in the present study we show that inhibition can augment necroptosis mediated kidney allograft injury. Importantly, loss of donor kidney RIPK3 promoted allograft survival in an allogeneic mouse kidney transplant model.

3.3 Materials and Methods

Animals

B6 (H-2^b), Balb/c (H-2^d) (Jackson Laboratories, Bar Harbor, ME), and B6-RIPK3$^{-/-}$ (H-2^b, generously provided by Genentech Inc (40)) were maintained in the animal facility at the University of Western Ontario using approved protocols and procedures. RIPK3$^{-/-}$ mice are phenotypically unremarkable, and have normal kidney function and breeding (40). All experimental procedures were approved by the University of Western Ontario Animal Care Committee.

Tubular epithelial cell (TEC) culture

Primary cultures were derived from B6 and B6-RIPK3$^{-/-}$ mouse kidney cortex and grown in sterile full media at 37°C in 5% CO_2. Primary culture TEC were trypsinized to release from plates and were used for up to 2 passages. Typical cobblestone appearance of renal epithelial cells was confirmed by visual analysis and expression of TEC markers (cytokeratin, CD13, CD26, and E-cadherin) was confirmed.

Stable expression and delivery of shRNA

Generation of shRNA targeting caspase-8 was as described (4). The expression vector, pHEX6300, was ligated to the oligonucleotide sequence for caspase-8 mRNA (5`-AAC CTC GGG GAT ACT GTC TGA) to generate caspase-8 shRNA. Empty vector or caspase-8 targeting vector (150µg of DNA) was delivered to the kidney in donor B6 via IVC injection as described (41) 48 hours prior to kidney transplantation.

Kidney ischemia reperfusion injury

A renal clamp was applied to the right kidney pedicle and removed after 45 minutes at 34°C and the left kidney was removed (4, 9, 39). Kidneys were collected at 24h, 48h, and 72h post-IRI after being flushed with normal saline until clear. Serum was tested for creatinine using an automated CX5 clinic analyzer (Beckman, Fullteron, CA).

Allogeneic (H-2^b to H-2^d) kidney transplantation

Male Balb/c recipient mice were bilaterally nephrectomized and transplanted with kidneys from male B6, caspase-8 shRNA treated B6, or RIPK3$^{-/-}$ mice in a single procedure (42). Total ischemic time was limited to 35–40 minutes. Mice with weight loss of 15% or clinical deterioration were euthanized according to animal care protocols. In addition, all recipients terminated prior to 100 days were assessed for rejection by elevation of serum creatinine (> 50 μmol/l) and histology. Serum and kidneys for histology were collected at time of sacrifice for all euthanized mice to establish rejection.

Western blot

Protein was isolated from tissue and cells using cytoplasmic lysis or nuclear lysis buffer respectively. Blots were incubated with polyclonal rabbit anti-RIPK3 (Abcam), rabbit anti-HMGB1 (Abcam), or mouse anti-β-actin (Sigma) and quantified by densitometry (Alphaview, ProteinSimple) using β-actin.

RNA isolation and real-time PCR

Total RNA was extracted from tissue and cells by Trizol (Invitrogen). cDNA was generated using Superscript II (Invitrogen) and quantified by real time PCR MX3005 (Stratagene) using SybrGreen (Bio-Rad). Primers (Invitrogen) used for Q-PCR include: RIPK3: 5'-GGGACCTCAAGCCCTCTAAC-3' and 5'-GATCCCTGATCCTGACCCTGA-3'. β-actin was used as the endogenous control. The normalized delta threshold cycle value and relative expression levels ($2^{\Delta\Delta Ct}$) were calculated according to the manufacturer's protocol.

Cell death assays

Primary renal TEC from B6 or RIPK3$^{-/-}$ mice were grown to confluent monolayers and treated with recombinant human TNFα (Peprotech), cycloheximide (Sigma), Z-VAD-fmk (BD Bioscience), and necrostatin-1 (Calbiochem) in serum-free media. Cell viability and necrosis death were assessed using propidium iodide (PI) (BD Bioscience) labeling and were analyzed by flow cytometry (Beckman Coulter). Assessment of apoptosis utilized Annexin-V (BD Bioscience) along with PI by flow cytometry.

Histology and immunohistochemistry

Tissue sections were stained with hematoxylin and eosin (H&E) and scored by a renal pathologist blinded to groups using a semi quantitative method as described (42). Scoring included tubular cell and glomerular necrosis, mononuclear cell infiltration, tubulitis, fibrosis, and vascular injury. To quantify necrosis in sections, ethidium homodimer (Invitrogen) was perfused into kidneys and areas of necrosis were assessed in frozen tissue sections (43). Briefly, 5μM ethidium homodimer was injected at 1mL/min. for 10 min. into the renal artery via the aorta and then flushed with perfusion buffer at 1mL/min for 5 min. Total nuclei were labeled by 4,6 diamidino-2 phenylindole (DAPI) in kidney sections. Sections were quantified using a fluorescent microscope and an automated analysis program (Nikon) that measures the area and fluorescent intensity of 5 random fields of the outer renal cortex per slide. Immunohistochemistry was performed using polyclonal rabbit anti-RIPK3 (Abcam) and anti-CD3 (DAKO) and standardized immunoperoxidase methods. Allograft fibrosis was assessed using Mason trichrome staining.

Statistical analysis

Shapiro-Wilk testing was used to assess data sets for normality. Parametric data was compared using Student's t-test for unpaired values and ANOVA for multiple groups while non-parametric data was compared using a Mann-Whitney test. Graft survival was analyzed by log-rank testing (Mantel-Cox) using GraphPad Prism software (GraphPad Software Inc., CA). Data is presented as mean ± SEM using $p < 0.05$ for significance.

3.4 Results

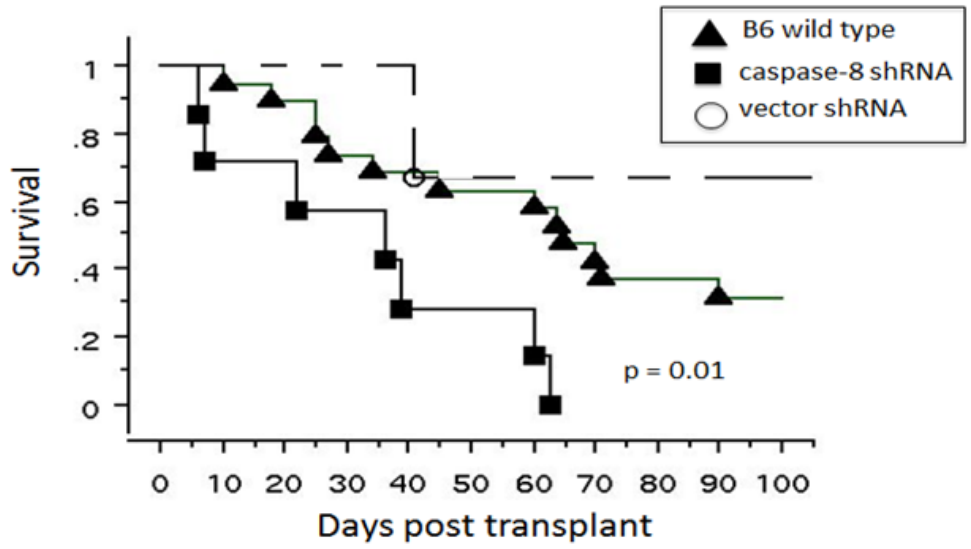
Inhibition of apoptosis by caspase-8 silencing reduced renal allograft survival

Apoptosis can be inhibited by c-FLIP or caspase-8 shRNA in TEC and treatment of kidneys *in vivo* by Fas or caspase-8 shRNA can attenuate kidney IRI (3, 4). However the potential benefit of caspase-8 inhibition has not been tested in an allogeneic renal transplantation model. Donor kidneys were treated with caspase-8 shRNA or empty vector shRNA via direct IVC injection 48 hours prior to being used for transplantation using a previously described method (4). While the duration of shRNA effect was not tested, the differential effect of caspase-8 shRNA in treated kidneys was clear compared to controls. Recipients of caspase-8 silenced donor kidneys had reduced survival compared to those that received B6 wild type donor kidneys (mean of 33.3±8.7 days, n=8 vs 68.3±10.9 days, n=17, p=0.01) (Figure 1A). Recipients treated with vector shRNA control kidneys had similar survival rates as unmanipulated B6 kidneys. One third of the recipients that received naïve donor kidneys demonstrated acceptance, consistent with previous reports of spontaneous acceptance (44). In marked contrast, none of the caspase-8 silenced allograft recipients here survived to day 100 post-transplant (p=0.01). Consistent with this shortened survival, increased mononuclear graft infiltration was evident in caspase-8 silenced grafts (Figure 1B).

Previous studies have demonstrated that TNFα triggers necroptosis rather than apoptosis when caspase-8 is inhibited and unable to block the RIPK1/3 complex, (22, 23, 30, 31, 45-47). Therefore we tested the possibility that caspase-8 shRNA inhibition augmented donor kidney necrosis to shorten survival. EHD, which labels necrotic cells with loss of cell membrane integrity, was used to quantitatively assess necrosis in kidneys (43, 48). There was increased tissue necrosis in caspase-8 silenced kidney allografts on day 4 post-transplant compared to shRNA controls as indicated by red fluorescence after EHD perfusion. Accordingly, increased release of non-nuclear HMGB1 was detected in caspase-8 shRNA treated kidneys compared to shRNA controls or naïve donor kidneys (Figure 1B). EHD fluorescence was also increased in day 8 caspase-8 shRNA treated allografts compared to controls (caspase-8 shRNA: 10±2 vs control shRNA: 1±0, p=0.04, n=3/group) (Figure 1C). Inhibition of caspase-8 in the donor renal allograft appears therefore to increase tissue necrosis and subsequent release of HMGB1. As increased mononuclear infiltrate in caspase

8-shRNA treated kidneys were observed, it is possible that reduced survival was related to augmented rejection due to increased necrosis and HMGB1.

A


B6 wild type
caspase-8 shRNA
vector shRNA
Survival
1
.8
.6
.4
.2
0
p = 0.01
0 10 20 30 40 50 60 70 80 90 100
Days post transplant

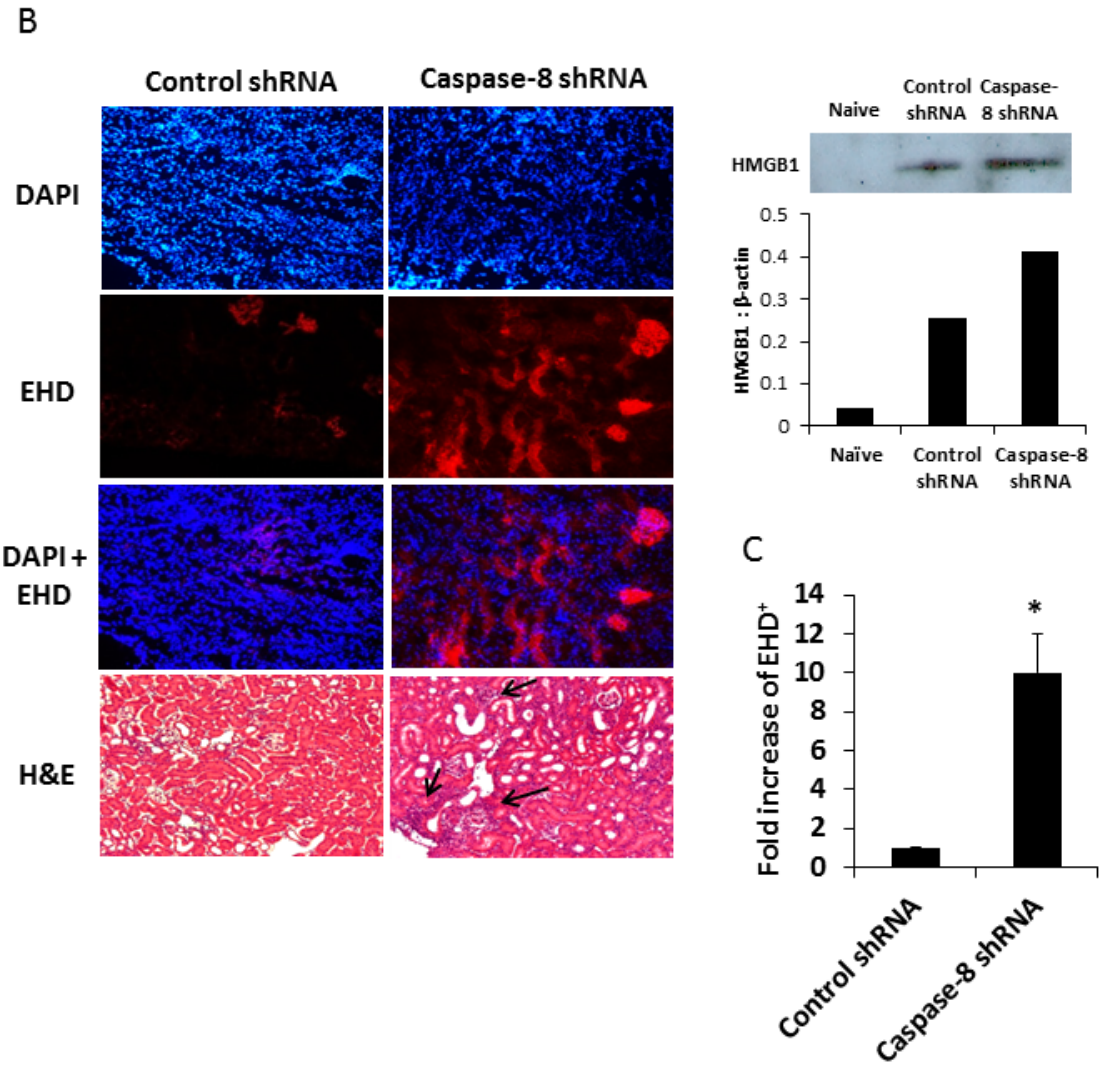

B
Control shRNA
Caspase-8 shRNA
DAPI
EHD
DAPI + EHD
H&E
Naive
Control shRNA
Caspase-8 shRNA
HMGB1
HMGB1 : β-actin
0.5
0.4
0.3
0.2
0.1
0
Naïve
Control shRNA
Caspase-8 shRNA
C
Fold increase of EHD+
14
12
10
8
6
4
2
0
*
Control shRNA
Caspase-8 shRNA

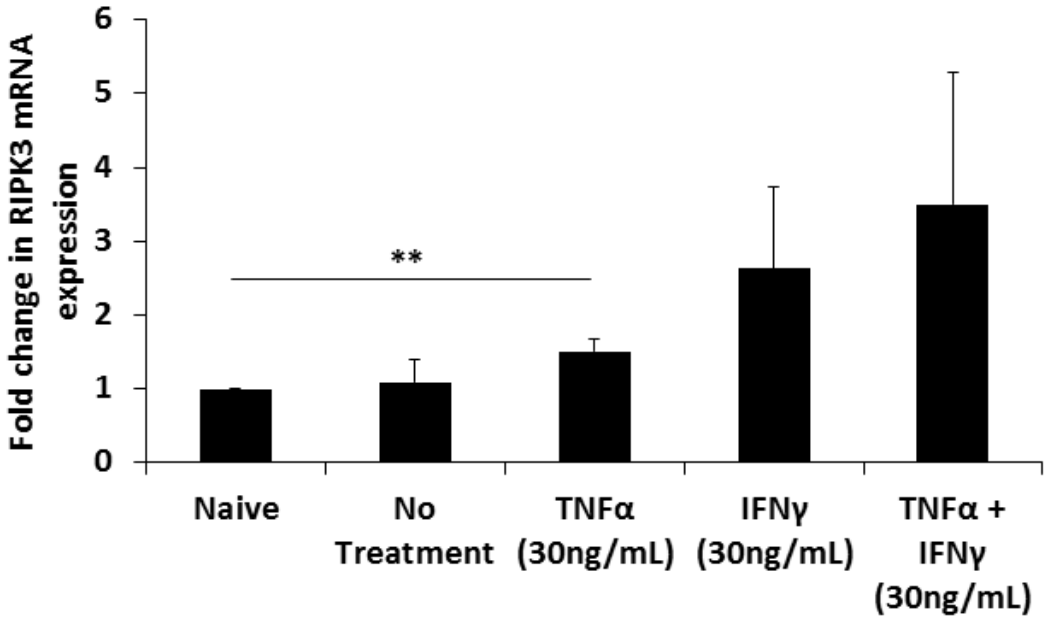
Figure 1. Caspase-8 silencing decreases renal allograft survival and increases tissue necrosis.

Bilaterally nephrectomised Balb/c (H-2^d) received a donor kidney from B6 (H-2^b) mice with or without shRNA caspase-8 silencing. Recipients were monitored as per methods. A) Renal allograft recipients were followed for survival. Recipients with wild type (B6) donor kidneys are denoted by triangles (▲) and recipients receiving caspase-8 shRNA silenced donor kidneys are denoted by squares (■).(p=0.01, log rank, n=8-17/group). Recipients receiving kidneys treated with control vector shRNA (n=3) are denoted by (○). B) Kidneys were perfused with ethidium homodimer (EHD) at 4 days post-transplant to visualize tissue necrosis (red fluorescence). Sections were stained with DAPI to identify nuclei. Sections were also stained with H&E to identify areas of graft infiltration (arrows). Images were taken at 100X magnification. Non-nuclear HMGB1 was analyzed in naïve kidney and renal allografts at 4 days post-transplant by immunoblot and semi-quantitated by densitometry (representative of 3 mice) C) EHD staining was quantified by fluorescent microscopy and analysis software in control vector and caspase-8 shRNA treated allografts at day4 post-transplant. (*: $p<0.05$, n=3/group)

RIPK3 is regulated by pro-inflammatory cytokines in renal TEC

TNFα is expressed by infiltrating cells as well as kidney parenchymal cells during acute kidney injury (50, 51). Soluble TNFα engagement with surface TNFR1 can therefore induce caspase-8 mediated apoptosis or necroptosis via RIPK1/3 if caspase-8 is inhibited (23, 24, 45, 47, 52-54). Expression of RIPK3 was confirmed in untreated primary culture TEC (Figure 2A) and following exposure to TNFα and IFNγ, which upregulated RIPK3 mRNA (1±0 vs TNFα: 1.5±0.2, p=0.007, n=3) and was maximal upon combined application of both. Similarly, RIPK3 protein was constitutively expressed in resting TEC but only modestly increased in cytokine exposed cells (Figure 2B). Therefore RIPK3 is basally expressed in renal TEC as in most cells, and expression is required for necroptosis (29, 32).

A


Fold change in RIPK3 mRNA expression
6
5
4
3
2
1
0
**
Naive
No Treatment
TNFα (30ng/mL)
IFNγ (30ng/mL)
TNFα + IFNγ (30ng/mL)

B

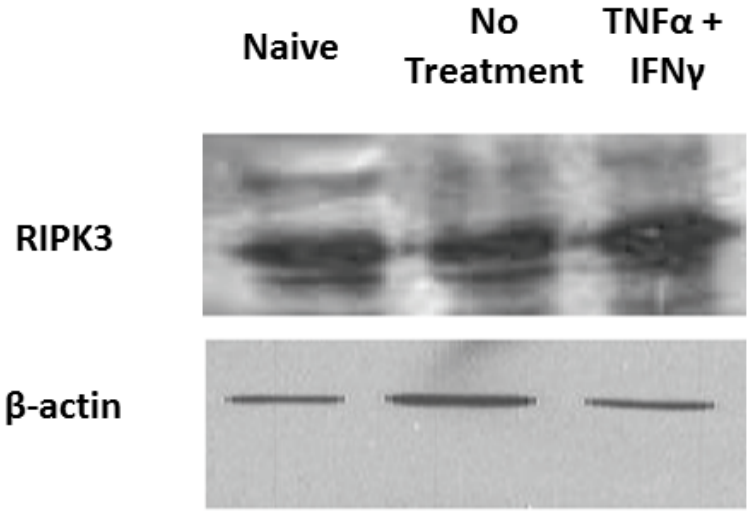
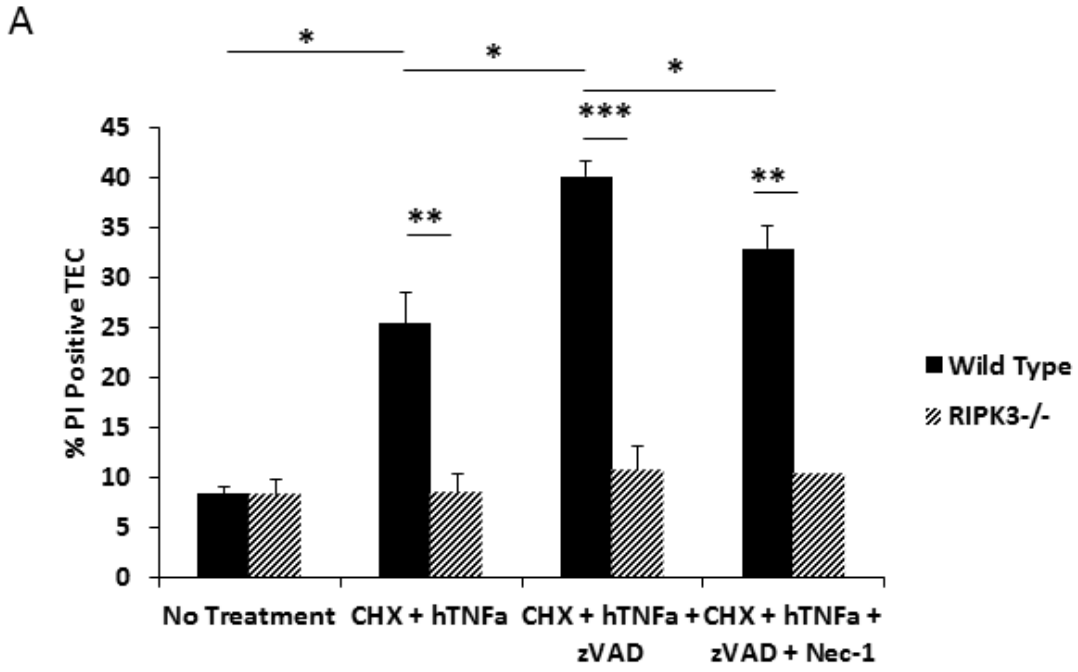

Naive
No Treatment
TNFα + IFNγ
RIPK3
β-actin

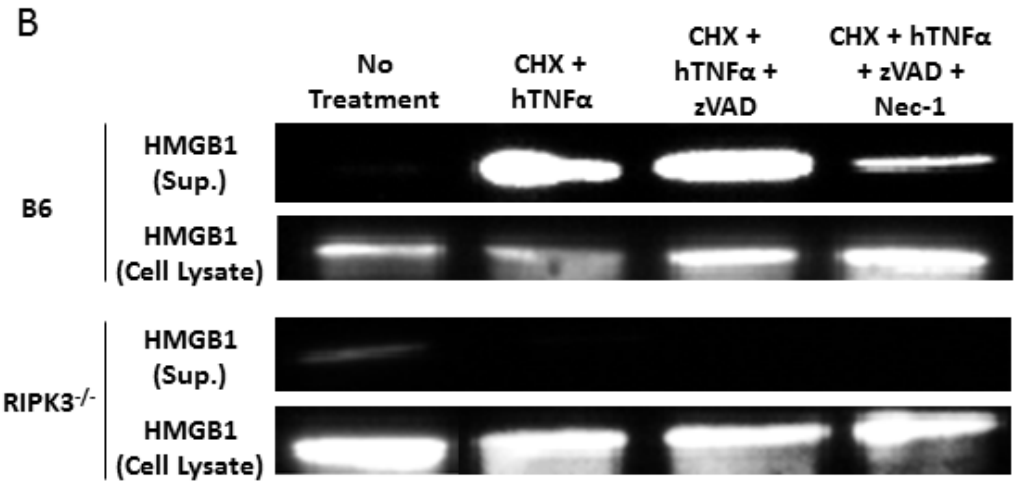
Figure 2. RIPK3 is regulated by pro-inflammatory cytokines in renal TEC.

Renal TEC were isolated from B6 and RIPK3$^{-/-}$ mice as previously described. TEC were grown to confluent monolayers and treated in serum free media. A) Wild type TEC were treated for 48 hours with 30ng/mL of TNFα and IFNγ and RIPK3 mRNA levels were quantified by Q-PCR. (**: $p<0.01$, $n=4$) B) Protein expression of RIPK3 was confirmed in wild type TEC from total cell lysate by immunoblotting using β-actin as a loading control (representative of 3 experiments)

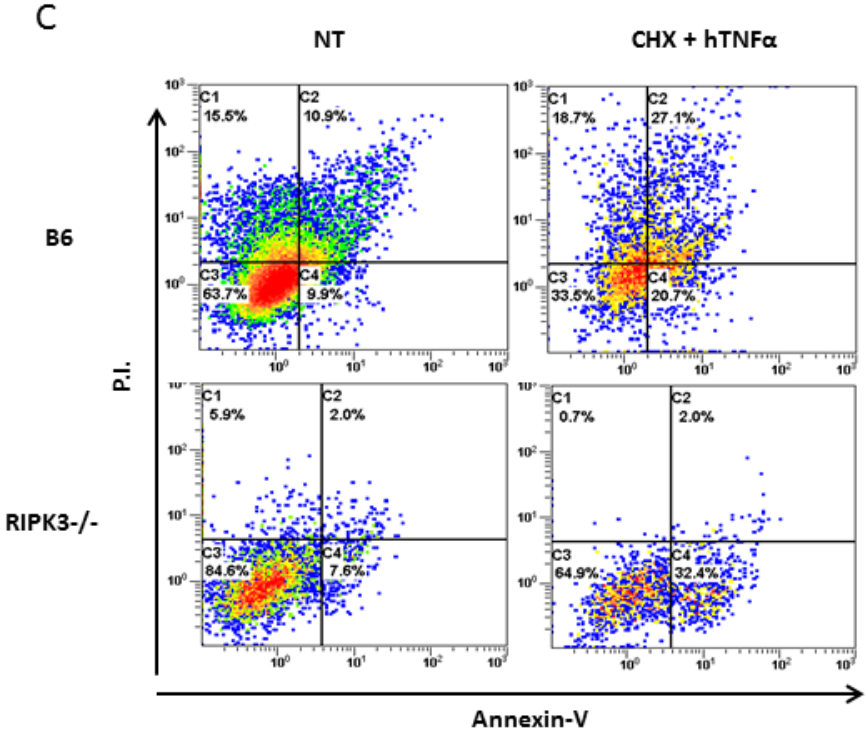
RIPK1/3 mediated necroptosis regulates HMGB1 release in TEC

RIPK3$^{-/-}$ and wild type TEC express both TNFR1 and TNFR2 surface receptors (not shown). To bias towards TNFR1 related death rather than enhanced survival via TNFR2 (55), we exposed TEC to human TNFα (hTNFα) which has a greater affinity for TNFR1 than TNFR2 in murine cells (40) as well as cycloheximide (CHX) to enhance CICD (34, 56). We also tested the RIPK1 inhibitor Nec-1 as well as RIPK3$^{-/-}$ TEC for survival and release of HMGB1 following TNFα with caspase inhibition (49, 57, 58). Wild type TEC increased PI positivity (8.3±0.7% vs. 25.4±3.2%, p=0.01, n=3) with CHX and hTNFα at 24h. z-VAD-fmk (zVAD) increased the number of PI positive cells (25.4±3.2% vs. 40.2±1.6%, p=0.01, n=3) (Figure 3A) consistent with caspase independent regulated necrosis (RN). Nec-1, which blocks necroptosis via RIPK1 (34, 59), modestly reduced PI positivity (40.2±1.6% vs. 32.9±2.2%, p=0.03, n=3). Addition of Nec-1 alone did not have an effect on TEC apoptosis in our hands (not shown), as shown in previous studies (34). In contrast, RIPK3$^{-/-}$ TEC were completely resistant to CHX and hTNFα induced necrosis compared to wild type TEC (10.8±2.3% vs. 40.2±1.6%, p=0.0005, n=3) and did not change with zVAD. Interestingly, death induced without caspase-inhibition, using only CHX and TNF□ treatment was also abolished in RIPK3$^{-/-}$ TEC. This latter finding is in line with *in vivo* reports of observed protective effects with Nec-1 without caspase-inhibition, as reviewed in Linkermann et al (60). Consistent with PI results and a necroptosis mechanism, HMGB1 release into supernatant was greater with zVAD treated TEC compared to hTNFα/CHX treated TEC and could not be detected in the supernatant from RIPK3$^{-/-}$ TEC. Importantly HMGB1 release from necrotic cells (58) was nearly completely absent in Nec-1 treated TEC (Figure 3B) and intracellular HMGB1 from cell lysates remained unchanged in all treatment groups. Exposure of RIPK3$^{-/-}$ tubular cells to extremely high concentrations of hTNF□ (300ng/mL) can induce apoptosis as detected by Annexin-V (Figure 3C), yet no detectable PI positivity was induced nor was HMGB1 was found in the supernatant (Figure 3D). This suggests that in the absence of necroptosis as a death pathway, apoptosis can occur in RIPK3 $^{-/-}$ TEC but without the release of HMGB1 or PI positivity. These data demonstrate that caspase inhibition in TEC results in necroptosis which could account for shRNA transplant results.


A
*
*
*
***
**
**
45
40
35
30
25
20
15
10
5
0
% PI Positive TEC
No Treatment
CHX + hTNFa
CHX + hTNFa + zVAD
CHX + hTNFa + zVAD + Nec-1
Wild Type
RIPK3-/-


B
No Treatment
CHX + hTNFα
CHX + hTNFα + zVAD
CHX + hTNFα + zVAD + Nec-1
B6
HMGB1 (Sup.)
HMGB1 (Cell Lysate)
RIPK3-/-
HMGB1 (Sup.)
HMGB1 (Cell Lysate)

C


NT
CHX + hTNFα
B6
RIPK3-/-
P.I.
Annexin-V
C1 15.5%
C2 10.9%
C3 63.7%
C4 9.9%
C1 18.7%
C2 27.1%
C3 33.5%
C4 20.7%
C1 5.9%
C2 2.0%
C3 84.6%
C4 7.6%
C1 0.7%
C2 2.0%
C3 64.9%
C4 32.4%

D

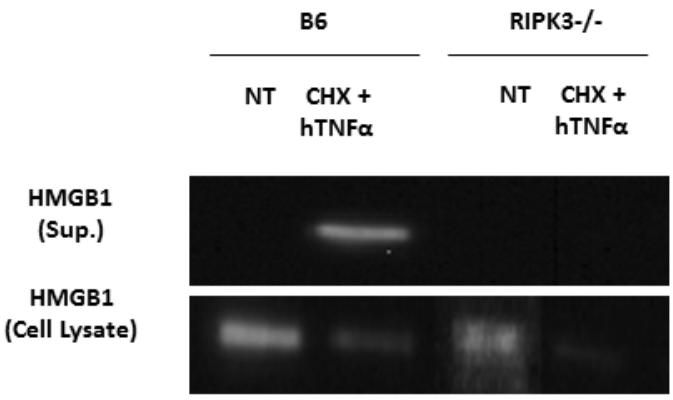
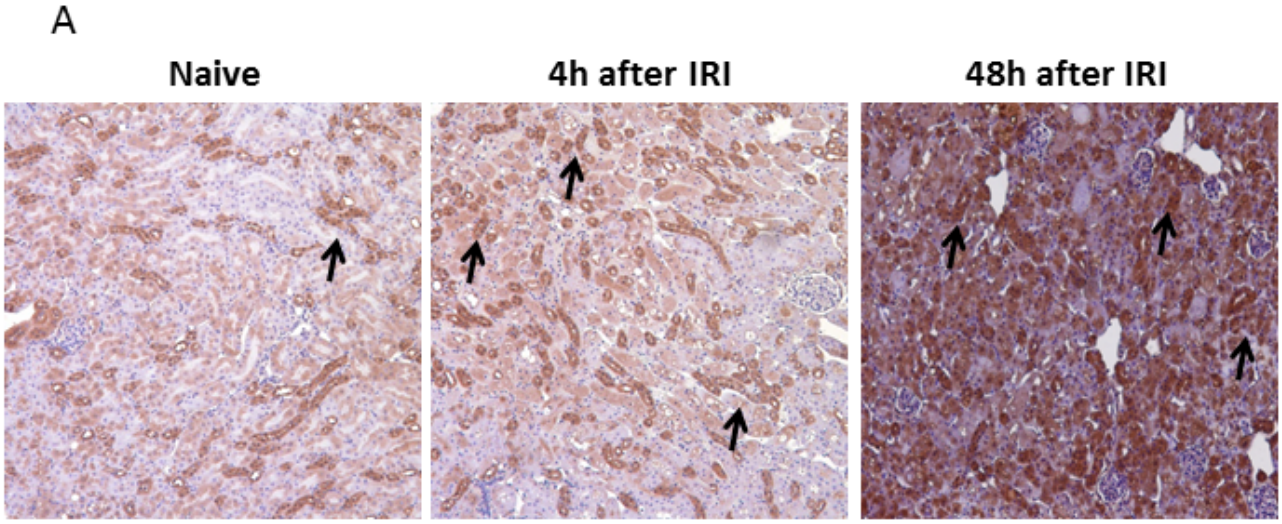

B6
RIPK3-/-
NT
CHX + hTNFα
NT
CHX + hTNFα
HMGB1 (Sup.)
HMGB1 (Cell Lysate)

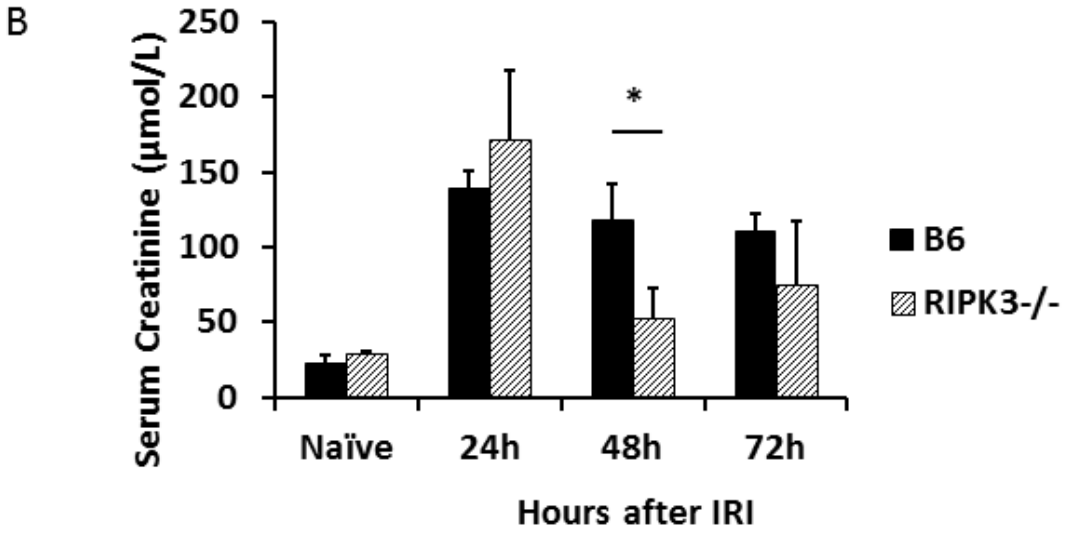
Figure 3. RIPK1/3 is a regulator of TNFα mediated necroptosis in renal TEC.

Renal TEC were isolated from B6 and RIPK3$^{-/-}$ mice as previously described. TEC were grown to confluent monlayers and treated in serum free media. A) Wild type and RIPK3$^{-/-}$ TEC were treated with CHX (1ug/mL), hTNFα (100ng/mL), Z-VAD-fmk (50μM), or Nec-1 (10μM) for 24h. Necroptosis was analyzed by PI labeling and flow cytometry. (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, n=3/group) B) Supernatants and total intracellular protein from cell lyastes collected from wild type and RIPK3$^{-/-}$ TEC treated with CHX (1ug/mL), TNFα (100ng/mL), Z-VAD-fmk (50μM), or Nec-1 (10μM) for 24h were analyzed for HMGB1 by immunoblotting (representative of 3 experiments). C) Wild type and RIPK3$^{-/-}$ TEC were treated with CHX (1ug/mL) and hTNFα (300ng/mL) for 24h and cell death was analyzed by Annexin-V and PI labeling and flow cytometry (representative of 3 experiments). D) Supernatants and total intracellular protein from cell lysates collected from TEC were analyzed for HMGB1 by immunoblotting (representative of 3 experiments).

RIPK3$^{-/-}$ mice are resistant to kidney injury after renal IRI

The RIPK1 inhibitor Nec-1 can ameliorate kidney injury in a mouse model (34). We therefore extended those studies to include the participation of RIPK3 in kidney injury. RIPK3 expression was detected at low levels in naive murine kidney sections but was expressed at higher levels at 4 hours and persisted as long as 48 hours post IRI (Figure 4A). RIPK3 expression was ubiquitously expressed in both proximal and distal tubules (indicated by arrows), which is consistent with our results using primary culture TEC. Kidney function post IRI was assessed by serum creatinine measurements at 24h, 48h, and 72h in both wild type (B6) and RIPK3 mice and compared to naïve mice. Interestingly, both were equivalently elevated at 24hours post IRI. However, a clear benefit of RIPK3 absence in renal IRI was observed at 48h (61 ± 24 vs 137 ± 26 μmol/L, p=0.03, n=7/group) (Figure 4B). In our model, 48 hours post IRI consistently represents a maximum injury time point, as mice have recovered sufficiently post procedure to exclude hydration as a variable. Wild type mice had increased acute tubular necrosis and a greater injury score as compared to RIPK3$^{-/-}$ after 48h of renal IRI (2.5 ± 0 vs. 1.5 ± 0.2, p=0.02, n=4-7/group) (Figure 4C). Our data demonstrates that inhibition of RIPK3 can ameliorate acute kidney injury similar to that observed with RIPK1 inhibition (34).


A
Naive
4h after IRI
48h after IRI


B
Serum Creatinine (µmol/L)
250
200
150
100
50
0
*
Naïve
24h
48h
72h
Hours after IRI
B6
RIPK3-/-

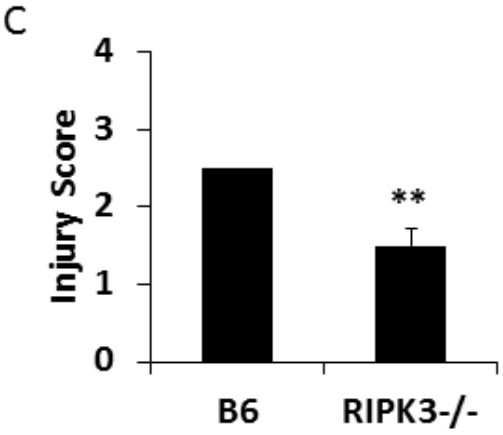

C
Injury Score
4
3
2
1
0
**
B6
RIPK3-/-

Figure 4. Absence of kidney RIPK3 improves renal function and ameliorates injury during renal IRI.

B6 controls and RIPK3$^{-/-}$ mice were subjected to acute ischemia for 45 min. using a renal clamp at 32° Celsius. Reperfusion injury occurred over a 48 hour period during which mice were sacrificed at various time points. A) Kidney sections were analyzed for RIPK3 by immunohistochemistry. Tubules positive for the presence of RIPK3 are indicated by arrows. Images were taken at 100X magnification. B) Renal function was determined by serum creatinine in naïve and at 24h, 48h, and 72h post-IRI. (*: $p<0.05$, n=7/group) C) Kidney sections were stained with H&E and scored by a pathologist blinded to groups. Areas of injury (arrows) are more evident in B6 kidneys compared to RIPK3$^{-/-}$ at 48h post-IRI. Slides were scored on a scale from 0-4 where 0=no injury and 4=area of injury >75% of kidney. (**: $p<0.01$, n=4-7/group).

Using EHD, tissue necrosis was easily detected in wild type kidneys 48h after renal IRI but was nearly undetectable in RIPK3$^{-/-}$ kidneys (Figure 5A). Kidney sections also showed more areas of tubular injury and necrosis (arrows) in wild type as compared to RIPK3$^{-/-}$. EHD was quantitated in Figure 5B and confirmed markedly decreased levels of necrosis in RIPK3$^{-/-}$ kidneys 48h after renal IRI (1±0 vs. 18±2, $p<0.0001$, n=4/group). HMGB1 increased in wild type kidneys at 48h post-IRI (0.1±0.01 vs. 0.3±0.04, $p=0.02$, n=3) (Figure 5C) but did not increase in RIPK3$^{-/-}$ kidneys.

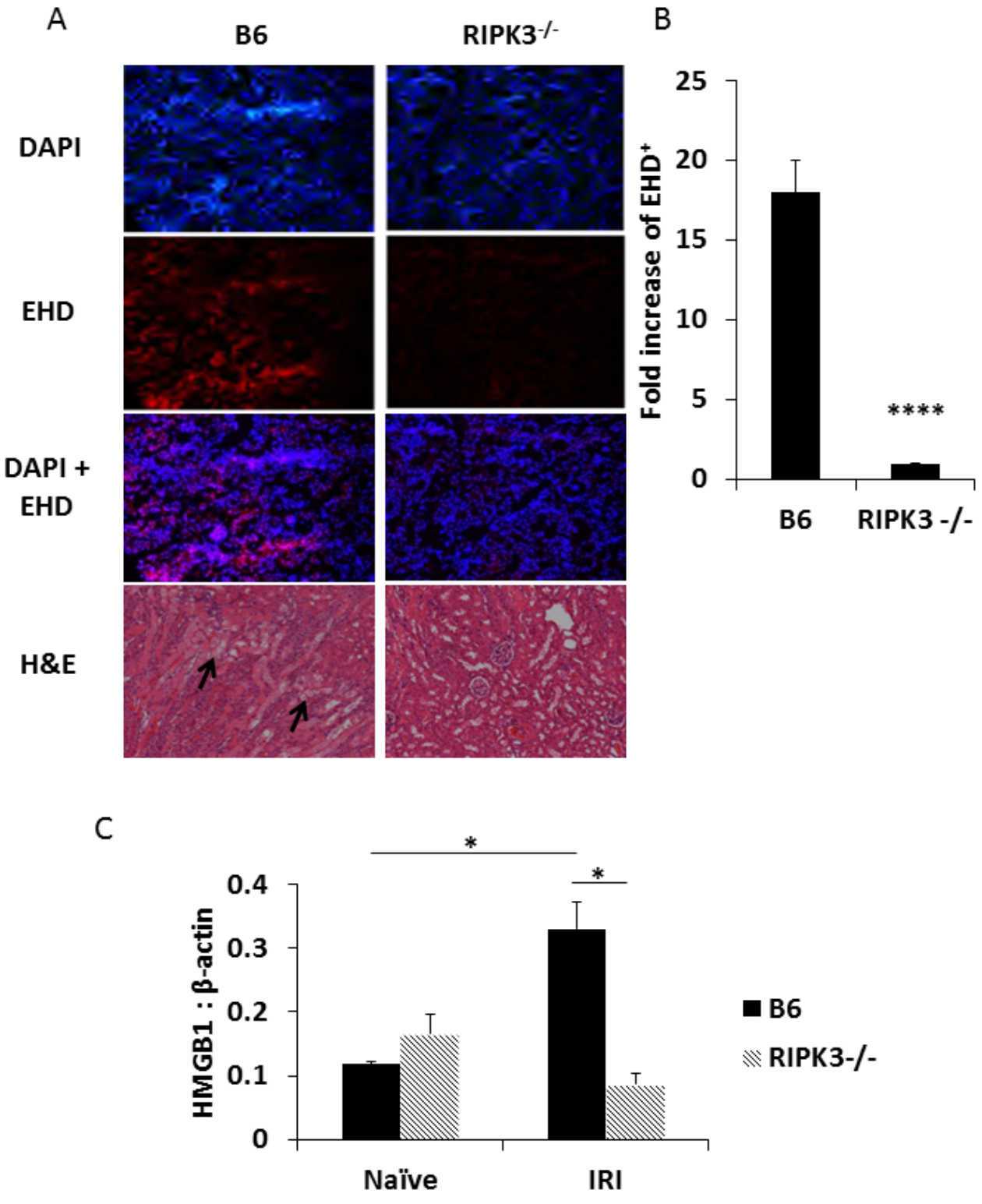
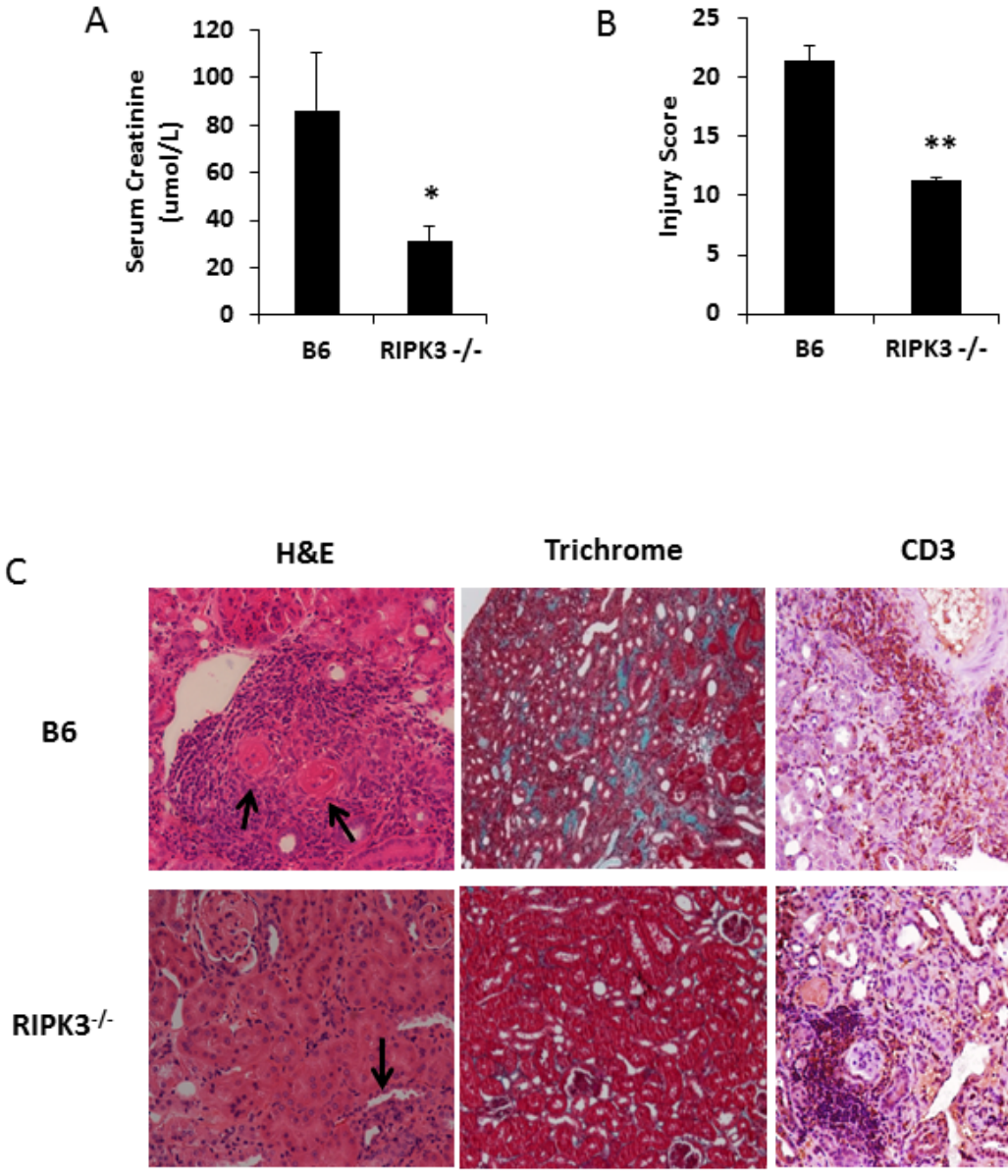

A
B6
RIPK3-/-
DAPI
EHD
DAPI +
EHD
H&E
B
Fold increase of EHD+
25
20
15
10
5
0
****
B6
RIPK3 -/-
C
*
*
HMGB1 : β-actin
0.4
0.3
0.2
0.1
0
Naïve
IRI
B6
RIPK3-/-

Figure 5. Absence of kidney RIPK3 reduces necrosis during renal IRI.

A) Kidneys were perfused with ethidium homodimer (EHD) after 48h of IRI and stained with DAPI to visualize nuclei. Images were taken at 40 X (DAPI, EHD) and 100 X (H&E) magnification (representative of 4 mice). B) EHD stained sections were quantified by fluorescent microscopy and scored by automated software analysis. (****: $p<0.0001$, n=4/group) C) Total non-nuclear protein was isolated from kidney tissue samples in wild type and RIPK3$^{-/-}$ 48h post-IRI. HMGB1 protein expression was analyzed by immunoblot using β-actin as a loading control. (*: $p<0.05$, n=3/group)

RIPK3$^{-/-}$ donor kidneys are resistant to allograft dysfunction and inflammation

Chronic allograft injury is a major complication associated with kidney transplantation (61) and previous studies have suggested that fibrosis with persistent inflammation is important (2, 62, 63). We therefore tested whether the absence of RIPK3 and its resultant effect on necroptosis in a donor kidney could improve function or long term survival following allo-transplantation. Serum creatinine levels of RIPK3$^{-/-}$ grafts were lower than wild type at study end (31 ± 0.6 vs 86 ± 24 μmol/L, p=0.03, n=8-9/group) (Figure 6A) and had reduced inflammation and histological injury (21.4±1.2 vs 11.2±0.2, p=0.006, n=4-5/group) (Figure 6B, 6C). Wild type grafts also had greater neutrophil infiltration, fibrosis, tubilitis, and vascular injury (Figure 6D). Sections from long term (>100 days) grafts were found to have equivalent expression of CD3 positive infiltrates in both wild type and RIPK3$^{-/-}$ kidneys (Figure 6C). Kidney HMGB1 was less in RIPK3$^{-/-}$ kidneys at both day 8 and d100 post-transplant (p=0.04, n=3/group) (Figure 7A).


A
Serum Creatinine (umol/L)
120
100
80
60
40
20
0
*
B6
RIPK3 -/-
B
Injury Score
25
20
15
10
5
0
**
B6
RIPK3 -/-
C
H&E
Trichrome
CD3
B6
RIPK3-/-

D

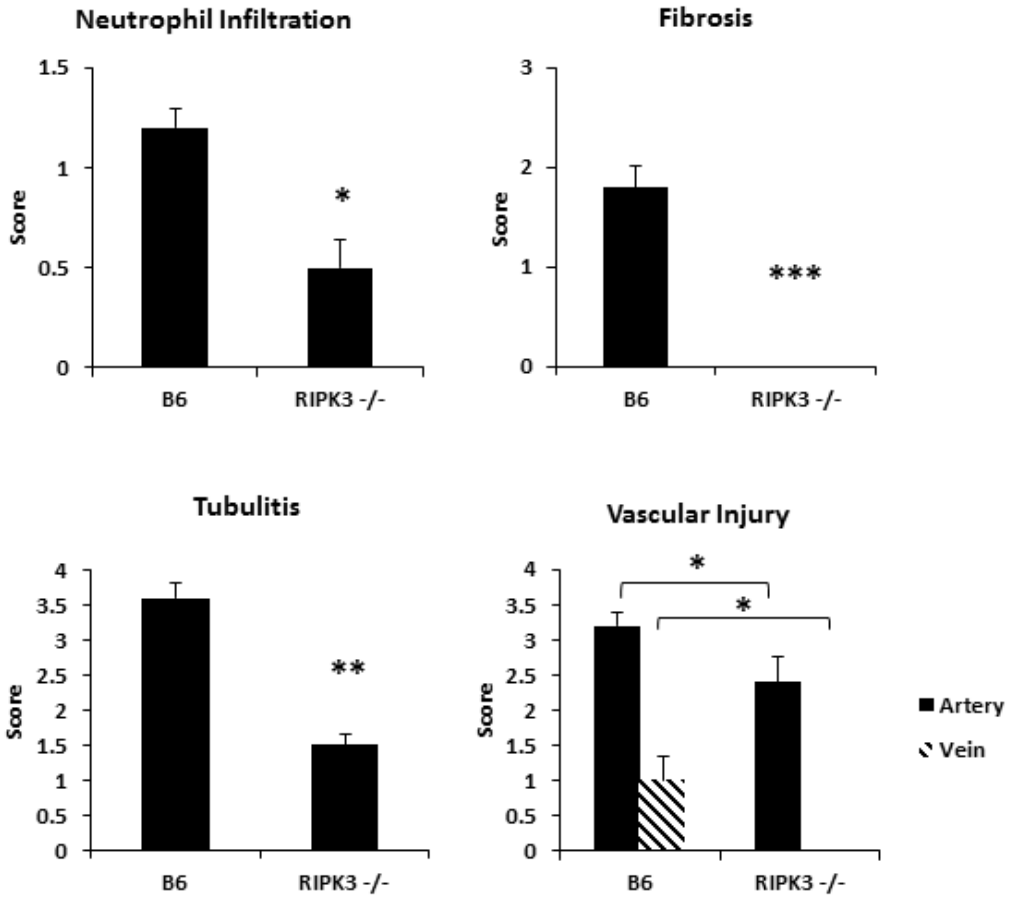
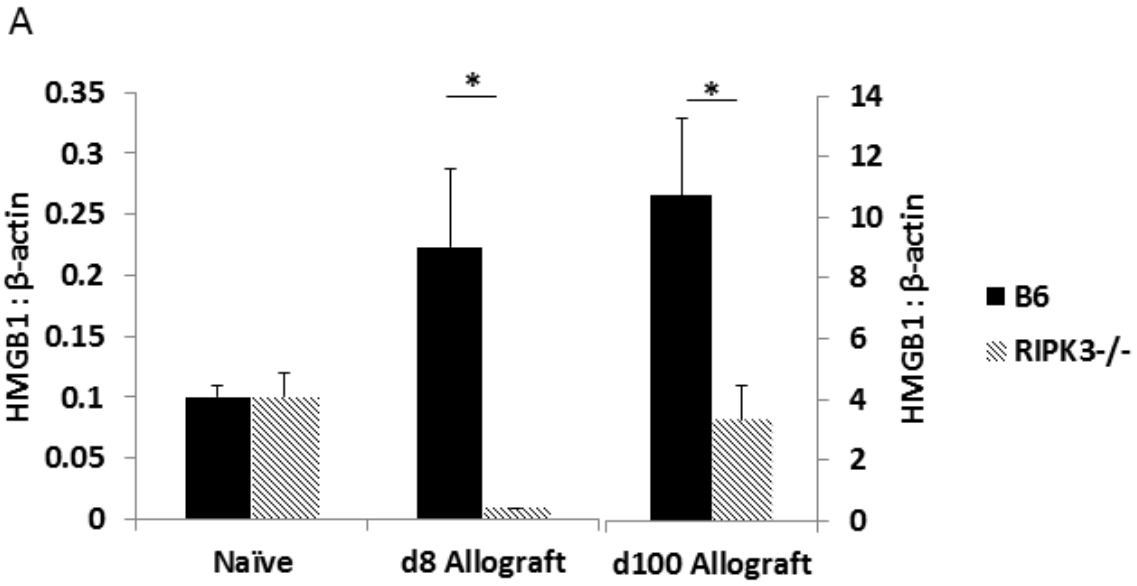

Neutrophil Infiltration
Score
1.5
1
0.5
0
*
B6
RIPK3 -/-
Fibrosis
Score
3
2
1
0
***
B6
RIPK3 -/-
Tubulitis
Score
4
3.5
3
2.5
2
1.5
1
0.5
0
**
B6
RIPK3 -/-
Vascular Injury
Score
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
B6
RIPK3 -/-
Artery
Vein

Figure 6. RIPK3$^{-/-}$ kidney allografts have better function and decreased inflammation.

Bilaterally nephrectomised Balb/c (H-2^{d}) received a donor kidney from B6 or RIPK3$^{-/-}$ (H-2^{b}) mice. Recipients were monitored as per methods. A) Renal function was determined by serum creatinine at the time of sacrifice (*: $p<0.05$, n=8-9/group). B, C, D) Kidney tissue was formalin fixed at time of sacrifice. Kidney sections were stained with H&E, Mason trichrome, and CD3 and were scored by a pathologist blinded to group. More infiltrating cells (CD3^{+}) are evident in wild type (B6) grafts than in RIPK3$^{-/-}$. Images were taken at 100 X (Trichrome) and 200 X (H&E, CD3) magnification and arrows (H&E) indicate representative areas of injury. Slides were scored on a scale from 0-4 where 0=no change and 4=changes in +75% of kidney for various pathological criteria and compiled to generate an overall injury score. (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, n=4-5/group).

Inhibition of RIPK3 in kidney allografts prolongs long term survival

RIPK3$^{-/-}$ kidney graft recipients achieved greater rejection free survival to day 100 (90 vs 23%, p=0.002, n=10-17/group) (Figure 7B) and survived longer than those that received wild type (B6) kidneys (56.7±9.4 vs 94.1±2.1 days, p=0.0006, n=10-17/group). Body weight loss was maintained in both groups of mice at day 100. Wild type mice terminated prior to day 100 however had weight loss from baseline that was greater than RIPK3$^{-/-}$ recipients (7.8±4.5% vs.1.2±1.2%, n=4-5/group) which was consistent with rejection, as terminated wild type kidney recipients had histological rejection and higher serum creatinine levels than RIPK3$^{-/-}$ kidney recipients (148 ± 51 vs. 29 ± 6 μmol/L, p=0.03, n=5-7/group). Mice that survived to day 100 had similar kidney function in each group (wild type: 53.2±17.7 vs. RIPK3$^{-/-}$: 27.2±6.6 μmol/L, n=5/group, p=ns). As CD3+ mononuclear cell infiltration and tubulitis was observed in RIPK3$^{-/-}$ kidneys, rejection was clearly not prevented. However there was a marked absence of fibrosis and reduction in vascular injury in RIPK3$^{-/-}$ kidneys (Figure 6C, 6D).


A
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
HMGB1 : β-actin
14
12
10
8
6
4
2
0
HMGB1 : β-actin
*
*
B6
RIPK3-/-
Naïve
d8 Allograft
d100 Allograft

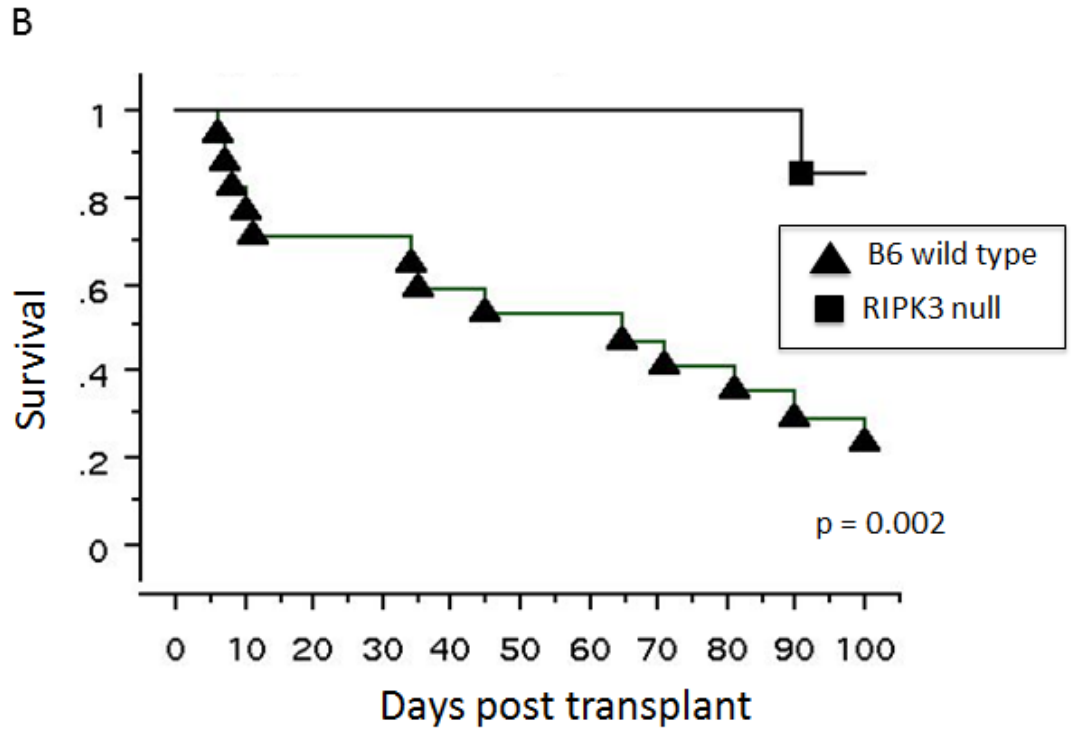

B
1
.8
.6
.4
.2
0
Survival
B6 wild type
RIPK3 null
p = 0.002
0 10 20 30 40 50 60 70 80 90 100
Days post transplant

Figure 7. Inhibition of RIPK3 in donor kidneys increased allograft survival in allogeneic kidney transplant recipients.

Bilaterally nephrectomised Balb/c (H-2^d) received a donor kidney from B6 or RIPK$3^{-/-}$ (H-2^b) mice. Recipients were monitored as in methods. A) Total non-nuclear protein was isolated from kidney tissue samples in wild type and RIPK$3^{-/-}$ kidneys and allografts. HMGB1 protein expression was detected by immunoblot using β-actin as a loading control. (*: $p<0.05$, n=3/group) Note the change in scale in the d100 allografts. B) Recipients with wild type (B6) donor kidneys are denoted by triangles (▲) and recipients with RIPK$3^{-/-}$ donor kidneys are denoted by squares (■). ($p=0.002$, log rank, n=10-17/group)

3.5 Discussion

Although acute rejection rates and one year graft survivals have improved in kidney transplantation, long-term survival has not substantially changed or is improving very slowly (64-66). Although reasons are most certainly multifactorial and biologically complex, there has been little attention directed to donor organ factors that might contribute to long-term graft loss. Kidney transplantation is invariably associated with organ damage, which includes IRI. IRI triggers a cascade of linked innate and adaptive immune responses that propagate injury, kill parenchymal cells, and promote antibody and cell mediated rejection (9, 63, 67, 68). The form of cell death may be an early variable that directs the outcome of immune responses. The current understanding of cell death mechanisms has greatly expanded beyond apoptosis to regulated necrosis, a broad category that includes necroptosis, pyroptosis, and others. As a result, various assays have been used to differentiate between cell death subroutines including cell viability, morphology, quality of DNA fragmentation, loss of membrane integrity assays, TUNEL-positivity, PARP1 cleavage, caspase activation, labeling with Annexin or PI (57, 69) and many others (70)**.** A powerful approach in labeling the type of cell death utilizes pharmacologic inhibitors that target specific pathways, RNA knockdown strategies, and animal models with genetic deletions (57).

Apoptosis is a classic form of programmed cell death (12, 13). In previous studies we have shown that caspase inhibition using shRNA to silence caspase-8 or transgenic overexpression of the endogenous caspase-8 inhibitor c-FLIP can protect renal TEC against TNF□ induced apoptosis *in vitro* or ischemic kidney injury *in vivo* (4, 36). Pan-caspase inhibition of both initiator and effector caspases can reduce cold preservation injury due to apoptosis in liver endothelial cells and transplanted islet cells (71, 72). However, reports in other models and in particular renal IRI, has not confirmed a benefit using caspase inhibition (34) perhaps as variability exists in the specificity of caspase inhibitors (73). Long term studies to evaluate the effect of donor organ apoptosis inhibition in kidney transplants have been hampered as the embryonic lethality of caspase-8 deficiency precludes the use of animal models (74) and the duration of gene silencing with siRNA is limited. Results in the present study in which donor caspase-8 RNA silencing worsened kidney transplants are consistent with recent insights into new forms of regulated cell death in which caspase 8 silencing triggers pro-

inflammatory necroptosis (47, 49, 75). These results support that short term outcomes in an IRI model can differ from long term outcomes in a transplant model, depending on targets of cell death.

While apoptosis generates membrane bound apoptotic bodies that sequester cellular contents (73, 76), necrosis results in loss of membrane integrity and the release of HMGB1 and other CCDAMP which promote inflammatory responses (49, 69) through interaction with TLR, as well as other innate receptors which are ubiquitously expressed within the kidney (77-79) and on a wide variety of immune cells including dendritic cells. Maximal protection in IRI has been observed when HMGB1 was not able to engage kidney TLR4 (80, 81). Thus, HMGB1 release may contribute to the propagation of kidney injury.

Ligand engagement of death receptor (DR) family members (CD95/Fas, TNFR1 and TRAIL) normally results in apoptosis and inhibition of caspase-8 might be expected to be of benefit in IRI in which acute loss of cells reduces organ function (4). However, recent studies using TNFα indicate that a primary function of caspase-8 may be also to block necroptosis (22, 23, 30). Genetic deletion of caspase-8 is embryonically lethal due to unrestricted necroptosis in the developing yolk sack (22). Similarly, caspase-8 inhibition could be detrimental in adult tissue if DR mediated apoptosis was 'replaced' by necroptosis (32,82). Necroptosis may be particularly relevant to kidney injury in that TEC produce TNF□ as well as respond to TNFα (83).

In this paper, we found pan-caspase inhibition resulted in necrosis of cytokine exposed TEC *in vitro*. As the presence of PI positive labeling by flow cytometry alone may not exclusively define necrotic cells as opposed to cells undergoing late apoptosis, we also measured HMGB1 release to confirm necrosis in TEC and grafts (57, 58). Loss of plasma membrane integrity during necrosis results in nuclear HMGB1 moving to the extracellular space. As HMGB1 is not actively synthesized during this process, the resulting HMGB1 found in supernatant is presumed to be due to release of HMGB1 from nuclei. In the present paper we confirm that TEC undergo necroptosis which can be abrogated by Nec-1. We also show that TEC express abundant levels of RIPK3 protein which increased within hours of ischemic injury. $RIPK3^{-/-}$ TEC exposed to extremely high concentrations of hTNFα to TEC can undergo Annexin-V positive apoptosis without PI positive necrosis or the release of

HMGB1. Our data suggests that in the absence of the necroptosis pathway, apoptosis can still occur in TEC.

Consistent with our *in vitro* results, the worsening of graft survival following caspase inhibition likely represents enhanced alloimmune responses following necroptosis and the release of CCDAMP from kidney cells (17, 84). In testing kidney samples, protein was isolated using a lysis buffer that excludes nuclear protein including HMGB1. Therefore, the HMGB1 we detected in whole kidney lysates was released either from kidney parenchymal cells or infiltrating cells. We utilized EHD to measure tissue necrosis which is reproducible and quantifiable but is limited by a lack of specific cellular detail. Increased HMGB1 and EHD positivity in shRNA caspase-8 silenced kidneys was consistent with necroptosis and observed worsened kidney function. While not tested here, the release of CCDAMP such as HMGB1 likely promoted alloimmune responses that reduced survival. In marked contrast, HMGB1 was lower, EHD was absent, and kidney function was better using RIPK3$^{-/-}$ mice in our IRI model. Unlike RIPK1, RIPK3 cannot participate in NFkB-signaling and kidney function and development are normal in RIPK3$^{-/-}$ mice. Protection in RIPK3$^{-/-}$ mice was very similar to IRI studies in which both kidney and infiltrating cells were exposed to Nec-1 (34) suggesting that the beneficial effect of RIPK1 and RIPK3 inhibition was due to elimination of kidney necroptosis.

These data provide the first unequivocal demonstration of RIPK3 mediated necroptosis in both renal IRI and transplantation. While both apoptosis and necrosis appear to contribute to kidney dysfunction in short term IRI models, our results suggest that there may be a transitional phase following IRI in which alloimmune activation can be promoted by necroptosis as well as loss of anti-inflammatory responses generated by apoptosis (12, 13, 76, 85, 86). HMGB1 and TLR4 expression peaks with 5-10 days in renal IRI (80, 81). In blocking caspase-8 and augmenting necroptosis, prolonged HMGB1 and CCDAMP release may have increased the participation of IRI relevant infiltrating T cells (87), NK cells (9), and other effectors. Additional studies will be required to delineate the immune effector cells involved. Furthermore, the relative impact of various forms of cell death may vary in different solid organs, generating organ-specific apoptosis-necroptosis ‘equilibriums’ and both may need to be targeted for maximal protection (88).

This paper provides the first report of the benefit of kidney RIPK3 deletion and necroptosis elimination on long term allograft survival. Despite preserved function reflected by serum creatinine and prolonged survival of $RIPK3^{-/-}$ kidney recipients, tissue injury with HMGB1 release and $CD3^{+}$ cellular infiltration were observed in the grafts of long term survivors. Clearly the elimination of necroptosis and reduced CCDAMP release early post-transplant did not result in tolerance *per se*. It therefore may be speculated that the benefit of RIPK3 deletion is related to an attenuation but not elimination of alloimmune responses. While HMGB1 levels were consistently less than wild type controls at every time point, significant levels of HMGB1 were detected in late day 100 $RIPK3^{-/-}$ allografts which may have been derived from parenchymal cells undergoing non-necroptosis death or secretion from viable infiltrating cells (89, 90). Notably however, the near complete absence of fibrosis in $RIPK3^{-/-}$ kidneys suggests that necroptosis may play a critical role in long-term allograft injury that results in scarring.

In summary, we show for the first time that RIPK3 regulates necroptosis in the kidney and that this has a major impact on renal IRI and kidney transplant survival. As well we have demonstrated that inhibition of caspase-8 within TEC eliminates a key regulatory role for this enzyme in controlling RIPK1/3 mediated necroptosis, and therapeutic strategies may require control of multiple pathways. We suggest that reduction of necroptosis in donor organs will have a profound benefit in graft function and survival. More efficacious forms of Nec-1 (91) and the possibility of targeting RIPK3 (25) will greatly advance such strategies and may represent a paradigm shift in modifying organ injury to dampen alloimmune responses.

3.6 Acknowledgements

We thank Genentech and Dr. Francis K.M. Chan for providing breeder pairs of RIPK3$^{-/-}$ mice to establish a colony and Ms. Pamela Gardner for administrative support. This study was supported by the Canadian Institutes of Health Research (XTW-90932, MOP-111180, MOP-115048), the Kidney Foundation of Canada (A.M.J., Z.X.Z), and the Program of Experimental Medicine (POEM) at Western University, London, Ontario, Canada.

3.7 Author Disclosure

The authors have no financial disclosures or conflict of interest in this study.

3.8 References

1. Pagtalunan ME, Olson JL, Tilney NL, Meyer TW. Late consequences of acute ischemic injury to a solitary kidney. Journal of the American Society of Nephrology : JASN 1999;10(2):366-373.

2. Bonventre JV. Dedifferentiation and proliferation of surviving epithelial cells in acute renal failure. Journal of the American Society of Nephrology : JASN 2003;14 Suppl 1:S55-61.

3. Du C, Guan Q, Yin Z, Masterson M, Zhong R, Jevnikar AM. Renal tubular epithelial cell apoptosis by Fas-FasL-dependent self-injury can augment renal allograft injury. Transplantation Proceedings 2003;35(7):2481-2482.

4. Du C, Wang S, Diao H, Guan Q, Zhong R, Jevnikar AM. Increasing resistance of tubular epithelial cells to apoptosis by shRNA therapy ameliorates renal ischemia-reperfusion injury. Am J Transplant 2006;6(10):2256-2267.

5. Noronha BT, Li JM, Wheatcroft SB, Shah AM, Kearney MT. Inducible nitric oxide synthase has divergent effects on vascular and metabolic function in obesity. Diabetes 2005;54(4):1082-1089.

6. Chen L, Ahmed E, Wang T, Wang Y, Ochando J, Chong AS et al. TLR signals promote IL-6/IL-17-dependent transplant rejection. Journal of immunology 2009;182(10):6217-6225.

7. Chen L, Wang T, Zhou P, Ma L, Yin D, Shen J et al. TLR engagement prevents transplantation tolerance. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2006;6(10):2282-2291.

8. Kinsey GR, Li L, Okusa MD. Inflammation in acute kidney injury. Nephron Experimental nephrology 2008;109(4):e102-107.

9. Zhang Z-X, Wang S, Huang X, Min W-P, Sun H, Liu W et al. NK cells induce

apoptosis in tubular epithelial cells and contribute to renal ischemia-reperfusion injury. J Immunol 2008;181(11):7489-7498.

10. Obata F, Yoshida K, Ohkubo M, Ikeda Y, Taoka Y, Takeuchi Y et al. Contribution of CD4+ and CD8+ T cells and interferon-gamma to the progress of chronic rejection of kidney allografts: the Th1 response mediates both acute and chronic rejection. Transpl Immunol 2005;14(1):21-25.

11. Ichimura T, Asseldonk EJ, Humphreys BD, Gunaratnam L, Duffield JS, Bonventre JV. Kidney injury molecule-1 is a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells. J Clin Invest 2008;118(5):1657-1668.

12. Pallet N, Dieudé M, Cailhier J, Hébert M. The molecular legacy of apoptosis in transplantation. American Journal of Transplantation 2012;12(6):1378-1384.

13. Ferguson TA, Choi J, Green DR. Armed response: how dying cells influence T-cell functions. Immunol Rev 2011;241(1):77-88.

14. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell death and differentiation 2009;16(1):3-11.

15. Schroder K, Tschopp J. The inflammasomes. Cell 2010;140(6):821-832.

16. Vilaysane A, Chun J, Seamone ME, Wang W, Chin R, Hirota S et al. The NLRP3 inflammasome promotes renal inflammation and contributes to CKD. J Am Soc Nephrol 2010;21(10):1732-1744.

17. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002;418(6894):191-195.

18. Beyer C, Stearns NA, Giessl A, Distler JH, Schett G, Pisetsky D. The extracellular release of DNA and HMGB1 from Jurkat T cells during in vitro necrotic cell death. Innate Immunity 2012.

19. Andrade-Oliveira V, Campos EF, Goncalves-Primo A, Grenzi PC, Medina-Pestana

JO, Tedesco-Silva H et al. TLR4 mRNA levels as tools to estimate risk for early posttransplantation kidney graft dysfunction. Transplantation 2012;94(6):589-595.

20. Goldstein DR, Tesar BM, Akira S, Lakkis FG. Critical role of the Toll-like receptor signal adaptor protein MyD88 in acute allograft rejection. J Clin Invest 2003;111(10):1571-1578.

21. Liu G, Zhao Y. Toll-like receptors and immune regulation: their direct and indirect modulation on regulatory CD4+ CD25+ T cells. Immunology 2007;122(2):149-156.

22. Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C et al. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 2011;471(7338):363-367.

23. Green DR, Oberst A, Dillon CP, Weinlich R, Salvesen GS. RIPK-dependent necrosis and its regulation by caspases: a mystery in five acts. Mol Cell 2011;44(1):9-16.

24. Zhang D-W, Shao J, Lin J, Zhang N, Lu B-J, Lin S-C et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 2009;325(5938):332-336.

25. Rebsamen M, Heinz LX, Meylan E, Michallet M-C, Schroder K, Hofmann K et al. DAI/ZBP1 recruits RIP1 and RIP3 through RIP homotypic interaction motifs to activate NF-kappaB. EMBO Rep 2009;10(8):916-922.

26. Declercq W, Vanden Berghe T, Vandenabeele P. RIP kinases at the crossroads of cell death and survival. Cell 2009;138(2):229-232.

27. Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X et al. Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 2008;4(5):313-321.

28. Festjens N, Vanden Berghe T, Cornelis S, Vandenabeele P. RIP1, a kinase on the crossroads of a cell's decision to live or die. Cell death and differentiation 2007;14(3):400-410.

29. Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ et al. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 2008;135(7):1311-1323.

30. O'Donnell MA, Perez-Jimenez E, Oberst A, Ng A, Massoumi R, Xavier R et al. Caspase 8 inhibits programmed necrosis by processing CYLD. Nat Cell Biol 2011;13(12):1437-1442.

31. Günther C, Martini E, Wittkopf N, Amann K, Weigmann B, Neumann H et al. Caspase-8 regulates TNF-α-induced epithelial necroptosis and terminal ileitis. Nature 2011;477(7364):335-339.

32. Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 2009;137(6):1112-1123.

33. Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 2005;1(2):112-119.

34. Linkermann A, Bräsen JH, Himmerkus N, Liu S, Huber TB, Kunzendorf U et al. Rip1 (receptor-interacting protein kinase 1) mediates necroptosis and contributes to renal ischemia/reperfusion injury. Kidney International 2012;81(8):751-761.

35. Linkermann A, Himmerkus N, Rolver L, Keyser KA, Steen P, Brasen JH et al. Renal tubular Fas ligand mediates fratricide in cisplatin-induced acute kidney failure. Kidney Int 2011;79(2):169-178.

36. Du C, Guan Q, Yin Z, Zhong R, Jevnikar AM. IL-2-mediated apoptosis of kidney tubular epithelial cells is regulated by the caspase-8 inhibitor c-FLIP. Kidney International 2005;67(4):1397-1409.

37. Wang S, Zhang Z-X, Yin Z, Liu W, Garcia B, Huang X et al. Anti-IL-2 receptor antibody decreases cytokine-induced apoptosis of human renal tubular epithelial cells (TEC). Nephrol Dial Transplant 2011;26(7):2144-2153.

38. Mohib K, Guan Q, Diao H, Du C, Jevnikar AM. Proapoptotic activity of indoleamine 2,3-dioxygenase expressed in renal tubular epithelial cells. Am J Physiol Renal Physiol 2007;293(3):F801-812.

39. Mohib K, Wang S, Guan Q, Mellor AL, Sun H, Du C et al. Indoleamine 2,3-dioxygenase expression promotes renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 2008;295(1):F226-234.

40. Newton K, Sun X, Dixit VM. Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. Mol Cell Biol 2004;24(4):1464-1469.

41. Wu X, Gao H, Pasupathy S, Tan PH, Ooi LL, Hui KM. Systemic administration of naked DNA with targeting specificity to mammalian kidneys. Gene Therapy 2005;12(6):477-486.

42. Du C, Jiang J, Guan Q, Diao H, Yin Z, Wang S et al. NOS2 (iNOS) Deficiency in Kidney Donor Accelerates Allograft Loss in a Murine Model. American Journal of Transplantation 2007;7(1):17-26.

43. Edwards JR, Diamantakos EA, Peuler JD, Lamar PC, Prozialeck WC. A novel method for the evaluation of proximal tubule epithelial cellular necrosis in the intact rat kidney using ethidium homodimer. BMC Physiol 2007;7.

44. Zhang Z, Zhu L, Quan D, Garcia B, Ozcay N, Duff J et al. Pattern of liver, kidney, heart, and intestine allograft rejection in different mouse strain combinations. Transplantation 1996;62(9):1267-1272.

45. Zhang D-W, Zheng M, Zhao J, Li Y-Y, Huang Z, Li Z et al. Multiple death pathways in TNF-treated fibroblasts: RIP3- and RIP1-dependent and independent routes. Cell Res 2011;21(2):368-371.

46. Welz P-S, Wullaert A, Vlantis K, Kondylis V, Fernández-Majada V, Ermolaeva M et al. FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation. Nature 2011;477(7364):330-334.

47. Linkermann A, De Zen F, Weinberg J, Kunzendorf U, Krautwald S. Programmed necrosis in acute kidney injury. Nephrol Dial Transplant 2012;27(9):3412-3419.

48. Prozialeck WC, Edwards JR, Lamar PC, Liu J, Vaidya VS, Bonventre JV. Expression of kidney injury molecule-1 (Kim-1) in relation to necrosis and apoptosis during the early stages of Cd-induced proximal tubule injury. Toxicology and applied pharmacology 2009;238(3):306-314.

49. Kaczmarek A, Vandenabeele P, Krysko DV. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity 2013;38(2):209-223.

50. Dong X, Swaminathan S, Bachman LA, Croatt AJ, Nath KA, Griffin MD. Resident dendritic cells are the predominant TNF-secreting cell in early renal ischemia-reperfusion injury. Kidney International 2007;71(7):619-628.

51. Misseri R, Meldrum DR, Dinarello CA, Dagher P, Hile KL, Rink RC et al. TNF-alpha mediates obstruction-induced renal tubular cell apoptosis and proapoptotic signaling. Am J Physiol Renal Physiol 2005;288(2):F406-411.

52. Lee TH, Huang Q, Oikemus S, Shank J, Ventura J-J, Cusson N et al. The death domain kinase RIP1 is essential for tumor necrosis factor alpha signaling to p38 mitogen-activated protein kinase. Mol Cell Biol 2003;23(22):8377-8385.

53. Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 2003;114(2):181-190.

54. He S, Wang L, Miao L, Wang T, Du F, Zhao L et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 2009;137(6):1100-1111.

55. Speeckaert MM, Speeckaert R, Laute M, Vanholder R, Delanghe JR. Tumor necrosis factor receptors: biology and therapeutic potential in kidney diseases. American journal of nephrology 2012;36(3):261-270.

56. Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S et al. Fas triggers an alternative, caspase-8–independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 2000;1(6):489-495.

57. Vanden Berghe T, Grootjans S, Goossens V, Dondelinger Y, Krysko DV, Takahashi N et al. Determination of apoptotic and necrotic cell death in vitro and in vivo. Methods 2013.

58. Makarov R, Geserick P, Feoktistova M, Leverkus M. Cell death in the skin: how to study its quality and quantity? Methods in molecular biology 2013;961:201-218.

59. Linkermann A, Bräsen JH, De Zen F, Weinlich R, Schwendener RA, Green DR et al. Dichotomy between RIP1- and RIP3-mediated necroptosis in tumor necrosis factor-α-induced shock. Mol Med 2012;18(1):577-586.

60. Linkermann A, Hackl M, Kunzendorf U, Walczak H, Krautwald S, Jevnikar AM. Necroptosis in Immunity and Ischemia-Reperfusion Injury. American Journal of Transplantation 2013;in this issue.

61. Cornell LD, Smith RN, Colvin RB. Kidney transplantation: mechanisms of rejection and acceptance. Annu Rev Pathol 2008;3:189-220.

62. Park WD, Griffin MD, Cornell LD, Cosio FG, Stegall MD. Fibrosis with inflammation at one year predicts transplant functional decline. Journal of the American Society of Nephrology : JASN 2010;21(11):1987-1997.

63. Boros P, Bromberg JS. New cellular and molecular immune pathways in ischemia/reperfusion injury. Am J Transplant 2006;6(4):652-658.

64. Lamb KE, Lodhi S, Meier-Kriesche HU. Long-term renal allograft survival in the United States: a critical reappraisal. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2011;11(3):450-462.

65. Chapman JR, O'Connell PJ, Nankivell BJ. Chronic renal allograft dysfunction. Journal of the American Society of Nephrology : JASN 2005;16(10):3015-3026.

66. Lodhi SA, Meier-Kriesche HU. Kidney allograft survival: the long and short of it. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 2011;26(1):15-17.

67. Feng L, Cheng F, Ye Z, Li S, He Y, Yao X et al. The effect of renal ischemia-reperfusion injury on expression of RAE-1 and H60 in mice kidney. Transplant Proc 2006;38(7):2195-2198.

68. Kouwenhoven EA, de Bruin RW, Bajema IM, Marquet RL, Ijzermans JN. Cold ischemia augments allogeneic-mediated injury in rat kidney allografts. Kidney international 2001;59(3):1142-1148.

69. Chan FK. Fueling the flames: Mammalian programmed necrosis in inflammatory diseases. Cold Spring Harbor perspectives in biology 2012;4(11).

70. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell death and differentiation 2012;19(1):107-120.

71. Emamaullee JA, Davis J, Pawlick R, Toso C, Merani S, Cai SX et al. Caspase inhibitor therapy synergizes with costimulation blockade to promote indefinite islet allograft survival. Diabetes 2010;59(6):1469-1477.

72. Baskin-Bey ES, Washburn K, Feng S, Oltersdorf T, Shapiro D, Huyghe M et al. Clinical Trial of the Pan-Caspase Inhibitor, IDN-6556, in Human Liver Preservation Injury. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2007;7(1):218-225.

73. Sirois I, Raymond MA, Brassard N, Cailhier JF, Fedjaev M, Hamelin K et al. Caspase-3-dependent export of TCTP: a novel pathway for antiapoptotic intercellular communication. Cell death and differentiation 2011;18(3):549-562.

74. Salmena L, Lemmers B, Hakem A, Matysiak-Zablocki E, Murakami K, Au PY et al. Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. Genes & development 2003;17(7):883-895.

75. Challa S, Chan FK. Going up in flames: necrotic cell injury and inflammatory diseases. Cellular and molecular life sciences : CMLS 2010;67(19):3241-3253.

76. Soulez M, Sirois I, Brassard N, Raymond M-A, Nicodème F, Noiseux N et al.

Epidermal growth factor and perlecan fragments produced by apoptotic endothelial cells co-ordinately activate ERK1/2-dependent antiapoptotic pathways in mesenchymal stem cells. Stem Cells 2010;28(4):810-820.

77. Shigeoka AA, Holscher TD, King AJ, Hall FW, Kiosses WB, Tobias PS et al. TLR2 is constitutively expressed within the kidney and participates in ischemic renal injury through both MyD88-dependent and -independent pathways. J Immunol 2007;178(10):6252-6258.

78. Pulskens WP, Teske GJ, Butter LM, Roelofs JJ, van der Poll T, Florquin S et al. Toll-like receptor-4 coordinates the innate immune response of the kidney to renal ischemia/reperfusion injury. PLoS ONE 2008;3(10).

79. Krüger B, Krick S, Dhillon N, Lerner SM, Ames S, Bromberg JS et al. Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. Proc Natl Acad Sci USA 2009;106(9):3390-3395.

80. Wu H, Chen G, Wyburn KR, Yin J, Bertolino P, Eris JM et al. TLR4 activation mediates kidney ischemia/reperfusion injury. J Clin Invest 2007;117(10):2847-2859.

81. Wu H, Ma J, Wang P, Corpuz TM, Panchapakesan U, Wyburn KR et al. HMGB1 contributes to kidney ischemia reperfusion injury. J Am Soc Nephrol 2010;21(11):1878-1890.

82. Sun X, Yin J, Starovasnik MA, Fairbrother WJ, Dixit VM. Identification of a novel homotypic interaction motif required for the phosphorylation of receptor-interacting protein (RIP) by RIP3. J Biol Chem 2002;277(11):9505-9511.

83. Jevnikar AM, Wuthrich RP, Brennan DC, Maslinski W, Glimcher LH, Rubin-Kelley VE. TNF-alpha is expressed on the surface of kidney proximal tubular cells. Transplant Proc 1991;23(1 Pt 1):231-232.

84. Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med 2000;191(3):423-434.

85. Kazama H, Ricci J-E, Herndon JM, Hoppe G, Green DR, Ferguson TA. Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. Immunity 2008;29(1):21-32.

86. Laplante P, Raymond M-A, Labelle A, Abe J-I, Iozzo RV, Hébert M-J. Perlecan proteolysis induces an alpha2beta1 integrin- and Src family kinase-dependent anti-apoptotic pathway in fibroblasts in the absence of focal adhesion kinase activation. J Biol Chem 2006;281(41):30383-30392.

87. Jang HR, Ko GJ, Wasowska BA, Rabb H. The interaction between ischemia-reperfusion and immune responses in the kidney. J Mol Med 2009;87(9):859-864.

88. Xu X, Chua KW, Chua CC, Liu CF, Hamdy RC, Chua BH. Synergistic protective effects of humanin and necrostatin-1 on hypoxia and ischemia/reperfusion injury. Brain research 2010;1355:189-194.

89. Dumitriu IE, Baruah P, Valentinis B, Voll RE, Herrmann M, Nawroth PP et al. Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products. Journal of immunology 2005;174(12):7506-7515.

90. Gardella S, Andrei C, Ferrera D, Lotti LV, Torrisi MR, Bianchi ME et al. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. EMBO Rep 2002;3(10):995-1001.

91. Takahashi N, Duprez L, Grootjans S, Cauwels A, Nerinckx W, DuHadaway JB et al. Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models. Cell Death Dis 2012;3.

Chapter 4

4 Glycyrrhizic acid (GZA) ameliorates HMGB1-mediated cell death and inflammation after renal ischemia reperfusion injury

Arthur Lau[1,3], Shuang Wang[2,4], Weihua Liu[1], Aaron Haig[3], Zhu-Xu Zhang[1,2,3,4, *], Anthony M. Jevnikar[1,2, 3,4, *]

[1]Matthew Mailing Centre for Translational Transplant Studies, London Health Sciences Centre; Departments of [2]Medicine and [3]Pathology, Western University; [4]Lawson Health Research Institute, London, Ontario, Canada

Citation:

Lau A, Wang S, Liu W, Haig A, Zhang ZX, Jevnikar AM. Glycyrrhizic acid (GZA) ameliorates HMGB1-mediated cell death and inflammation after renal ischemia reperfusion injury. *American Journal of Nephrology* 2014: In press. S. Karger AG©

4.1 Abstract

Background: Renal ischemia reperfusion injury (IRI) leads to acute kidney injury (AKI) and the death of tubular epithelial cells (TEC). The release of High Mobility Group Box-1 (HMGB1) and other damage associated molecular pattern moieties from dying cells may promote organ dysfunction and inflammation by effects on TEC. Glycyrrhizic acid (GZA) is a functional inhibitor of HMGB1 but its ability to attenuate HMGB1 mediated injury of TEC has not been tested.

Methods/Results: *In vitro*, hypoxia and cytokine treatment killed TEC and resulted in progressive release of HMGB1 into the supernatant. GZA reduced hypoxia induced TEC death as measured by Annexin-V/PI. Hypoxia increased expression of MCP-1 and CXCL1 in TEC which was reduced by GZA in a dose dependent manner. Similarly, HMGB1 activation of effector NK cells was inhibited by GZA. To test the effect of HMGB1 neutralization by GZA *in vivo*, mice were subjected to renal IRI. HMGB1 protein expression increased progressively in kidneys from 4 to 24 hours post ischemia and was detected in tubular cells by 4 hours using immunohistochemistry. GZA preserved renal function after IRI and reduced tubular necrosis and neutrophil infiltration by histological analyses and ethidium homodimer staining.

Conclusions: Importantly, these data demonstrate for the first time that AKI following hypoxia and renal IRI may be promoted by HMGB1 release which can reduce survival of TEC and augment inflammation. Inhibition of HMGB1 interaction with TEC by GZA may represent a therapeutic strategy for attenuation of renal injury following IRI and transplantation.

4.2 Introduction

Ischemia reperfusion injury (IRI) occurs invariably in kidney transplantation and contributes to graft dysfunction and rejection in recipients [1–3]. The initial ischemic insult induces widespread death of kidney parenchymal cells and in particular tubular epithelial cells (TEC) [4–8], which results in organ dysfunction and the release of damage associated molecular pattern (CDAMP) proteins into the extracellular space [10-13]. High Mobility Group Box-1 (HMGB1) and other CDAMP moieties may further contribute to pro-inflammatory injury [13–16]. However, their effects on the survival or pro-inflammatory functions of TEC remain unknown and could alter allograft survival.

HMGB1 is a ubiquitous nuclear protein that is highly conserved throughout many species. Physiologically, it binds to DNA within the nucleus and is involved in essential processes such as DNA replication and transcription [17]. HMGB1 has been previously identified as a CDAMP molecule in different injury models in the liver [18], lungs [19], and heart [20]. Furthermore, previous studies in acute injury models have suggested that HMGB1 is not only released passively following cell death [21-23] but may be actively secreted [24,25] by some cell types even while viable. The pro-inflammatory nature of HMGB1 is related to increased expression of chemokines and cytokines that attract and activate diverse immune cells. This inflammatory response is typically mediated through Toll-like receptors (TLR) which has been implicated in having a major role in propagating tissue injury and inflammation [26-30]. TLR signalling following the binding of CDAMP ligands such as HMGB1 [31] results in the recruitment of various adapter proteins (MyD88) leading to the activation of pro-inflammatory mediators such as TNFα, IL-6, CCL2, CXCL8, and CX3CL1 [32,33].

Although targeting of HMGB1 has been suggested to limit acute renal injury, studies have been limited by clear understanding of effects on renal parenchymal cells as well as clinically feasible reagents other than neutralizing antibodies [34,35]. Glycyrrhizic acid (GZA), a functional inhibitor of HMGB1, has been tested clinically in patients with Hepatitis C [36]and appears to ameliorate both liver and kidney injury [37-40]. It is plausible that GZA may have the potential to reduce organ storage injury and IRI following kidney transplantation as well as diminishing inflammation with immune rejection.

In the present study, we tested the effect of HMGB1 released from injured cells on TEC survival and function and whether GZA altered the effects of HMGB1 on kidney cells *in vitro* and *in vivo* with IRI. We have demonstrated that GZA can inhibit TEC death by blocking HMGB1 which may directly contribute to kidney injury *in vivo* as well as indirectly by the production of pro-inflammatory molecules such as monocyte chemotactic protein-1 (MCP-1) and CXCL1, and IL-6. Importantly, GZA neutralization blocks deleterious effects of HMGB1 on kidney cells, suggesting it may be useful to attenuate IRI and other forms of inflammatory kidney injury.

4.3 Materials and Methods

Animals

C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in the animal facility at Western University using approved protocols and procedures.

Cell Cultures

The TEC line NG1.1 was developed from proximal tubular epithelial cells from C57BL/6J mice by SV40 transformation as previously described [4]. NK cells were purified from C57BL/6J mice spleen using anti-CD49a MACS beads selection (MiltenyiBiotec) and were grown in the presence of IL-2 (1000IU/mL) in RPMI-1640. Purity of NK cells was confirmed by flow cytometry and >90% of cells were CD3-CD49b+ for each experiment. NK cells were treated with endotoxin free recombinant HMGB1 (R&D Systems).

Kidney IRI

Renal IRI was performed as previously described [8]. Briefly, a renal clamp was applied to the right kidney pedicle and removed after 45 minutes while the left kidney was nephrectomised. Serum was collected at 48h post IRI for creatinine detection by Jaffe reaction method using an automated CX5 clinic analyzer (Beckman).

GZA (Sigma) was reconstituted in a minimum volume of DMSO and diluted with saline and injected 2h pre-operatively and post-operatively at 8h and 24h in mice undergoing IRI (1mg GZA, <1% DMSO). Control mice were subjected to IRI and equivalent doses of DMSO as vehicle control.

Hypoxia Treatment

TEC were made hypoxic with deoxygenated serum and glucose free media in a hypoxia chamber for 20 minutes. Oxygen in the chamber was displaced by a gas mixture of 3% H2, 5% CO2, and a balance of N2 (Praxair) at a rate of 0.1L/min. Cells were collected at various

time points for FACS analysis of apoptosis and necrosis by Annexin-V and propidium iodide (PI) (BD Bioscience) respectively.

Immunoblotting and Real-time PCR

Protein was isolated from tissue and cells using a non-nuclear protein lysis buffer that excluded nuclear proteins. Protein from supernatant was concentrated by centrifugation (Millipore). Membranes were probed with anti-HMGB1 (Abcam) or mouse anti-β-actin (Sigma).

Total RNA was extracted from tissue and cells by Trizol (Invitrogen) as described by the manufacturer. cDNA was generated from RNA using Superscript II (Invitrogen) as described by the manufacturer. cDNA was quantified by real time PCR using SybrGreen (Bio-Rad) as described by the manufacturer. Primers (Invitrogen) used for Q-PCR include: HMGB1: 5`-TAAAAAGCCGAGAGGCAAAA-3`, 5`-GCAGACATGGTCTTCCACGT-3`; MCP-1: 5`-AGCACCAGCCAACTCTCACT-3`, 5`- CGTTAACTGCATCTGGCTGA-3`; RANTES: 5`-ATATGGCTCGGACACCACTC-3`, 5`-TCCTTCGAGTGACAAACACG-3`; CXCL1: 5' – AGACTGCTCTGATGGCACCT-3', 5'-TGCACTTCTTTTCGCACAAC-3'; IL-6: 5′-GAGGATACCACTCCCAACAGACC-3′, 5′-AAGTGCATCATCGTTGTTCATACA-3′; IFN-γ: 5′-CAT TGAAAGCCTAGAAAGTCTGA-3′, 5′-TAGCGATGCAAATGCTTGATATC-3′; Perforin: 5'-GAAGACCTATCAGGACCAGTACAACTT-3', 5'-CAAGGTGGAGTGGAGGTTTTTG-3'; Granzyme B: 5'-CGATCAAGGATCAGCAGCC-3', 5'-CTGGGTCTTCTCCTGTTCT-3'. β-actin was used as the endogenous control. The normalized delta threshold cycle value and relative expression levels (2ΔΔCt) were calculated according to the manufacturer's protocol.

Histology and Immunohistochemistry

Tissue sections were H&E stained and scored by a pathologist in a blinded fashion using an injury scoring method as described [7]. Criteria for kidney injury include tubular necrosis, immune cell infiltration, lumen casts, and glomerular cell necrosis. Immunohistochemistry was performed using anti-HMGB1 (Abcam). To visualize and quantify kidney tissue necrosis in vivo, frozen tissue sections were scored from mice having renal artery infusion of

ethidium homodimer (Invitrogen) as previously described [41]. Briefly, 5μM ethidium homodimer was injected at 1mL/min. for 10 min. into the renal artery through the aorta and then flushed with perfusion buffer at 1mL/min for 5 min. Sections were analyzed and quantified score using a fluorescent microscope and an automated image analysis program (Nikon) measuring area and fluorescent intensity.

Statistical Analysis

Data was compared using Student's t-test for unpaired values and one way ANOVA for multiple comparisons. Data was presented as mean ± SEM and $p< 0.05$ was considered to be significantly different.

4.4 Results

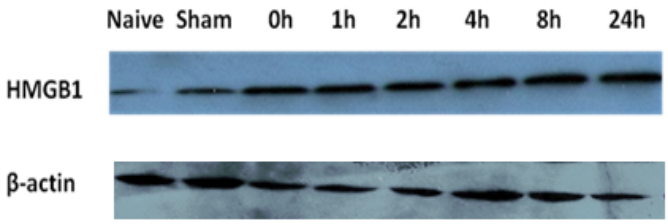
HMGB1 protein expression is upregulated during renal IRI

Renal ischemia reperfusion injury causes severe tissue injury and various forms of cell death [4–8] including apoptosis, necrosis, autophagy, and other non-classical forms of cell death [42]. During necrotic cell death, cells invariably lose membrane integrity and eventual lyse, resulting in the release of intracellular contents and various CDAMPs such as HMGB1.

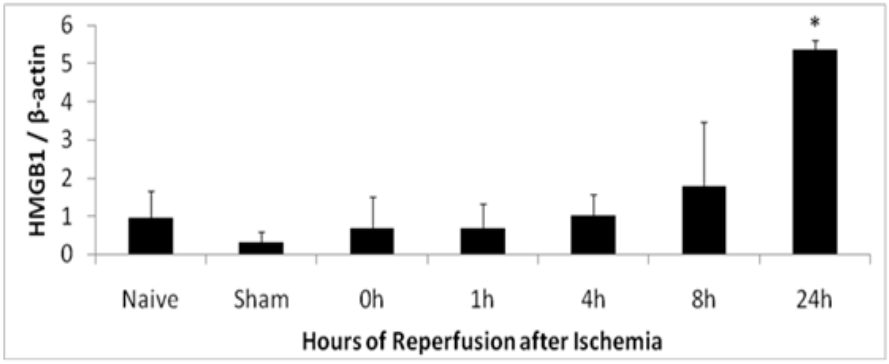
To first demonstrate the kinetics of HMGB1 release in renal IRI, we tested mRNA and non-nuclear protein levels in kidneys for up to 24 hours following IRI. As shown in Figure 1A and 1B, non-nuclear HMGB1 protein increased in the kidney progressively for up to 24 hours of reperfusion after ischemia (control density ratio: 0.953±0.707 vs. 24h post IRI 5.368±0.239). In contrast, mRNA expression of HMGB1 was decreased after ischemia as compared to controls (sham: 1±0 vs. 0h post-ischemia: 0.17±0.04) and remained at low levels for up to 24 hours of reperfusion as shown in Figure 1C. Together, these results indicate that the presence of HMGB1 protein outside the nucleus increased over the course of renal IRI but was not due to increased transcription.

As expected, mice undergoing IRI demonstrated decreased kidney function as indicated by an increase in serum creatinine 24 hours following reperfusion (Figure 1D). HMGB1 expression analyses by immunohistochemistry showed that HMGB1 expression was detected in tubules as early as 4 hours after IRI (Fig. 1E). While TEC expressing HMGB1 appear to be located in cortical areas, these data do not distinguish their identity as proximal or distal tubules. However *in vitro* results using previously characterized NG TEC, suggest that proximal tubular cells are likely to be a prominent source of HMGB1 *in vivo*.

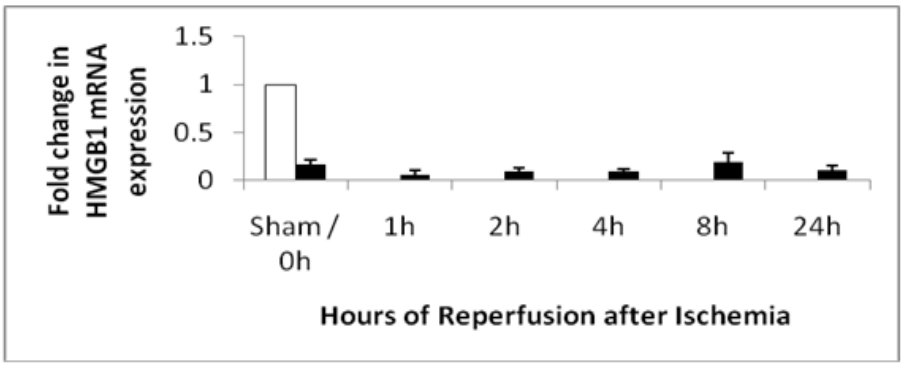
A


Naive Sham 0h 1h 2h 4h 8h 24h
HMGB1
β-actin

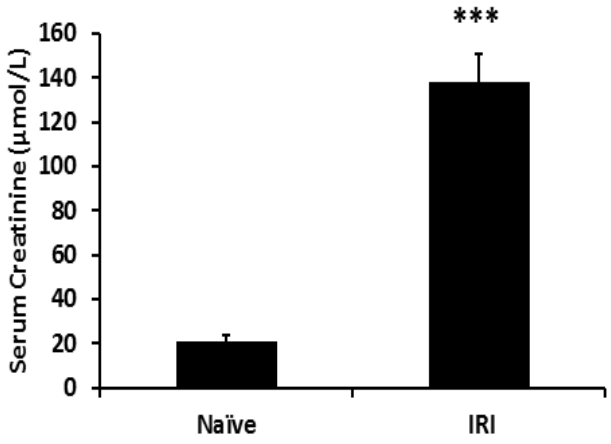
B


HMGB1 / β-actin
6
5
4
3
2
1
0
*
Naive
Sham
0h
1h
4h
8h
24h
Hours of Reperfusion after Ischemia

C


Fold change in HMGB1 mRNA expression
1.5
1
0.5
0
Sham / 0h
1h
2h
4h
8h
24h
Hours of Reperfusion after Ischemia

D


***
160
140
120
100
80
60
40
20
0
Serum Creatinine (µmol/L)
Naïve
IRI

E

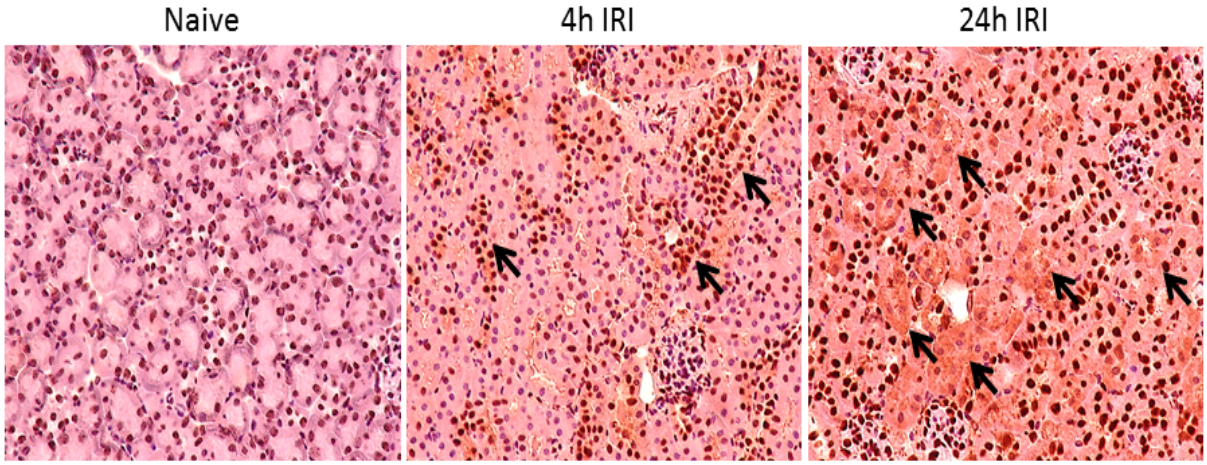

Naive
4h IRI
24h IRI

Figure 1. Characterization of HMGB1 expression in the kidney after renal IRI.

C57BL/6 mice were subjected to acute ischemia for 45 min. using a renal clamp at 32° Celsius. Sham mice did not have a renal clamp applied. Reperfusion injury occurred over a 24 hour period during which mice were sacrificed at various time points. A, B) Non-nuclear kidney protein was isolated and analyzed by immunoblot using anti-HMGB1 (representative of 3 independent experiments). Relative protein concentration was determined by semi-quantitative densitometry and normalized by β-actin (*: $p < 0.01$, n=3/group) C) mRNA expression of HMGB1 after renal IRI was measured by real time PCR. Fold change mRNA expression was normalized by β-actin. (n=3/group) D) Kidney function was determined by serum creatinine in naïve and IRI treated mice at 24 hours. (***: $p<0.001$, n=4-5/group) E) Kidney sections were analyzed for HMGB1 by immunohistochemistry. Arrows indicate tubules positive for HMGB1. Images were taken at 200X magnification. (n=3/group)

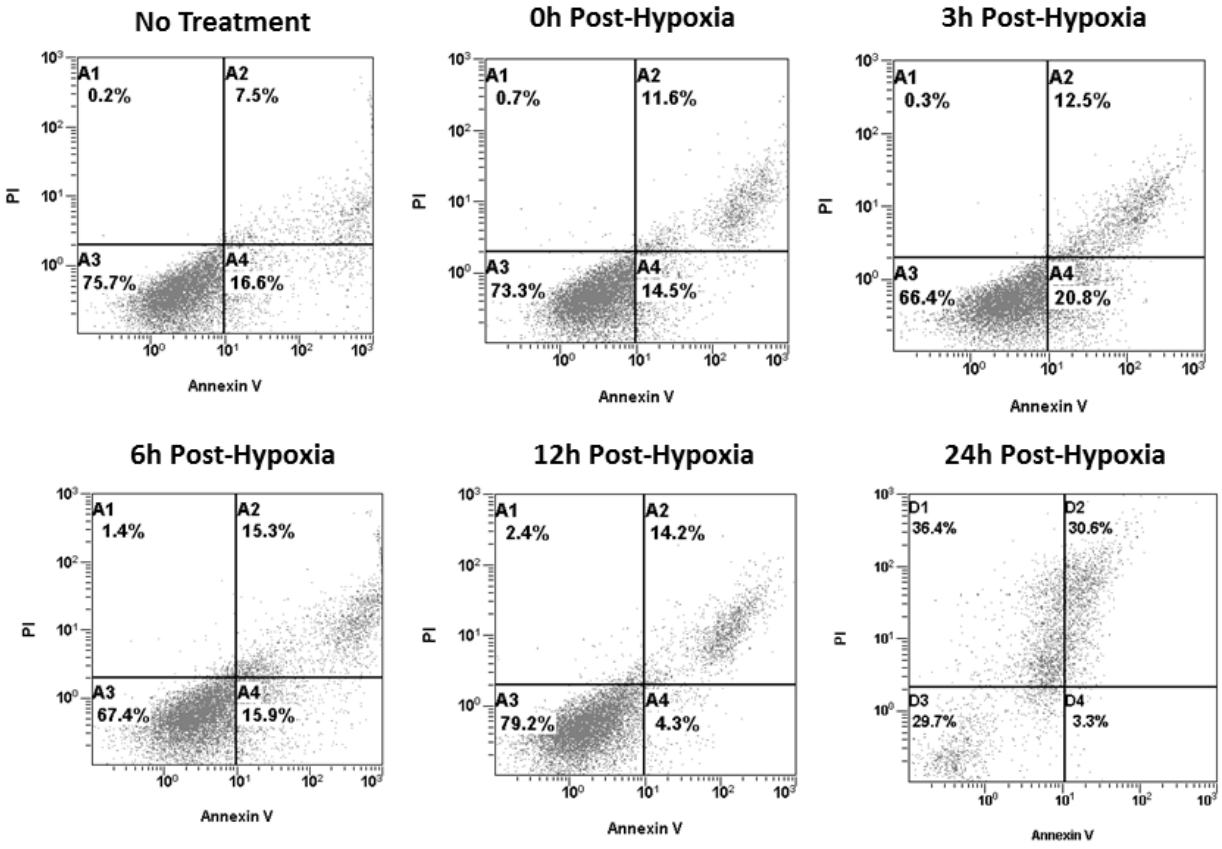
GZA neutralization of HMGB1 released from hypoxic TEC can inhibit cell death

It has been previously reported that cells release HMGB1 during necrotic cell death [13,22]. As hypoxia similarly results in TEC death, we tested their capacity to release HMGB1 after hypoxia. As shown in Figure 2A, TEC cultures underwent increasingly higher levels of cell death and were primarily and maximally Annexin-V/PI positive at 24 hours post hypoxia (7.5% vs. 30.6%). In addition, the percentage of viable cells (Annexin-V/PI negative) decreased 24 hours post hypoxia (75.7% vs. 29.7%).

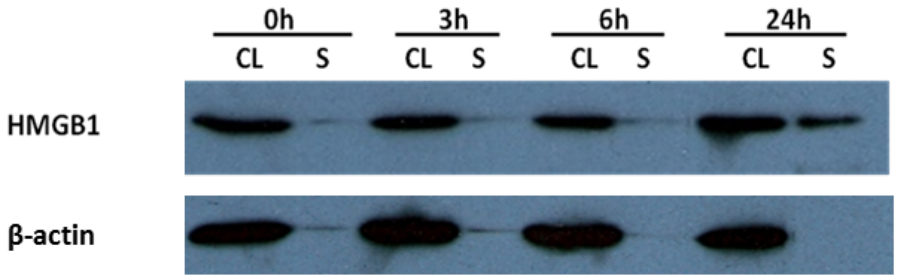
Next, we tested whether HMGB1 was released from TEC after hypoxic cell death. Analysis of supernatant from TEC following hypoxia over a 24 hour period (Figure 2B, 2C) clearly demonstrated that HMGB1 was released from killed and remaining TEC with detectable levels immediately after hypoxia treatment (0.574 with no treatment vs. 8.876 24 hours post hypoxia). In addition, it was also observed that the lysate fraction containing protein from both the nuclear and cytoplasmic compartments from remaining adherent TEC did not similarly show increased levels of HMGB1.

As supernatant from hypoxia treated cells contains a complex number of mediators that could affect cell death or viability, we attempted to clarify the role of HMGB1 by the addition of GZA which specifically inhibits HMGB1 [40]. As shown in Figure 2D and 2E, viability of TEC was reduced from 81.00±1.87% (Annexin-V/PI negative) to 41.73±7.26% with hypoxia ($p=0.003$). The addition of 1000ng/mL of GZA to TEC during hypoxia modestly increased cell viability from 41.73±7.26% to 58.03±7.39% ($p=0.05$). Although many TEC expressed mediators in the conditioned media have an effect on cell viability, this data suggests HMGB1 has a role in cell death that might be inhibited by GZA in vivo.

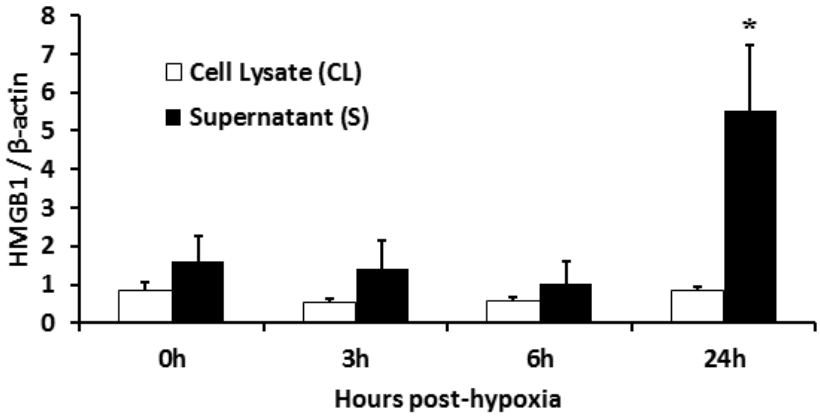
A


No Treatment
A1
0.2%
A2
7.5%
A3
75.7%
A4
16.6%
0h Post-Hypoxia
A1
0.7%
A2
11.6%
A3
73.3%
A4
14.5%
3h Post-Hypoxia
A1
0.3%
A2
12.5%
A3
66.4%
A4
20.8%
6h Post-Hypoxia
A1
1.4%
A2
15.3%
A3
67.4%
A4
15.9%
12h Post-Hypoxia
A1
2.4%
A2
14.2%
A3
79.2%
A4
4.3%
24h Post-Hypoxia
D1
36.4%
D2
30.6%
D3
29.7%
D4
3.3%
PI
Annexin V

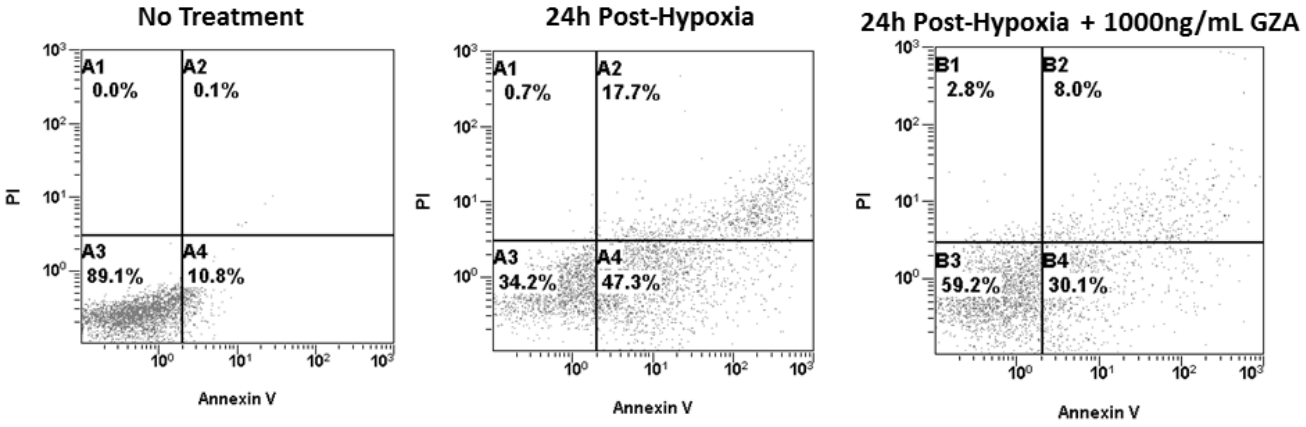
B


0h
3h
6h
24h
CL
S
CL
S
CL
S
CL
S
HMGB1
β-actin

C


HMGB1 / β-actin
8
7
6
5
4
3
2
1
0
Cell Lysate (CL)
Supernatant (S)
*
0h
3h
6h
24h
Hours post-hypoxia

D


No Treatment
A1
0.0%
A2
0.1%
A3
89.1%
A4
10.8%
PI
Annexin V
24h Post-Hypoxia
A1
0.7%
A2
17.7%
A3
34.2%
A4
47.3%
PI
Annexin V
24h Post-Hypoxia + 1000ng/mL GZA
B1
2.8%
B2
8.0%
B3
59.2%
B4
30.1%
PI
Annexin V

E

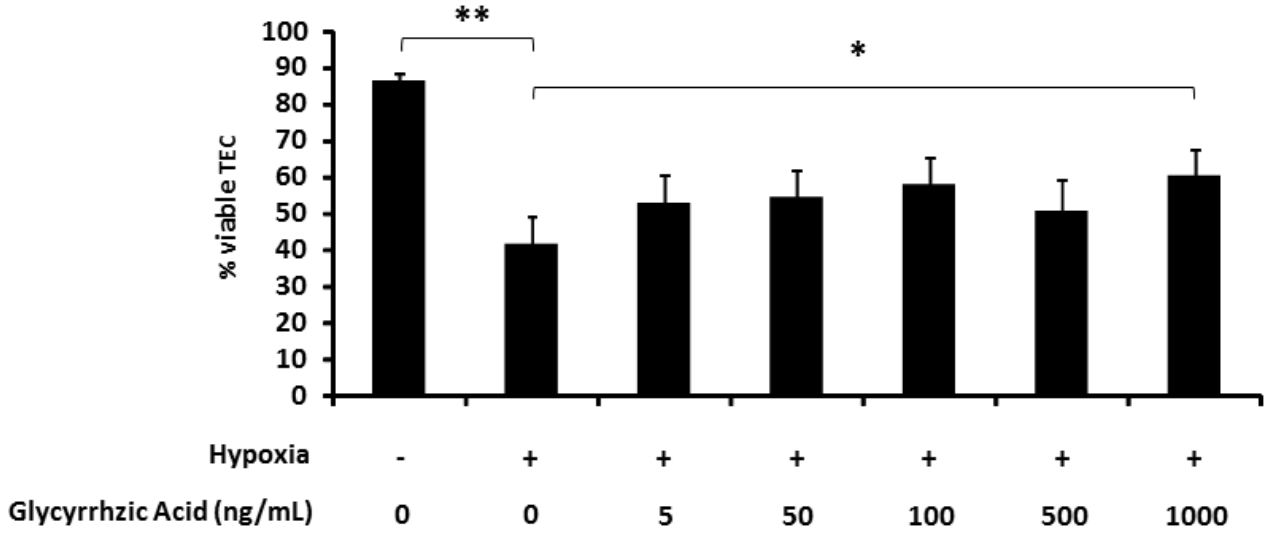

**
*
% viable TEC
100
90
80
70
60
50
40
30
20
10
0
Hypoxia - + + + + + +
Glycyrrhzic Acid (ng/mL) 0 0 5 50 100 500 1000

Figure 2. GZA neutralization of HMGB1 released from hypoxic TEC can inhibit cell death.

A) TEC were subjected to hypoxia and cell death was measured by Annexin-V/PI for apoptosis and necrosis respectively at various time points. (representative of 3 independent experiments) B, C) Total cell lysate and supernatants were collected from TEC and HMGB1 was detected by immunoblot. Relative protein concentrations were determined by semi-quantitative densitometry and normalized by β-actin. (n=3/group) D, E) TEC were treated with hypoxia and various concentrations of GZA and cell death was measured at 24 hours using Annexin-V/PI. Viable TEC were negative for Annexin-V/PI labeling. (*: $p<0.05$, **: $p<0.01$, n=4/group)

Increased TEC expression of pro-inflammatory cytokines and NK cell activation is inhibited by GZA

Renal IRI is associated with upregulation of local pro-inflammatory mediators within the kidney which promotes further injury from the influx of effector cells including neutrophils, T cells and NK cells [43]. The release of HMGB1 into the micro-environment with subsequent activation of several key TLR pathways likely accounts for much of the upregulation of pro-inflammatory molecules. It has been demonstrated that GZA can induce an anti-inflammatory effect in an ischemic spinal cord injury model with downregulation of cytokines and chemokines [44]. Therefore, we tested whether inflammation could similarly be ameliorated by neutralizing HMGB1 through the use of GZA.

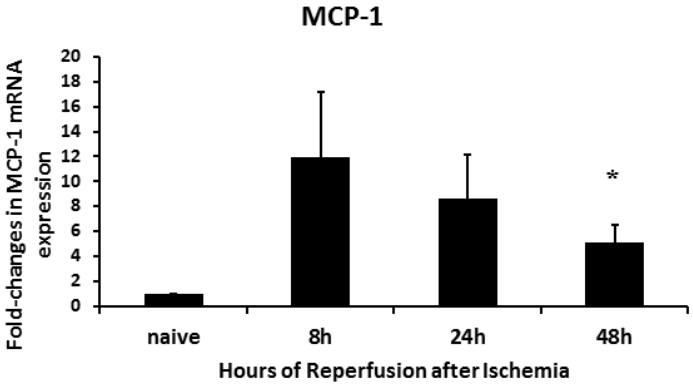
We first confirmed that inflammatory chemokine mRNA expression increased in the total kidney after IRI. As shown in Figure 3A and 3B, there was upregulation of MCP-1 (naive: 1±0 vs. 48h IRI: 5.13±1.39, p=0.03, n=3) and CXCL1 (naive: 1±0 vs. 48 IRI: 5.22±1.03, p=0.04, n=3) peaking by 8 hours but persisting for up to 48 hours after reperfusion. In addition, the pro-inflammatory cytokine IL-6 was upregulated in kidney after renal IRI (naïve: 1±0 vs. 48h IRI: 20.17±5.48, p=0.04, n=3) as shown in Figure 3F. In contrast, RANTES mRNA expression (Figure 3C) peaked by 24 hours but was downregulated by 48h after IRI (naive: 1±0 vs. 48h IRI: 0.30±0.05, p=0004, n=3). Other chemokines tested included MIP-1α and CX3CL1but did not demonstrate increased expression after renal IRI (data not shown). This data confirmed that there is an increase in the production of pro-inflammatory molecules during IRI.

To determine whether the observed increased chemokine expression during renal IRI might involve TEC, total mRNA from 24h post hypoxia TEC were tested for MCP-1, CXCL1, and IL-6 mRNA expression. As shown in Figure 3D and 3E, there was an increase in both MCP-1 (no treatment: 1±0 vs. 24h post hypoxia: 9.32±2.85) and CXCL1 (no treatment: 1±0 vs. 24h post hypoxia: 10.22±0.52) 24 hours after hypoxia. RANTES mRNA expression was unchanged after hypoxia treatment (not shown). However, when GZA was added to TEC cultures during hypoxia, it had a dose dependent inhibitory effect as determined by one-way ANOVA on MCP-1 ($F(5,11)=4.13$, $p=0.02$) and CXCL1 ($F(4,10)=12.83$, $p=0.001$) expression which resulted in a return to untreated control levels. TEC expression of pro-

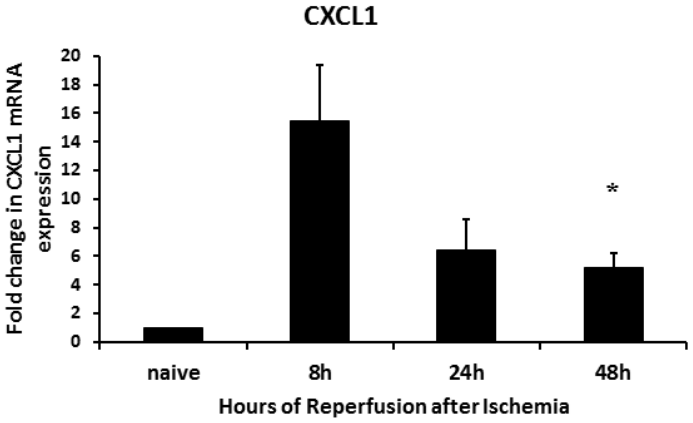
inflammatory IL-6 mRNA was clearly inhibited by the addition of GZA following hypoxia (12.61±2.7 vs 0.71±0.22, ($F(2,6)=18.88$, $p=0.002$) as shown in Figure 3G. Given that HMGB1 neutralization by GZA reduces pro-inflammatory chemokine and cytokine expression in TEC, GZA may potentially ameliorate inflammation during renal IRI.

We have recently demonstrated the significance of NK cell contribution towards TEC injury as a result of inflammation during renal IRI [8]. As NK cells can be activated HMGB1, we attempted to elucidate the effects of HMGB1 inhibition by GZA on NK cell activation. As shown in Figure 3H, addition of HMGB1 activated NK cells and resulted in upregulation of IFNγ, perforin, and granzyme B mRNA. In the presence of GZA, HMGB1 activation was inhibited and led to the downregulation of IFNγ (2.9±0.5 vs. 0.8±0.1, $p=0.03$), perforin (2.2±0.4 vs. 0.3±0.1, $p=0.02$), and granzyme B (2.1±0.6 vs. 0.5±0.4, $p=0.04$) mRNA. This data suggests that GZA may prevent HMGB1 mediated activation of NK cells and thereby reduce TEC injury during renal IRI.

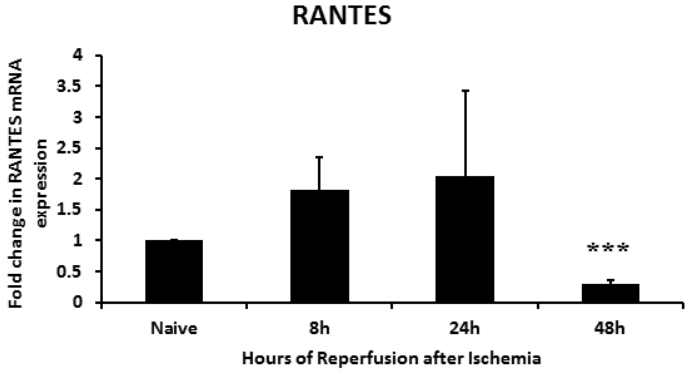
A


MCP-1
Fold-changes in MCP-1 mRNA expression
20
18
16
14
12
10
8
6
4
2
0
*
naive
8h
24h
48h
Hours of Reperfusion after Ischemia

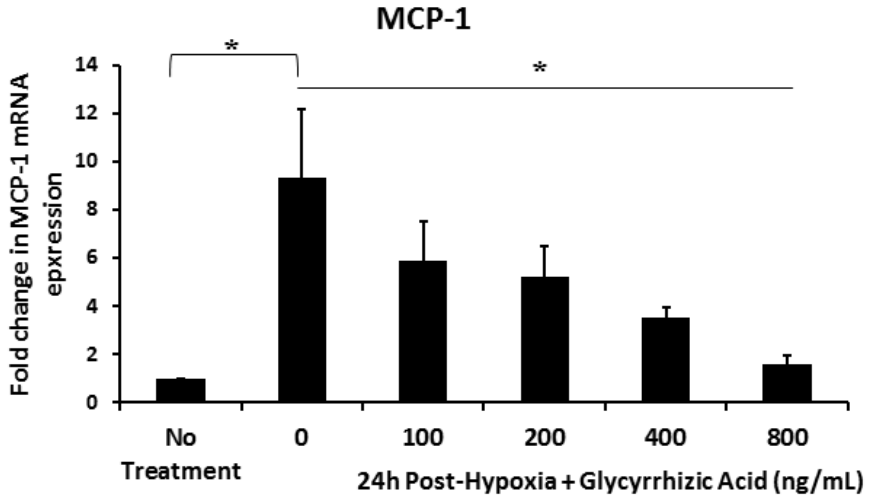
B


CXCL1
Fold change in CXCL1 mRNA expression
20
18
16
14
12
10
8
6
4
2
0
*
naive
8h
24h
48h
Hours of Reperfusion after Ischemia

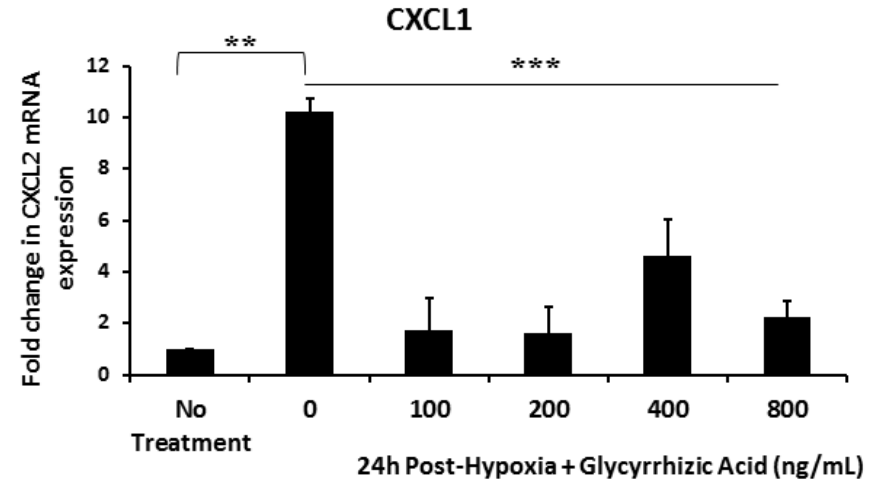
C


RANTES
Fold change in RANTES mRNA expression
4
3.5
3
2.5
2
1.5
1
0.5
0
***
Naive
8h
24h
48h
Hours of Reperfusion after Ischemia

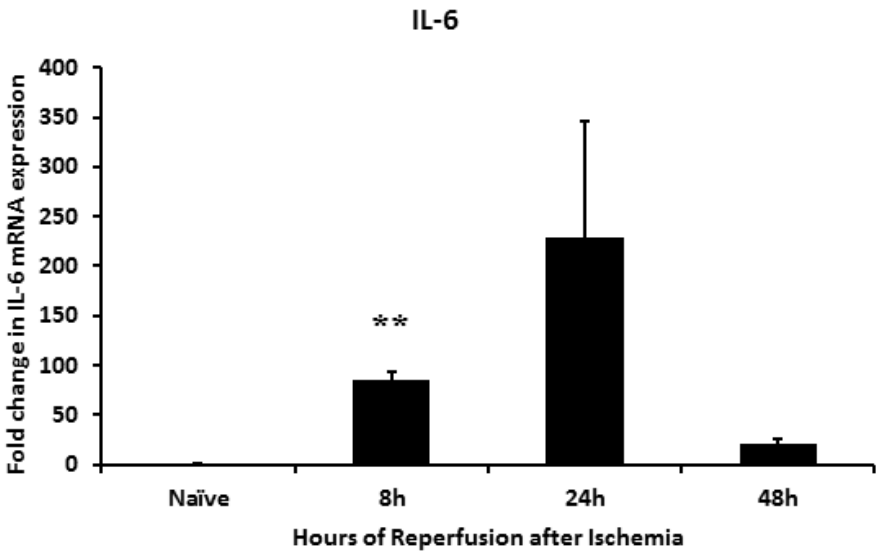
D


MCP-1
*
*
Fold change in MCP-1 mRNA epxression
14
12
10
8
6
4
2
0
No Treatment
0
100
200
400
800
24h Post-Hypoxia + Glycyrrhizic Acid (ng/mL)

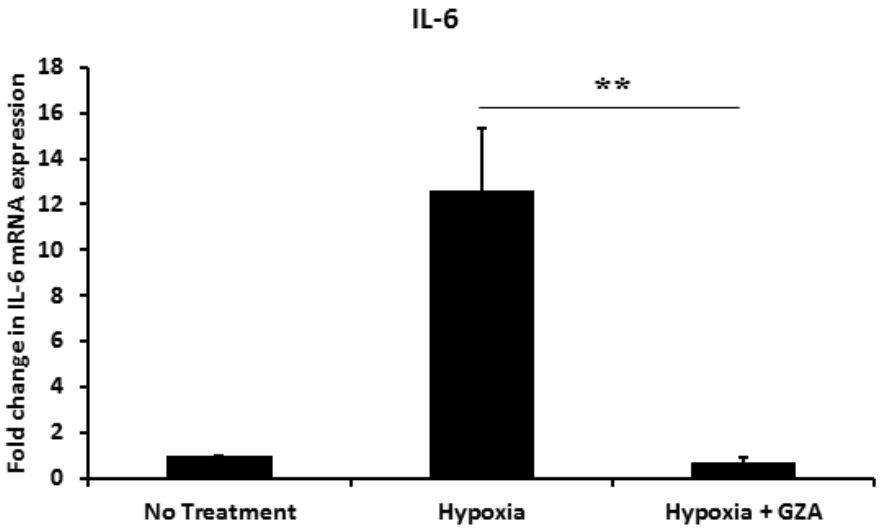
E


CXCL1
**
***
Fold change in CXCL2 mRNA expression
12
10
8
6
4
2
0
No Treatment
0
100
200
400
800
24h Post-Hypoxia + Glycyrrhizic Acid (ng/mL)

F


IL-6
Fold change in IL-6 mRNA expression
400
350
300
250
200
150
100
50
0
**
Naïve
8h
24h
48h
Hours of Reperfusion after Ischemia

G


IL-6
Fold change in IL-6 mRNA expression
18
16
14
12
10
8
6
4
2
0
**
No Treatment
Hypoxia
Hypoxia + GZA

H

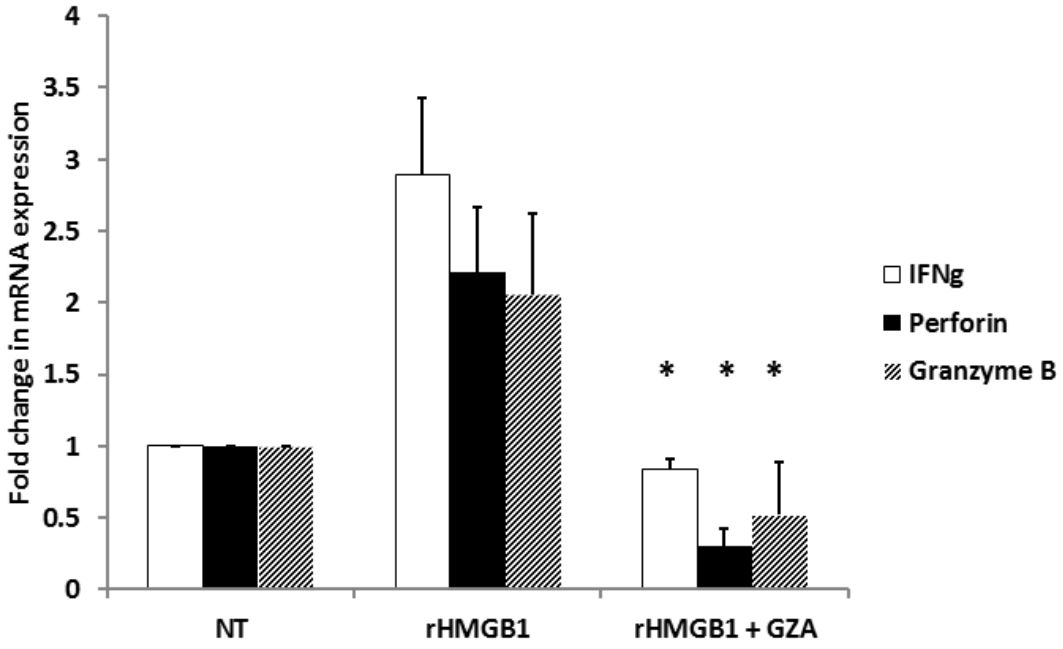
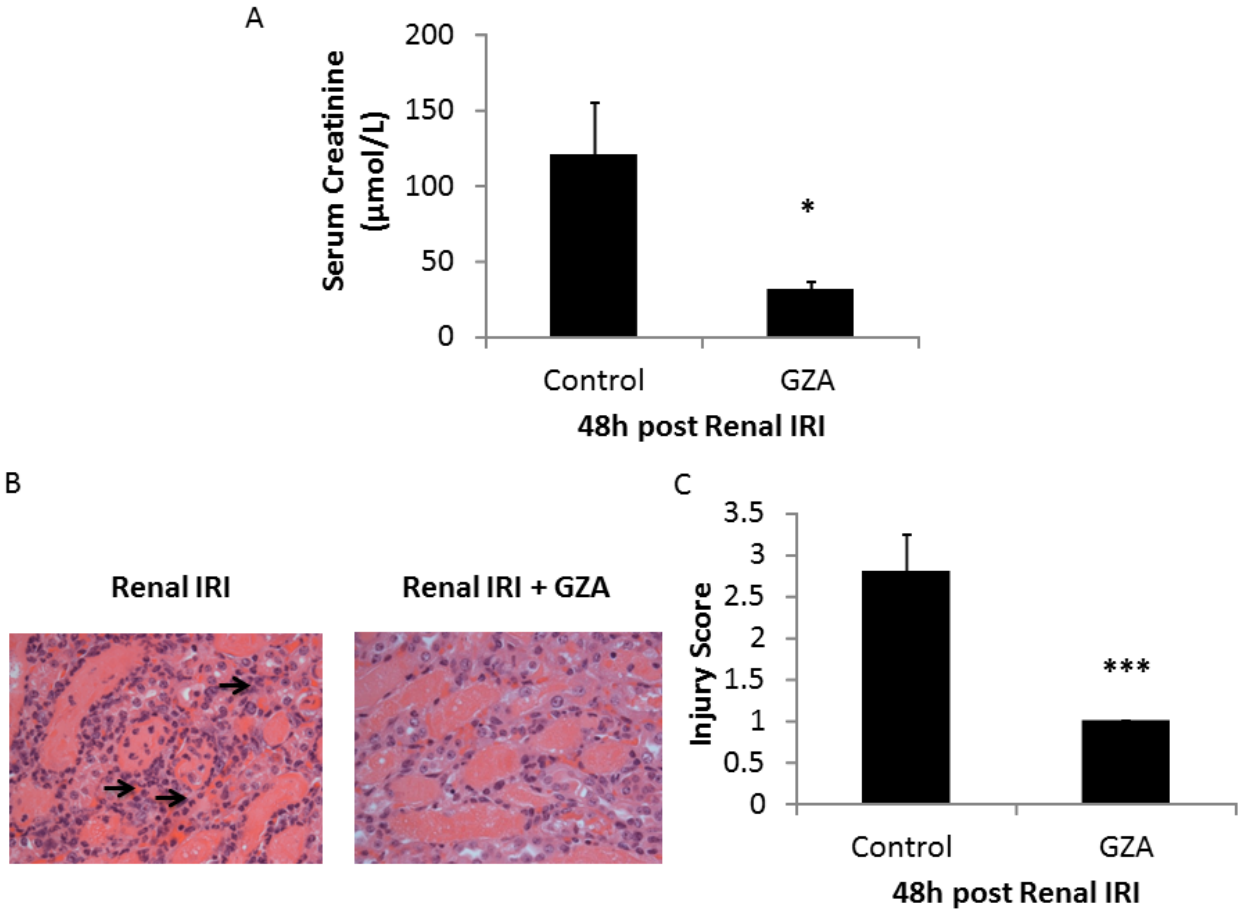

Fold change in mRNA expression
4
3.5
3
2.5
2
1.5
1
0.5
0
NT
rHMGB1
rHMGB1 + GZA
* * *
IFNg
Perforin
Granzyme B

Figure 3. Increased TEC expression of pro-inflammatory cytokines and NK cell activation is inhibited by GZA.

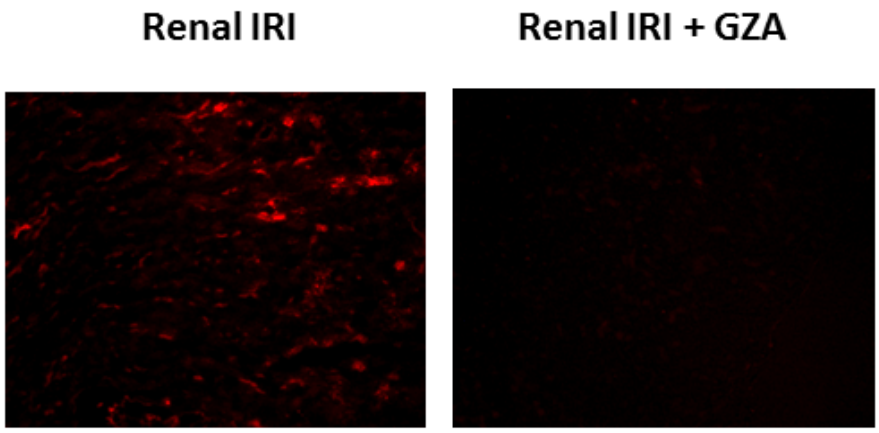
A, B, C) C57BL/6 mice were subjected to renal IRI. Total renal mRNA was analyzed for MCP-1, CXCL1, and RANTES expression by real time PCR. (*: $p<0.05$, ***: $p<0.001$, n=3/group) D, E) TEC were subjected to hypoxia and were treated with various concentrations of GZA. Total mRNA was analyzed at 24 hours for MCP-1 and CXCL1 expression by real time PCR. (*: $p<0.05$, **: $p<0.01$, ***: $p<0.01$, n=3-4/group) F, G) Total mRNA expression of IL-6 was analyzed in C57BL/6 mice kidneys subjected to renal IRI or 24h post-hypoxia treated TEC with 800ng/mL GZA (**: $p<0.01$, n=3/group). H) NK cells were treated with 1000ng/mL rHMGB1 and 1000ng/mL GZA for 24 hours. Total mRNA from NK cells were analyzed for IFNγ, perforin, and granzyme B by real-time PCR. (*: $p<0.05$, n=3/group)

HMGB1 inhibition by GZA improves renal function after kidney IRI

Based on the anti-inflammatory and pro-survival effects of GZA on TEC *in vitro*, we tested the capacity of GZA to inhibit HMGB1 *in vivo* and its potential to attenuate renal dysfunction after IRI. Mice were subjected to IRI with or without GZA pre-treatment and renal function was assessed at 48h. As shown in Figure 4A, mice with renal IRI had markedly elevated serum creatinine levels as compared to the renal IRI and GZA treated mice (48h IRI: 120±35μmol/L vs. 48h IRI with GZA: 31.4±5 μmol/L, p=0.03). Consistent with renal function, (Figure 4C) blinded injury scores were higher in renal IRI treated mice compared to GZA treated mice (48h IRI: 2.8±0.45 vs. 48h IRI with GZA: 1±0, p=0.0004). In particular, more neutrophil infiltration was observed (indicated by arrows) in control mice as compared to the GZA treated mice (Figure 4B). Unlike apoptosis which can be quantified in tissues by TUNEL and other methods, quantitative assessment of necrosis in tissue to date has been difficult to quantitate, relying on histological patterns and electron micrographs. To address this, we have modified a method that quantifies the release of an easily measured fluorochrome (ethidium homodimer) from intact cells following organ perfusion to measure tissue necrosis [41]. Consistent with histology and functional data, we observed greater necrosis in renal IRI treated mice compared to the GZA treated mice as shown in Figure 4D and 4E (necrosis score at 48h IRI: 27.54±5.94 vs. 48h IRI with GZA: 12.04±5.21, p=0.04). Collectively these *in vitro* and *in vivo* data demonstrates that GZA treatment can improve cell viability and reduce renal IRI. These data suggest that the deleterious effects of IRI can be mediated by HMGB1 released from dying cells, and that GZA may neutralize HMGB1 therapeutically.


A
Serum Creatinine (µmol/L)
200
150
100
50
0
*
Control
GZA
48h post Renal IRI
B
Renal IRI
Renal IRI + GZA
C
Injury Score
3.5
3
2.5
2
1.5
1
0.5
0
***
Control
GZA
48h post Renal IRI

D


Renal IRI
Renal IRI + GZA

E

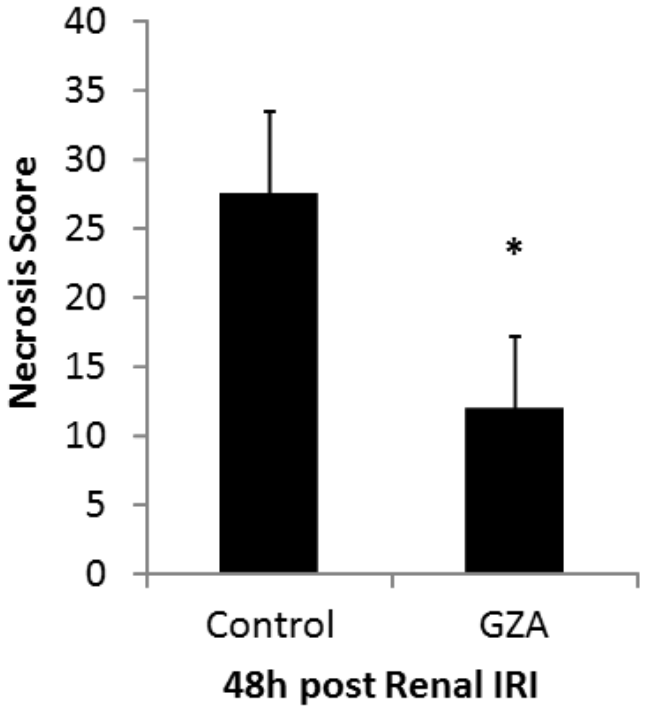

40
35
30
25
20
15
10
5
0
Necrosis Score
*
Control
GZA
48h post Renal IRI

Figure 4. GZA can improve renal function and prevent tissue necrosis during renal IRI.

C57BL/6 mice were subjected to renal IRI. GZA was injected intraperitoneally pre and post ischemia. Control mice were injected with only DMSO vehicle. A) Renal function was determined by serum creatinine at 48h (*: $p<0.05$, n=5/group). B, C) Kidney tissue was collected at 48h post ischemia and stained by H&E. Arrows indicate areas of neutrophil infiltration. Images were taken at 100X magnification. Sections were scored for injury by an unbiased blinded pathologist. (***: $p<0.001$, n=5/group) D, E) Kidneys were perfused with ethidium homodimer after 48h of IRI. Sections were analyzed by fluoresecent microscopy and scored by automated software analysis. Images were taken at 40x magnification. (*: $p<0.05$, n=5/group).

4.5 Discussion

Despite our considerable knowledge of the adaptive immune system and the effectiveness of current immunosuppressive therapies (directed against namely T and B cells), kidney allografts have a limited survival. It has been suggested that innate immunity by its ability to promote inflammation through CDAMPs during IRI may have a large but perhaps underappreciated role in limiting allograft survival**.** In particular, TEC which represent the majority cell type of the renal parenchyma are particularly sensitive to ischemia, inflammation and AKI. During renal IRI, cells undergo prominent forms of cell death, namely apoptosis and necrosis. As a result of cell death, CDAMPs are released to the extracellular compartment allowing for interaction with TLR which can be found on a variety of cell types including renal parenchyma. Recognition of CDAMP by TLR results in downstream activation of various adaptor molecules which mediates pro-inflammatory responses. The release of CDAMPs during renal IRI and their interaction with TLRs on the kidney may play a role in propagating pro-inflammatory responses and result in acute and chronic renal allograft rejection.

Kidney cell death results in the release of endogenous CDAMPs including HMGB1, heat shock proteins, hyaluronic acid, and others. HMGB1 can be released through necrotic cells or actively secreted by activated immune cells such as dendritic cells and macrophages. We have demonstrated that renal tubular epithelial cells (TEC) can release HMGB1 into the extracellular space after hypoxic injury (Figure 2). This supports previous studies that have suggested that HMGB1 is expressed in renal TEC by immunohistochemistry [35]. Previously, our studies have demonstrated that death receptor mediated TEC death can not only result in apoptosis but also a form of programmed necrosis known as necroptosis resulting in HMGB1 release from TEC [45,46]. This is consistent with the pro-inflammatory damage seen in kidney IRI in the form of acute tubular necrosis. Although our results of HMGB1 release from hypoxic TEC are consistent with previous reports of release from necrotic cells, it is possible that some HMGB1 was partially released from apoptotic TEC or viable activated TEC. However, this seems unlikely as we did not detect HMGB1 in the supernatant in viable cell cultures or those that were subjected to sub-lethal treatments (data

not shown). Our characterization of HMGB1 release from necrotic TEC after hypoxia highlights the importance of HMGB1 signalling within the kidney after IRI.

HMGB1 is known to upregulate various pro-inflammatory molecules such as TNFα, interleukin-1β, interleukin-6, and CXCL12. There is currently no therapeutic that targets HMGB1 mediated inflammation. We used GZA, a specific functional inhibitor of HMGB1, to block the production of pro-inflammatory molecules in hypoxic TEC (Figure 3) which was consistent with previous reports [44]. In addition, NK cell activation by HMGB1 was inhibited by the addition of GZA indicating an additional role in preventing inflammation (Figure 3). GZA was able to reduce cell death following hypoxia suggesting that HMGB1 could have a direct effect *in vivo* on propagating injury, although this has not been shown. The addition of recombinant HMGB1 (1-2µg/mL) was unable to induce cell death as measured by Annexin-V/PI labeling (not shown). Recent studies have shown that HMGB1 is post-translationally modified through oxidation during cell death, which can result in greater activation of pro-inflammatory pathways [47]. Furthermore, hyper-acetylation of HMGB1 allows for translocation from the nucleus to the cytosol and is therefore likely to be the form released during cell death [13]. Oxidation and acetylation of HMGB1 via cell death may thus account for discrepancy of results using recombinant HMGB1 as compared to native HMGB1. Consistent with a role of modified HMGB1 in injury *in vivo*, anti-HMGB1 antibody in previous studies [34] and GZA in the present study can attenuate IRI. Our *in vitro* results showing the protective effect of GZA was extended to *in vivo* and demonstrated that GZA treatment can also block IRI induced tissue necrosis through a novel quantifiable method using ethidium homodimer perfusion (Figure 4). The inhibition of HMGB1 mediated inflammation and necrosis during renal IRI was also reflected in significantly improved renal function. Based upon our findings, GZA may act as a viable therapeutic for acute kidney injury (AKI) after IRI due to its nephro-protective effects through the inhibition of HMGB1-mediated injury.

From a practical and therapeutic standpoint, GZA appears to be a promising candidate for targeting HMGB1 as loss of function is related to neutralization rather than blocking the candidate receptors including TLR and RAGE. Studies in other models of organ injury have demonstrated GZA can downregulate a panel of pro-inflammatory mediators which suggests it might be an effective broad anti-inflammatory agent in a variety of acute injury and

inflammatory disease models [48,49]. Currently, its clinical uses have been primarily for the treatment of hepatitis B and C at high doses [36]. GZA binds stably to both boxes of HMGB1 without interfering with its physiological DNA-binding functions or causing release of HMGB1 from chromatin thereby causing very little to no cytotoxic effects [40]. In addition, the ability of GZA to be efficiently perfused into the donor organ is also greater than anti-HMGB1 antibodies due to its smaller molecular weight. Due to the protein based nature of antibodies, their affinity towards their target molecules would be ineffective in the low temperature perfusion solution. Therefore, our work demonstrates the significance of inhibiting HMGB1 mediated injury to ameliorate renal IRI through novel therapeutics such as GZA.

Acute kidney injury resulting from IRI is presumed to be temporary and self-limiting. However, our results show that HMGB1 can directly injury TEC which suggests that progressive AKI may result from HMGB1 and perhaps other CDAMPs. The application of our HMGB1 inhibitor, GZA, had a beneficial effect on the kidney after IRI indicating that HMGB1 alone does have a large role in the pro-inflammatory response. Studies have demonstrated that HMGB1-like effects can also be generated by endotoxin contamination which could also generate the type of inflammation seen by TLR signalling [50]. In our case, this is unlikely since the anti-HMGB1 effects of GZA are not specific towards LPS and thus would only ameliorate injury due to HMGB1 signalling. Further characterization of other CDAMP molecules in AKI are required to thoroughly understand the complex mechanisms of CDAMP release and signaling and will provide greater insight into the role of innate immunity during IRI.

In summary, we have identified that HMGB1 released from dying TEC can mediate pro-inflammatory responses and cell death in TEC and can also impair renal function in kidney IRI. We have also demonstrated the therapeutic potential of GZA as a functional inhibitor of HMGB1 mediated injury during renal IRI through the inhibition of tissue necrosis and the downregulation of pro-inflammatory molecules. Inhibition of HMGB1 reduced cell death both *in vitro* and *in vivo* and may lead to a greater understanding of the mechanisms behind the propagation of injury during AKI. Inclusion of the role of HMGB1 in both inflammation and cell death during renal IRI may further our current understanding of transplant rejection

elicited by these pathways. Importantly GZA regulation of parenchymal cell death may also be useful in solid organ transplantation.

4.6 Acknowledgements

We thank Ms. Pamela Gardner for administrative support. This study was supported by the Canadian Institutes of Health Research (XTW-90932, MOP-111180, MOP-115048), the Kidney Foundation of Canada (A.M.J., Z.X.Z), and the Program of Experimental Medicine (POEM) at Western University, London, Ontario, Canada.

4.7 Author Disclosure

The authors have no financial disclosures or conflict of interest in this study.

4.8 References

1. Shoskes, D. A. & Halloran, P. F. Delayed graft function in renal transplantation: etiology, management and long-term significance. *J. Urol.* **155**, 1831–1840 (1996).

2. Paller, M. S. Free radical-mediated postischemic injury in renal transplantation. *Ren Fail* **14**, 257–260 (1992).

3. Homer-Vanniasinkam, S., Crinnion, J. N. & Gough, M. J. Post-ischaemic organ dysfunction: a review. *Eur J Vasc Endovasc Surg* **14**, 195–203 (1997).

4. Mohib, K., Guan, Q., Diao, H., Du, C. & Jevnikar, A. M. Proapoptotic activity of indoleamine 2,3-dioxygenase expressed in renal tubular epithelial cells. *Am. J. Physiol. Renal Physiol.* **293**, F801–812 (2007).

5. Gobé, G., Willgoss, D., Hogg, N., Schoch, E. & Endre, Z. Cell survival or death in renal tubular epithelium after ischemia-reperfusion injury. *Kidney Int.* **56**, 1299–1304 (1999).

6. Nogae, S. *et al.* Induction of apoptosis in ischemia-reperfusion model of mouse kidney: possible involvement of Fas. *J. Am. Soc. Nephrol* **9**, 620–631 (1998).

7. Du, C. *et al.* Renal tubular epithelial cell apoptosis by Fas-FasL-dependent self-injury can augment renal allograft injury. *Transplant. Proc* **35**, 2481–2482 (2003).

8. Zhang, Z.-X. *et al.* NK cells induce apoptosis in tubular epithelial cells and contribute to renal ischemia-reperfusion injury. *J. Immunol* **181**, 7489–7498 (2008).

9. Rosin, D. L. & Okusa, M. D. Dangers within: CDAMP responses to damage and cell death in kidney disease. *J. Am. Soc. Nephrol.* **22**, 416–425 (2011).

10. Pisetsky, D. Cell death in the pathogenesis of immune-mediated diseases: the role of HMGB1 and CDAMP-PAMP complexes. *Swiss Med Wkly* **141**, w13256 (2011).

11. Land, W. G. Injury to allografts: innate immune pathways to acute and chronic rejection. *Saudi J Kidney Dis Transpl* **16**, 520–539 (2005).

12. Klune, J. R., Dhupar, R., Cardinal, J., Billiar, T. R. & Tsung, A. HMGB1: endogenous danger signaling. *Mol. Med* **14**, 476–484 (2008).

13. Scaffidi, P., Misteli, T. & Bianchi, M. E. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* **418**, 191–195 (2002).

14. Nogueira-Machado, J. A., Volpe, C. M. de O., Veloso, C. A. & Chaves, M. M. HMGB1, TLR and RAGE: a functional tripod that leads to diabetic inflammation. *Expert Opin. Ther. Targets* **15**, 1023–1035 (2011).

15. Abraham, E., Arcaroli, J., Carmody, A., Wang, H. & Tracey, K. J. HMG-1 as a mediator of acute lung inflammation. *J. Immunol* **165**, 2950–2954 (2000).

16. Andersson, U. & Tracey, K. J. HMGB1 is a therapeutic target for sterile inflammation and infection. *Annu. Rev. Immunol.* **29**, 139–162 (2011).

17. Czura, C. J., Wang, H. & Tracey, K. J. Dual roles for HMGB1: DNA binding and cytokine. *J. Endotoxin Res.* **7**, 315–321 (2001).

18. Tsung, A. *et al.* The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J. Exp. Med* **201**, 1135–1143 (2005).

19. Ueno, H. *et al.* Contributions of high mobility group box protein in experimental and clinical acute lung injury. *Am. J. Respir. Crit. Care Med.* **170**, 1310–1316 (2004).

20. Andrassy, M. *et al.* High-mobility group box-1 in ischemia-reperfusion injury of the heart. *Circulation* **117**, 3216–3226 (2008).

21. Rovere-Querini, P. *et al.* HMGB1 is an endogenous immune adjuvant released by necrotic cells. *EMBO Rep* **5**, 825–830 (2004).

22. Beyer, C. *et al.* The extracellular release of DNA and HMGB1 from Jurkat T cells during in vitro necrotic cell death. *Innate Immunity* (2012).doi:10.1177/1753425912437981

23. Bell, C. W., Jiang, W., Reich, C. F. & Pisetsky, D. S. The extracellular release of HMGB1 during apoptotic cell death. *Am. J. Physiol., Cell Physiol* **291**, C1318–1325 (2006).

24. Gardella, S. *et al.* The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Rep* **3**, 995–1001 (2002).

25. Zhu, H. *et al.* An efficient delivery of CDAMPs on the cell surface by the unconventional secretion pathway. *Biochem. Biophys. Res. Commun.* **404**, 790–795 (2011).

26. Kaczorowski, D. J. *et al.* Mechanisms of Toll-like receptor 4 (TLR4)-mediated inflammation after cold ischemia/reperfusion in the heart. *Transplantation* **87**, 1455–1463 (2009).

27. Chen, L. *et al.* TLR engagement prevents transplantation tolerance. *Am. J. Transplant* **6**, 2282–2291 (2006).

28. Yu, M. *et al.* HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock* **26**, 174–179 (2006).

29. Krüger, B. *et al.* Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. *Proc. Natl. Acad. Sci. U.S.A* **106**, 3390–3395 (2009).

30. Wu, H. *et al.* TLR4 activation mediates kidney ischemia/reperfusion injury. *J. Clin. Invest* **117**, 2847–2859 (2007).

31. Park, J. S. *et al.* High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am. J. Physiol., Cell Physiol* **290**, C917–924 (2006).

32. Andersson, U. *et al.* High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. *J. Exp. Med* **192**, 565–570 (2000).

33. Tsuboi, N. *et al.* Roles of Toll-Like Receptors in C-C Chemokine Production by Renal Tubular Epithelial Cells. *J Immunol* **169**, 2026–2033 (2002).

34. Wu, H. *et al.* HMGB1 contributes to kidney ischemia reperfusion injury. *J. Am. Soc. Nephrol.* **21**, 1878–1890 (2010).

35. Junhua, L. *et al.* Neutralization of the extracellular HMGB1 released by ischaemic damaged renal cells protects against renal ischemic reperfusion injury. *Nephrol Dial Transplant* **26**,469-478 (2011).

36. Veldt, B. J. *et al.* Long-term clinical outcome and effect of glycyrrhizin in 1093 chronic hepatitis C patients with non-response or relapse to interferon. *Scand. J. Gastroenterol.* **41**, 1087–1094 (2006).

37. Mabuchi, A., Wake, K., Marlini, M., Watanabe, H. & Wheatley, A. M. Protection by glycyrrhizin against warm ischemia-reperfusion-induced cellular injury and derangement of the microcirculatory blood flow in the rat liver. *Microcirculation* **16**, 364–376 (2009).

38. Ogiku, M., Kono, H., Hara, M., Tsuchiya, M. & Fujii, H. Glycyrrhizin prevents liver injury by inhibition of high-mobility group box 1 production by Kupffer cells after ischemia-reperfusion in rats. *J. Pharmacol. Exp. Ther.* **339**, 93–98 (2011).

39. Kang, D.-G., Sohn, E.-J., Mun, Y.-J., Woo, W.-H. & Lee, H.-S. Glycyrrhizin ameliorates renal function defects in the early-phase of ischemia-induced acute renal failure. *Phytother Res* **17**, 947–951 (2003).

40. Mollica, L. *et al.* Glycyrrhizin binds to high-mobility group box 1 protein and inhibits its cytokine activities. *Chem. Biol* **14**, 431–441 (2007).

41. Edwards, J. R., Diamantakos, E. A., Peuler, J. D., Lamar, P. C. & Prozialeck, W. C. A novel method for the evaluation of proximal tubule epithelial cellular necrosis in the intact rat kidney using ethidium homodimer. *BMC Physiol.* **7**, 1 (2007).

42. Suzuki, C. *et al.* Participation of autophagy in renal ischemia/reperfusion injury. *Biochem. Biophys. Res. Commun.* **368**, 100–106 (2008).

43. Prodjosudjadi, W. *et al.* Production and cytokine-mediated regulation of monocyte chemoattractant protein-1 by human proximal tubular epithelial cells. *Kidney Int.* **48**, 1477–1486 (1995).

44. Gong, G. *et al.* Glycyrrhizin attenuates rat ischemic spinal cord injury by suppressing inflammatory cytokines and HMGB1. *Acta Pharmacol. Sin.* **33**, 11–18 (2012).

45. Linkermann, A. *et al.* Rip1 (receptor-interacting protein kinase 1) mediates necroptosis and contributes to renal ischemia/reperfusion injury. *Kidney Int.* **81**, 751–761 (2012).

46. Lau, A. *et al.* RIPK3-mediated necroptosis promotes donor kidney inflammatory injury and

reduces allograft survival. *Am J Transplant* **11**, 2805-2818 (2013).

47. Kazama, H. *et al.* Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity* **29** (1), 21-32 (2008).

48. Mabuchi, A. *et al.*, Protection by glycyrrhizin against warm ischemia-reperfusion-induced cellular injury and derangement of the microcirculatory blood flow in the rat liver. *Microcirculation* **16** (4), 364-76 (2009).

49. Zhai, CL. *et al.*, Glycyrrhizin protects rat heart against ischemia-reperfusion injury through blockade of HMGB1-dependent phospho-JNK/Bax pathway. *Acta Pharmacol. Sin.* **33** (12), 1477-87 (2012).

50. Hung, C.-C. *et al.* Upregulation of chemokine CXCL1/KC by leptospiral membrane lipoprotein preparation in renal tubule epithelial cells. *Kidney Int* **69**, 1814–1822 (2006).

Chapter 5

5 Discussion

5.1 Kidney injury and rejection is promoted by the form of cell death and subsequent pro-inflammatory responses

These studies have demonstrated the importance of cell death and highlighted that the role of death regulates immune responses during renal IRI and transplantation (Figure 5.1). The kidney possesses a somewhat unique ability to resist inflammatory forms of tissue injury through the expression of various anti-apoptosis molecules, including SPI-6, which inhibits granzyme B-mediated cell death[8,109,110] and pro-survival molecules such as c-FLIP and erythropoietin (EPO), TGFβ, and others. During renal IRI as well as in transplantation, cytotoxic infiltrating immune cells play a large role in determining outcomes in kidney function and survival via effects on target parenchymal cells such as TEC. Cytotoxicity is mediated through several pathways including perforin/granzyme B which has been demonstrated to effectively induce cell death in TEC *in vitro*[49,50]. Under pro-inflammatory conditions, TEC can upregulate expression of SPI-6 and effectively resist granzyme mediated cytotoxicity. Loss of SPI-6 in the kidney graft clearly results in greater cell death and graft dysfunction as well as decreased allograft survival. This also supports a role for apoptotic cell death in promoting allograft dysfunction and reduced survival as SPI-6 inhibition of granzyme B normally prevents caspase-3 activation and subsequent apoptosis. Although apoptosis is not associated with augmenting inflammation such as with necrosis, sufficient loss of parenchymal cells without regeneration will inevitably result in loss of organ function. Therefore, limiting apoptosis remains an attractive therapeutic target, provided other forms of cell death remain in balance and are not affected.

Thus, previous studies in renal IRI using mouse models have demonstrated a benefit to inhibiting apoptosis with siRNA directed towards caspase-3 and 8[61]. However, in our model of kidney transplantation, inhibition of caspase-8 in the donor graft led to increased tissue injury and decreased allograft survival and disruption of this particular caspase had unintended consequences. This observation was confirmed *in vitro* in TEC as necrosis was

induced by TNFα with caspase-8 inhibition. As either Nec-1 or RIPK3 deletion recovered cell viability, this form of necrosis was by definition necroptosis. Release of HMGB1 was also demonstrated in TEC undergoing necroptosis, suggesting that this form of programmed necrosis had the potential to be pro-inflammatory by the release of CDAMP. Necroptosis was linked to AKI as kidney function was preserved and tissue injury was consistently reduced in RIPK3$^{-/-}$ mice with conditions that induced renal IRI in wild type mice. This benefit was extended tokidney transplantation as mouse recipients receiving RIPK3$^{-/-}$ allografts had preserved kidney function and prolonged graft survival.

The role of CDAMPs in inflammatory diseases has been well described in various models of acute injury including renal IRI[101]. Among those that are studied, HMGB1 has been the most extensively characterized as a regulator of the pro-inflammatory response. In our mouse model of renal IRI, HMGB1 is released from TEC undergoing necrotic cell death *in vitro*. Extended to *in vivo*, it is likely that extracellular HMGB1 can signal through TLRs on adjacent TEC to induce expression of pro-inflammatory cytokines that can promote further injury through greater chemoattraction and activation of immune cells at the site of injury. Consistent with this, use of the small molecule inhibitor GZA allows for functional inhibition of extracellular HMGB1[111] and provided protection from renal IRI *in vivo*. Without inhibition, HMGB1 that can bind to TLR can propagate inflammatory injury as activated infiltrating immune cells can cause further injury through the aforementioned death receptor pathways.

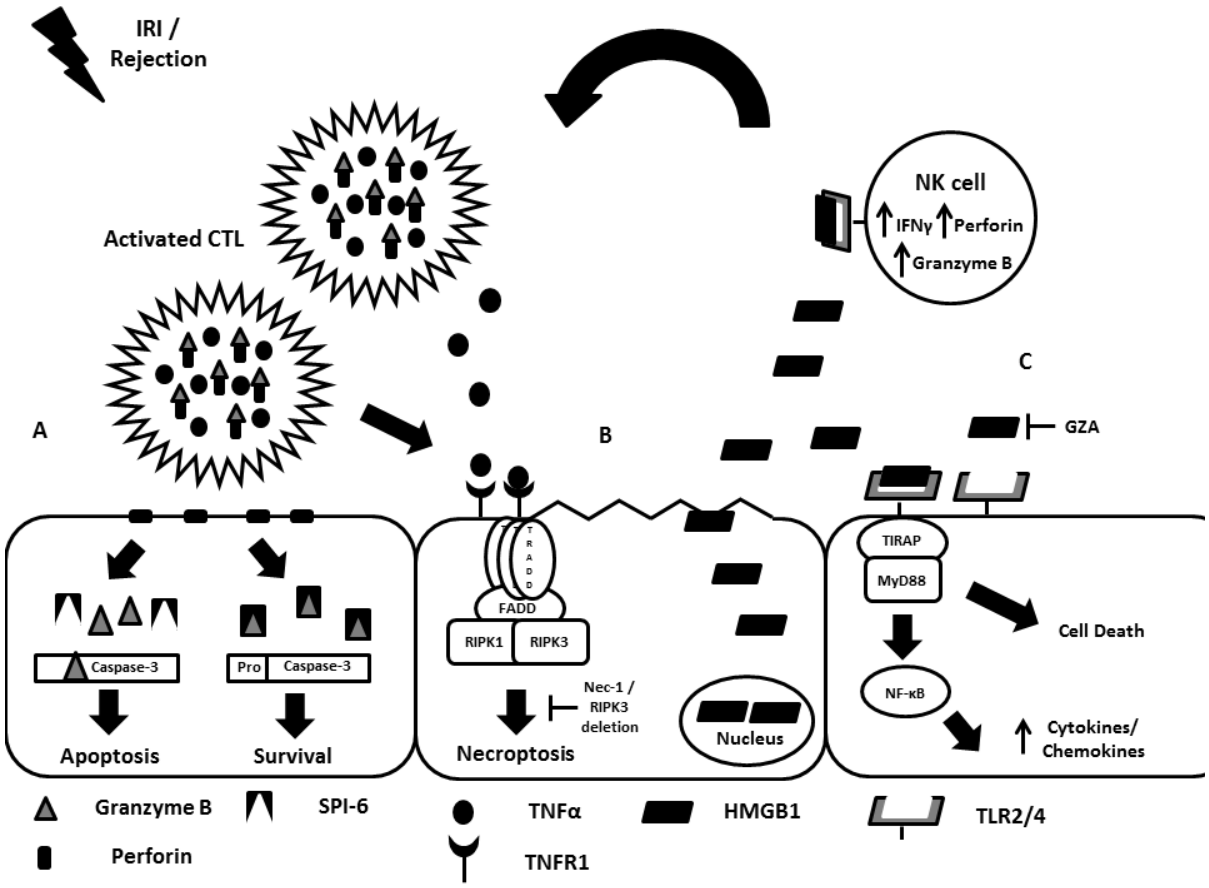

IRI /
Rejection
Activated CTL
NK cell
IFNγ
Perforin
Granzyme B
A
B
C
GZA
TRADD
FADD
RIPK1
RIPK3
TIRAP
MyD88
Caspase-3
Pro
Caspase-3
Apoptosis
Survival
Nec-1 /
RIPK3
deletion
Necroptosis
Nucleus
Cell Death
NF-κB
Cytokines/
Chemokines
Granzyme B
SPI-6
Perforin
TNFα
TNFR1
HMGB1
TLR2/4

Figure 5.1 Cell death regulates severity of tissue injury and inflammation in renal IRI and transplantation.

During renal IRI and transplant rejection, pro-inflammatory responses activate various CTL which infiltrate the kidney and induce cell death in TEC. A) Activated CTL upregulate perforin/granzyme B complexes and TNFα and secrete them into the extracellular space. Perforin forms pores on the surface of TEC allowing for granzyme B to enter the target cell and cleave caspase-3, inducing apoptosis. However, TEC can limit the extent of granzyme B mediated cytotoxicity through expression of SPI-6, an inhibitor of granzyme B. B) Soluble TNFα binds to TNFR1 expressed on the surface of TEC and activates formation of cell death complexes. If caspase-8 activity is inhibited, the necrosome complex consisting of FADD, RIPK1, and RIPK3 is formed and induces necroptosis in the TEC. The resulting necroptotic death releases HMGB1 into the extracellular space. However, if RIPK1/3 activity is blocked by Nec-1 or RIPK3 deletion, necroptosis is inhibited. C) Extracellular HMGB1 can bind to TLR2 or TLR4 found on the surface of TEC and immune cells. Activation of TLR signaling by HMGB1 in TEC leads to upregulation of pro-inflammatory responses and cell death. This response can perpetuate immune mediated cytotoxicity in the kidney due to further activation of naïve CTL. Intervention of HMGB1 mediated inflammation by GZA can protect TEC and prevent further tissue injury during renal IRI.

5.2 Regulation of renal IRI and allograft injury by donor organ factors

Current clinical practices for controlling rejection in kidney transplantation is primarily focused on limiting the aggressiveness of adaptive immune responses specifically in the form of T and B cells. These strategies have shown to be effective in preventing and even treating acute rejection episodes. However, chronic rejection appears to be unaffected by current immunosuppression as long term survival has not changed nearly as dramatically as short term survivals[35,36]. Previous studies have characterized the expression of various cell survival and anti-inflammatory factors in renal tissue. In particular, PI-9/SPI-6 has been identified in renal allografts and is highly expressed during episodes of subclinical rejection suggesting TEC expression of this molecule may be the major determinant that controls progression to overt clinically detectable rejection[54]. This study has clarified the role of TEC expressed SPI-6 during allograft transplantation, particularly demonstrating the importance of this molecule in regulating granzyme B-mediated cytotoxicity during rejection resulting in increased graft survival[64]. Interestingly, other studies using non-life supporting murine kidney transplantation models have shown that the loss of perforin/granzyme in the recipient does not significantly improve transplant injury, suggesting alternative mechanisms of cytotoxicity including Fas-FasL interactions may play a more important role[112]. However, our study has clearly shown that altering susceptibility to granzyme cytotoxicity on the target/donor cell side via SPI-6 can alter injury and thus this may be a strategy for greater protection against transplant injury.

Along with our study, others have provided evidence that the kidney can attenuate injury through the production of additional cell survival and anti-inflammatory factors. TEC can also express c-FLIP, an anti-apoptotic molecule that is similar to caspase-8 but lacks the catalytic enzyme site and prevents the downstream catalytic function of the death inducing signaling complex (DISC)[110]. Furthermore, recent studies have shown that not only can c-FLIP prevent apoptotic death but complexed with caspease-8 can regulate necroptosis[66]. In contrast, TEC can also directly promote cell death through surface expression of both Fas and Fas-L during inflammation[8]. The increased expression of these surface molecules not only allows for death receptor mediated cell death of TEC by immune cells but also between

activated TEC in a process referred to as fratricide. As specialized cells of the nephron, TEC are highly metabolically active and under pro-inflammatory stress, are capable of upregulating and secreting cytokines. This was supported *in vitro* and *in vivo* in our renal IRI studies that demonstrated upregulation of a number of key pro-inflammatory cytokines within the kidney. However, the kidney is also capable of producing anti-inflammatory cytokines such as interleukin-10 (IL-10) and TGFβ[113,114] which promotes proliferation of $CD4^+FoxP3^+$ T-regulatory cells (Tregs) that can suppress $CD4^+$ and $CD8^+$ T cell populations. Interestingly despite alteration of donor SPI-6 or RIPK3 expression, infiltrating cytotoxic cells were still able to infiltrate the graft indicating that recipient immune function per se was not impaired. This provides supportive evidence that the modification of donor factors can play an important role in regulating the severity of rejection without comprising the systemic immune function in contrast to immunosuppressive therapies that have a generalized effect on immune response.

Currently, SPI-6 has been identified in various tissue and cell types and is particularly highly expressed in cytotoxic cells as they require protection from self-directed granzyme B mediated apoptosis[52]. This must be taken into consideration if expression of SPI-6 is upregulated systemically in the recipient for the purposes of protecting the graft from cytotoxic attack as increased SPI-6 in immune cells would also make them more resistant to cell death. Activation induced cell death following T cell activation leads to cell-to-cell fratricide by apoptosis as a mechanism to limit expansion. Perturbing immune cell death pathways could result in the increased activity of cytotoxic immune cells. As well, CTL forms of Tregs that would not be able to suppress their function through granzyme B mediated cytotoxicity, potentially leading to more severe rejection. Thus far, the only compound that has been identified to upregulate PI-9, or the mouse homolog SPI-6, is genistein, a naturally occurring component of soybean. In studies using cancer cell lines, genistein induced expression of PI-9 induced resistance against cytotoxic NK cells[115]. Nonetheless, upregulation of donor PI-9 could be adapted to transplantation therapy through the use of donor organ perfusion solutions containing genistein that can upregulate PI-9 in TEC as well as other kidney parenchyma, without affecting recipient immune cells.

5.3 Apoptosis and necroptosis are counterbalanced forms of cell death in renal IRI and allograft rejection

Kidney transplantation injury is triggered by a cascade of linked innate and adaptive immune responses that propagate injury, kill parenchymal cells, and promote antibody and cell mediated rejection. The form of cell death may be an early variable that directs the outcome of alloimmune responses and graft survival (Figure 5.2). The current understanding of cell death mechanisms has greatly expanded beyond apoptosis to include forms of regulated necrosis. This study has revealed a newly recognized form of programmed necrosis in both TEC and renal tissue and can affect AKI and allograft transplant survival[22]. Necroptosis, as a result of pro-inflammatory stimuli, is negatively regulated by active caspase-8 and is mediated by RIPK1/3. During AKI such as IRI and allograft rejection, acute tubular necrosis is a histological hallmark of renal injury which in its severest form results in the progression of fibrosis leading to kidney dysfunction and potentially graft loss[37]. Necroptotic death appears to contribute to overall necrosis during kidney injury as demonstrated by ethidium homodimer perfusion which visualizes and quantifies tissue necrosis *in vivo*. Importantly, prevention of necroptosis by genetic RIPK3 deletion in the donor graft prolongs allograft survival and preserves kidney function. Other studies have also observed necroptotic cell death in diverse forms of inflammatory disease including IRI in various organs, toxic injury, infectious disease, and autoimmune disorders[70,81,83,84]. In concordance with our observations in kidney transplantation, inhibition of necroptosis in these studies was also able to ameliorate inflammatory injury and reduce pathogenicity of the disease.

Blockade of multiple cell death pathways such as necroptosis and apoptosis may collectively represent a viable and novel strategy for the prevention of kidney injury. Previous reports have also demonstrated a benefit to the kidney after inhibition of apoptosis through caspase-3 and 8 by siRNA in AKI[9]. Surprisingly, our preliminary *in vivo* studies using combined caspase-$8^{-/-}$/$RIPK3^{-/-}$ mice[66] have demonstrated that the absence of caspase-8 along with loss of RIPK3 counteracts the benefit of RIPK3 deletion during renal IRI (not shown). Indeed, serum creatinine and histological analyses indicate that there was no difference in renal function and injury after renal IRI between wild type and caspase-$8^{-/-}$/$RIPK3^{-/-}$. In addition, when kidney allografts from caspase-$8^{-/-}$/$RIPK3^{-/-}$ mice are transplanted, a similar blunting of

benefit was seen as grafts did not survive longer than wild type allografts. Further elucidation of the mechanism behind this observation will be required in order to understand the complex interplay between caspase-8 and RIPK3 and their effects on other pathways. One possibility includes that lack of apoptosis, due to lost effect on tolerance induction, in the allograft may actually be deleterious to graft survival, although this does entirely explain the IRI results. Apoptosis inhibition has been demonstrated to be protective in a short term AKI model. Permanent apoptosis inhibition by genetic deletion in any model followed for longer times may have different effects. Studies support that apoptosis may have a role in stimulating an anti-inflammatory and pro-survival responses which may impact beneficially on the progression of rejection in the allograft[92,116,117]. Endothelial cells undergoing apoptosis can program anti-inflammatory macrophages[118] or proteolytically remodel surrounding extracellular matrix (ECM) to release anti-apoptotic molecules such as C-terminal fragment of perlecan (LG3)[119]. A more likely possibility may be that caspase-8 and RIPK3 may also regulate other cell death pathways such as intrinsic apoptosis and autophagy. The combined loss of these important molecules may have caused dysregulation of these pathways leading to greater kidney injury[120,121]. Nonetheless, as new cell death pathways and their regulators are defined, it will be crucial to consider effects on other pathways as cell death regulation involves high levels of crosstalk and overlap in function and outcome.

The benefits of necroptosis inhibition appear to be two-fold: prevention of loss of TEC and other renal parenchyma that are essential to kidney function, which was reflected by low levels of serum creatinine and secondly, a reduction in inflammation and subsequent immune response as noted by decreased neutrophil infiltration and less tubilitis. As expected, RIPK3 inhibition prevented necrosis in the graft as it is essential for this form of necrosis. It also appeared to induce resistance to apoptosis as well, which has been observed other studies, albeit with no clear mechanism[122]. Dampening of immune responses may be due to decreased CDAMP release as was seen in both *in vitro* and *in vivo* when necroptosis was inhibited. As lack of HMGB1 release from necroptotic cells is unable to trigger innate immune responses, this may in turn dampen the adaptive immune responses which in turn leads to less cell death and inflammation overall. This was well demonstrated in our preliminary studies on TEC production of pro-inflammatory cytokines using supernatants obtained from wild type and RIPK3$^{-/-}$ TEC undergoing hypoxic death. As expected, supernatants from wild type TEC

undergoing cell death were able to upregulate pro-inflammatory responses in TEC whereas supernatant from RIPK3 TEC undergoing cell death did not (data not shown). The presence of HMGB1 can thus enhance pro-inflammatory responses in TEC as well as immune cells. However, necroptosis inhibition does not completely eliminate the occurrence of rejection as noted by obvious CD3+ infiltrates in both wild type and RIPK3$^{-/-}$ grafts. The model we used does not add immunosuppression and so perhaps this was expected as there would be no agents to suppress the proliferation of T cells. However, the presence of infiltrating cells suggests that even in RIPK3$^{-/-}$ allografts there is ongoing injury sufficient to induce chemotactic signals. While these results provide important insights into the potential for necroptosis inhibitors to protect allografts from rejection without adversely affecting recipient immune responses, it is clear that additional therapy will be required, as loss of necroptosis does not generate tolerance per se.

Genetic deletion of RIPK3-mediated necroptosis in the donor graft prevented allograft loss but this is not generally feasible in clinical scenarios.Thus consideration of the effects of small molecule inhibitors to regulate this pathway is attractive if this is to be translated into a therapeutic. Nec-1, a RIPK1 inhibitor, has been demonstrated as an efficient *in vitro* inhibitor of necroptosis in TEC in our study, while others have demonstrated its effectiveness during AKI *in vivo*[47].Importantly, RIPK3 deletion in our *in vitro* and *in vivo* models had greater inhibition of necroptosis than Nec-1, suggesting targeting of RIPK3 directly may be of larger clinical benefit. While more complete inhibition may be accomplished with genetic deletion and RIPK3 inhibitors remain to be created, the regulation of necroptosis via Nec-1may not be straightforward in patients. For example, stimulation of the necroptotic pathway can occur in the absence of RIPK1 when signaled through either IFN receptors or TLRs[69,123]. It would follow that the use of RIPK3 inhibitors may be a more suitable necroptosis inhibitor in the future. Currently, the only known inhibitor of RIPK3 is the M45 viral peptide produced by cytomegalovirus (CMV), used by this pathogen to evade immune surveillance by preventing pro-inflammatory necroptosis in infected cells[120]. Alternatively targeting of MLKL, a molecule downstream of RIPK3 may be the most efficient approach to block necroptosis. Necrosulfonamide, a small molecule inhibitor of MLKL, has been shown to effectively block necroptosis as MLKL is required for the initiation of the final pathway of necroptosis[75]. Further studies are required to determine the effective dose and length of treatment with

either RIPK3 or MLKL inhibitors in preventing inflammation and promotion of rejection responses. Again, it is important to consider in translation strategies using these agents, that although inhibition of necroptosis in the donor graft appears to be beneficial to allograft survival, when given to the recipient in the form of a systemic therapeutic, it may have unintended adverse effects on allograft survival through effects on immune cell proliferation and possibly cause more severe rejection[124]. Treatment of the donor graft alone and for short duration should not experience this concern.

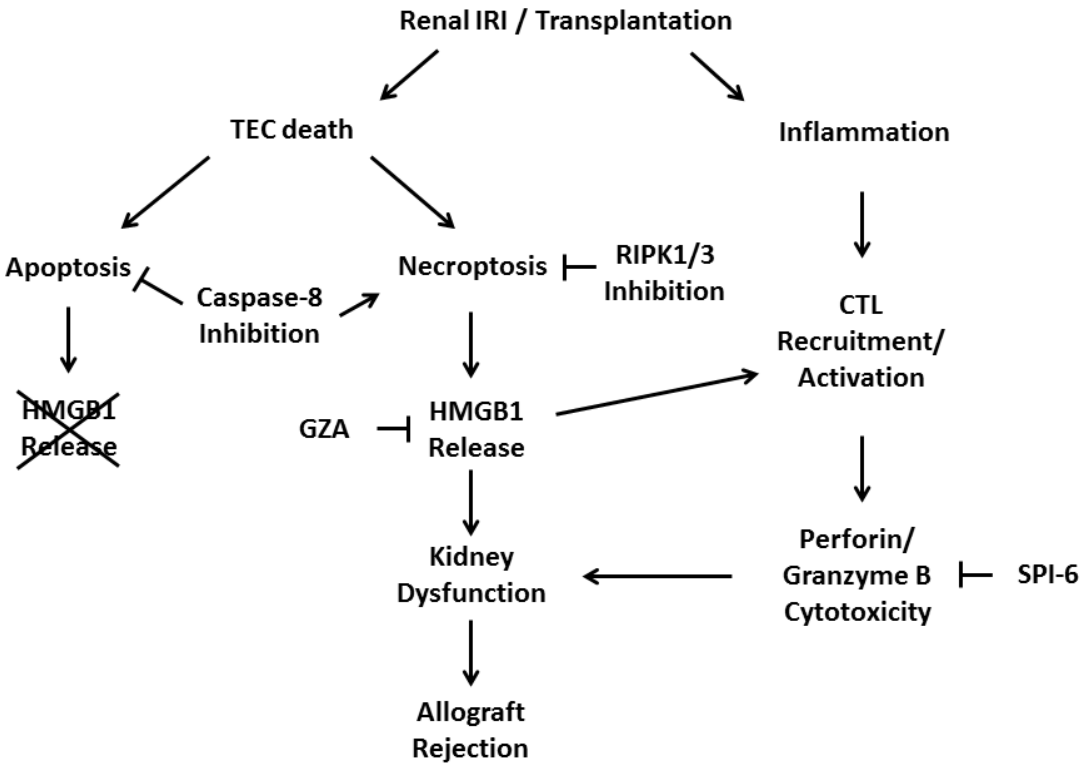

Renal IRI / Transplantation
TEC death
Inflammation
Apoptosis
Caspase-8 Inhibition
Necroptosis
RIPK1/3 Inhibition
CTL Recruitment/ Activation
HMGB1 Release
GZA
HMGB1 Release
Kidney Dysfunction
Perforin/ Granzyme B Cytotoxicity
SPI-6
Allograft Rejection

Figure 5.2 Apoptosis and necroptosis regulates outcomes of long term allograft function and survival.

During kidney transplantation, renal tubular epithelial cell (TEC) death and intra-graft inflammation occurs resulting in acute and chronic rejection. Renal TEC undergo various forms of cell death including apoptosis and necroptosis, each with contrasting immunological consequences. Under apoptotic conditions, TEC form apoptotic bodies which prevent the release of high mobility group box-1 (HMGB1) and other cellular death associated molecular patterns (CDAMPs), thus promoting an anti-inflammatory response. However, when caspase-8 is inhibited, receptor interacting protein kinase 1 and 3 (RIPK1/3) mediated necroptosis is upregulated and results in release of HMGB1 and other CDAMPs. Unless the pro-inflammatory function of HMGB1 is inhibited (i.e. glycyrrhizic acid (GZA)), a pro-inflammatory response is initiated involving the recruitment and activation of infiltrating immune cells, some of which have cytotoxic ability. Cytotoxic lymphocytes (CTL) can then induce apoptosis in target TEC through the perforin/granzyme B unless inhibited by TEC expression of serine protease inhibitor-6 (SPI-6). If renal TEC death and inflammation persist within the allograft, this leads to graft dysfunction and eventually graft rejection/loss.

Other cell death modalities in AKI and renal transplantation

Although apoptosis and necroptosis appear to be major cell death pathways that can regulate kidney injury and inflammation, numerous studies have also revealed other forms of cell death that can affect kidney function and survival. Studies in AKI have revealed that renal cells undergo autophagy which has also been observed during cold preservation injury, which is very relevant to current organ storage[125]. In addition, rapamycin, a commonly used immunosuppressant in transplant recipients, can promote autophagy due to its effects on the mammalian target of rapamycin (mTOR) pathway which may account for its adverse effects on podocyte injury, proteinuria, and glomerulonephritis[126]. Autophagy is typically initiated when a cell undergoes metabolic stress caused by nutrient deprivation or oxidative stress. This process will induce intracellular formation of autophagosomes which begin to break down components of the cell and reuse them in order to maintain cellular energy and prolong cell survival. However, in some cases, autophagy may promote cell death rather than survival and thus may contribute to promoting immune responses similar to apoptosis and necrosis although the manner in which it does this is currently unclear[121]. Interestingly, autophagy also appears to be linked with apoptosis as active caspase-8 inhibits autophagy related proteins (ATG) thereby promoting apoptosis over autophagy[127]. In contrast, when RIPK1 is silenced, necroptosis decreased but autophagy was upregulated. The diverse regulatory functions of caspase-8 and RIPK1 may allow for known crosstalk between apoptosis, necrosis, and autophagy and this ultimately will have effects on inflammation and organ injury.

Another form of programmed necrosis is known as pyroptosis which occurs in response to the presence of pathogens and PAMPs by TLR and nucleotide-binding oligomerization domain receptors (NLR) signaling leading to the activation of the inflammasome. When the inflammasome is activated by caspase-1, upregulation of IL-1β and IL-18 occurs as well as HMGB1 release. Collectively, this results in a pro-inflammatory response as seen in other form of necrosis, including necroptosis[128]. Formation of pores on the cell surface can also occur resulting in cell lysis and pyroptosis. This pathway has been identified in a chronic inflammatory kidney model where inhibition of inflammasome was able to protect against renal injury and decrease inflammation[129]. In human renal allografts, TLR expression on TEC is increased in grafts undergoing acute rejection, suggesting a role for necrosis,

HMGB1, and inflammasome-mediated injury[130]. As previously mentioned, TLRs can also be activated by CDAMP ligands which we and others have demonstrated to be present during AKI and renal allograft injury. Furthermore, our studies have suggested that CDAMPs may directly induce cell death, as inhibition of HMGB1 function by GZA was able to inhibit TEC death which suggests pyroptosis may be another mechanism of TEC death in our studies. Emerging studies have indicated that in the absence of caspase-8, RIPK1/3 may be involved in inflammasome signaling as inhibition of these molecules by Nec-1 or genetic knockout results in reduced active IL-1β production[128]. In addition, it has been shown that the RHIM binding site located on RIPK3 is also found on TIR-domain-containing adaptor-inducing interferon-β (TRIF), an adaptor protein that complexes with TLRs when activated[131]. Therefore, CDAMP activation of TLRs could allow for TRIF and RIPK3 to complex through RHIM interactions leading to necroptosis in addition to pro-inflammatory Nfκb and inflammasome signaling. It remains unclear as to the role of caspase-8, RIPK1, and RIPK3 in inflammasome activation however, one possibility is that necroptosis induces inflammasome activation through the release of HMGB1 and other CDAMPs.

5.4 HMGB1-mediated inflammatory injury in AKI and rejection

Emerging studies in the roles for innate immunity in AKI have demonstrated the importance of immune surveillance by TLRs for the presence injured tissue through CDAMP signaling. In particular, TLR2 and TLR4 have been the most well described of the TLR family in regards to its involvement in propagating renal IRI[16,132]. It has been observed that TLR4 on both renal parenchyma as well as immune cells are crucial to activating the pro-inflammatory response during renal IRI and that inhibition of these receptors on either side are capable of reducing kidney injury. However, the greater benefit was associated with loss of TLR4 on TEC[16]. In keeping with this, human studies in TLR expression on tubular cells from renal allograft biopsies have shown that not only is TLR4 expressed on these cells but increased expression was correlated with woresened graft function[133]. This provides insights into the importance of TLR expression in the kidney in regards to its ability to regulate inflammatory injury during AKI and potentially during renal transplantation.

Although many studies on renal inflammatory injury have focused on the importance of the expression of TLRs and other innate receptors, the ligands for these receptors have also been an area of interest. In a practical sense, it may be more feasible to target circulating ligands rather than TLR clinically. A variety of CDAMPs have been characterized as stimulators of pro-inflammmatory responses including HMGB1, heat shock proteins, uric acid, and other proteins. Our studies have demonstrated that HMGB1 release as a result of necrotic cell death can lead to upregulation of pro-inflammatory molecules in TEC as well as activation of immune cells. Interestingly, HMGB1 was capable of directly inducing cell death *in vitro*, as well as tissue necrosis *in vivo* as supported by the protective effects of GZA, a functional inhibitor of HMGB1 effect. There is growing evidence for the possibility TLR-mediated death generally as adaptors of the TLR family of receptors are able to interact with and activate cell death regulators such as RIPK3, resulting in necroptotic death[131]. Conceptually this would potentially allow for a continuous cycle of HMGB1 induced necroptosis leading to HMGB1 release and further activation of necroptosis in surrounding cells. In addition, with the activation of cytotoxic cells by HMGB1 and other upregulated pro-inflammatory cytokines, this would result in additional receptor mediated necroptosis (by TNFα or Fas-L) and apoptosis, further propagating inflammatory injury and loss of functional parenchymal cells. Our study highlights the feasibility and impact of HMGB1 blockade *in vivo* as its

multifaceted effects on kidney injury during AKI and renal transplantation may play a crucial role in regulating the severity and extent of inflammation.

Although HMGB1 is ubiquitously expressed in all eukaryotic cells and can be released through necrotic cell death, differences in the status of post-translational modifications can alter its immunogenicity. Early studies identified HMGB1 as an 'immunogenic' molecule when it was observed to be secreted from various mononuclear immune cells during inflammation, similar to the role of pro-inflammatory cytokines[95]. As previously noted, HMGB1 modifications can occur via acetylation[94] and oxidation[92] primarily, which are involved in translocation and immunogenicity respectively. In this study, we did not characterize isoforms and modifications of HMGB1 after release from necrotic TEC and kidney tissue. However GZA was capable of globally inhibiting the pro-inflammatory effects of extracellular HMGB1. Again, the complexity of these pathways is reflected in that it has been noted in other studies that the presence of extracellular oxidized HMGB1 may promote a tolerogenic effect[92]. It has been proposed that apoptotic death resulting in production of ROS was potentially responsible for this modification of the HMGB1 molecule. We extended our findings of pro-inflammatory HMGB1 from TEC death in preliminary studies using *in vitro* modification of the oxidative status of recombinant HMGB1 through the addition of hydrogen peroxide (H_2O_2) and dithiothreitol (DTT) to produce oxidative and reducing conditions respectively. When added to NK cells, IFNγ was more highly upregulated in NK cells exposed to reduced HMGB1 compared to oxidized HMGB1 (data not shown). When viable TEC were exposed to HMGB1 containing 'oxidized' and 'reduced' supernatant from hypoxia treated TEC, there was greater TEC death in those treated with 'reduced' supernatant compared to controls (data not shown). These findings highlight the complex biology of HMGB1 and that the neutralization of HMGB1 for the prevention of renal inflammatory may require consideration of altered forms of this molecule.

Other immunogenic molecules released during tissue injury and cell death

Modulation of the innate immune system may provide us with new strategies for ameliorating inflammation and injury during renal IRI and alloimmune rejection[12,134]. It has only been recently that reagents with effects on innate responses have been created or defined for this role. Although HMGB1 is the most well characterized CDAMP molecule, other

CDAMP molecules may also equally contribute to propagating the inflammatory response in the kidney. Our preliminary studies have revealed that although HMGB1 release is increased during renal IRI from TEC and other parenchyma, increased levels of HSP60 and HSP70 can also be detected. This is in agreement with previous studies that have demonstrated HSPs, along with HMGB1, are stimulators of pro-inflammatory responses through TLR signaling in various immune cells[102]. In addition, extracellular matrix (ECM) proteins from tissue injury can act as CDAMPs due to inflammation leading to enzymatic breakdown of these proteins which include fibronectin, hyaluronan, and heparin sulfate[135]. Renal injury studies have demonstrated that ECM generated CDAMP accumulation is observed during AKI and allograft rejection. Inflammation can then be triggered by these pro-inflammatory peptides when bound to their respective TLR or other receptors. As research progresses to define the roles of CDAMP molecules in regulating inflammation relevant to alloimmune responses, consideration of the entire spectrum of CDAMPs released from necrotic cells and the surrounding microenvironment will likely need to be considered when applying therapeutic strategies to transplant rejection.

Cellular death can result in the release of various immunogenic molecules including CDAMPs either freely to the microenvironment as well as being contained in membrane vesicles (MV). Our current understanding is that MV are primarily generated by cells undergoing apoptosis and that these vesicles which are composed of plasma membrane that encapsulate intracellular 'cargo' also expresses surface adhesion molecules that allow for receptor specific binding[136]. MV currently are classified by their diameter which can range from 50nm-1000nm in size, as well as by morphology. Functionally, MV can have effects on inflammation and immune responses depending on the contents contained within as well as their ability to bind to specific receptors on specific target cells[136]. Thus the effects of MV can range from induction of cell death by expression of Fas-L, activation of immune cells by antigen presentation, or inhibition of apoptosis along with smooth muscle cell proliferation by the C-terminal fragment of perlecan (LG3)[117]. Recent research has also suggested that MV may be useful biomarkers as well as potentially therapeutic targets in transplantation. Indeed, MV have been used therapeutically in rat bone marrow and heart transplants and a tolerogenic effect improved allograft function and survival[137]. These studies support the hypothesis while the loss of caspase-8 induces necroptosis, there may be reduced generation

of tolerogenic MV. Collectively, these and our studies provide greater insights into the variable immunogenicity of cell death and their effects on alloimmune responses during transplantation.

5.5 Future directions

Therapeutic applications

Our studies have highlighted the importance of donor graft factors in the regulation of tissue injury and alloimmunity during renal transplantation. In particular, cell death pathways in the allograft regulate not only the viability of parenchymal cells critical to renal function but also affects the severity of inflammatory responses that is reflected in allograft function and survival[22,64]. As we have demonstrated, inhibition of granzyme-mediated apoptosis by SPI-6 or necroptosis by RIPK3 genetic deletion in the donor allograft effectively prolonged allograft survival. As systemic anti-rejection therapeutics will have outcomes on the recipient immune system, further research is required to determine what effect inhibition of cell death pathways would have on components of innate and adaptive immunity. Our murine studies on renal transplantation have allowed us to alter donor specific graft factors in the absence of immunosuppression. This allowed us to more clearly isolate the role of each graft factor (SPI-6, RIPK3) on allograft function and survival. However, without further research into the effects of SPI-6 and RIPK1/3 manipulation on recipient immunity, it would be more prudent to apply inhibitors of cell death (i.e. genistein or solubilized Nec-1) to the donor graft only. Previous studies have demonstrated that application of small molecules to perfusion solution can effectively target donor graft factors specifically which was demonstrated using siRNA delivery into murine cardiac allografts via UW solution[138]. Translational studies in murine kidney transplantation demonstrating the application of these small molecules to renal allografts during cold preservation prior to transplantation as a method for inhibiting granzyme-mediated apoptosis or necroptosis would provide great insight into the feasibility of these compounds as a clinical therapeutic. While effects of cell death inhibitors are likely to be temporary, use of perfusion solution as a delivery system allows for donor graft specific treatment and would also be easier to manage than the typical systemic immunosuppression regimen that must be constantly monitored in recipients. In addition, the benefits of cell death inhibition through this method may allow for reduced immunosuppression post-transplantation as they may work synergistically with one another as shown in our heart transplant studies[139].

Enhancing cytotoxicity resistance in the renal allograft

Our study has demonstrated that donor SPI-6 regulates transplant injury by resisting granzyme-mediated cytotoxic attacks from infiltrating immune cells during renal allograft rejection[64]. In our murine model of kidney transplantation, recipients receiving donor grafts with SPI-6 deletion had reduced allograft survival as compared to those receiving wild type grafts. While our studies have also shown that caspase-8 inhibition is detrimental to long term graft survival, apoptosis inhibition by SPI-6 expression can still be a viable therapeutic option through prevention of granzyme-mediated apoptosis in renal parenchymal cells. In our next study, we will upregulate SPI-6 expression in the donor allograft to enhance long term allograft survival. Although wild type grafts survived significantly longer than SPI-$6^{-/-}$ grafts, only 35% achieved long term survival. While SPI-6 is highly expressed in renal TEC, our study has demonstrated that it can be further upregulated as seen with high doses of IFNγ. We hypothesize that enhancing renal specific expression of SPI-6 can be accomplished through the use of genistein in perfusion solution pre-transplant which will promote long term allograft survival. In addition, further studies will be conducted to determine the effects of SPI-6 resistance towards other cytotoxic cells relevant to transplant rejection such as NK cells and $CD4^+$ T cells. Our SPI-6 study has also indicated that although expression of SPI-6 can protect against $CD8^+$ cytotoxicity, TEC death can still occur through other mechanisms. Parallel studies involving cytotoxicity assay and mouse transplants involving inhibitors of other cytotoxicity pathways including Fas-Fas-L interactions in combination with SPI-6 upregulation will also be conducted. Lastly, even in with expression of SPI-6, as our model of transplantation does not involve the use of immunosuppression, we were unable to prevent the infiltration of immune cells into the graft which may have effects on allograft survival. Future studies will examine the effects of immunosuppression in combination with enhanced resistance to cytotoxicity on graft function and survival. By preventing both the proliferation and function of CTL in the renal allograft, it may have a synergistic effect on enhancing long term allograft survival.

Alternative inhibition of necroptosis during renal allograft injury

Emerging research on new programmed cell death pathways and their regulators has led to the characterization of these pathways in different disease models including transplantation rejection. Our studies have demonstrated that necroptosis plays an important role in promoting kidney injury and decreasing allograft survival however the mechanism and

regulation of this pathway may require greater elucidation[22]. In particular, the roles of caspase-8 and RIPK1 in regulating different programmed cell death pathways are still not clear cut as their complex interactions with many death pathway molecules and complexes can result in opposing outcomes depending on which pathways are activated. Many studies, including our own, have demonstrated that RIPK1 inhibition leads to inhibition of TNFα-mediated necroptosis. Conversely, if necroptosis is activated by IFNγ signaling, RIPK1 inhibition promotes necroptosis which makes RIPK1 a difficult target for blocking necroptosis due to complex TNFα and IFNγ signaling during inflammation[123,140]. In addition, caspase-8 is not only responsible for regulation of the 'necrosome' but has been found to regulate pathways involved in other forms of programmed necrosis such as pyroptosis[141] and other programmed cell death pathways including autophagy[121]. Therefore, despite inhibition of necroptosis, blocking caspase-8 and RIPK3 activity may enhance alternate forms of programmed cell death and still inevitably lead to graft dysfunction and loss. It will be crucial to achieve a greater understanding of these key molecules in cell death and their roles in the complex signaling pathways occurring during inflammatory injury and rejection in order to more specifically promote allograft survival.

As the role of RIPK1 regulation of different necroptosis pathways remains to be clarified, future studies will focus on the inhibition of downstream molecules, RIPK3 and MLKL, to more specifically block necroptotic death in renal allografts. Although there are no RIPK3 inhibitors currently available commercially, the viral peptide M45 produced by cytomegalovirus (CMV) is known to inhibit RIPK3 and necroptosis[70] and could potentially be used as a basis for development of a small molecule inhibitor similar to Nec-1. Our future studies will also focus on the use of MLKL inhibition as a method of blocking necroptosis in kidney inflammatory injury. MLKL is a target of RIPK3 phosphorylation and is necessary for receptor mediated necroptosis[75]. It is currently unclear how MLKL induces a necrotic phenotype but it has been suggested that MLKL moves to the plasma membrane of cells and forms pores resulting in the influx of Ca^{2+} leading to cell lysis[73]. Necrosulfonamide, an inhibitor of human MLKL[75], will be used for *in vitro* experiments to determine its efficacy in blocking receptor-mediated necroptosis in human renal TEC. These studies will be mirrored *in vivo* using our murine renal transplantation model through the use of $MLKL^{-/-}$ donor grafts to determine effects on long term allograft survival. As previously mentioned, our

preliminary studies in blocking both apoptosis and necroptosis through the use caspase-8$^{-/-}$/RIPK3$^{-/-}$ mice have shown that the benefit of RIPK3 inhibition is lost during renal IRI and kidney transplantation. Through the characterization of other programmed cell death pathways such as pyroptosis and autophagy *in vitro* via phenotypic changes and use of inhibitors, we will determine whether they are being non-specifically activated due to loss of caspase-8 and/or RIPK3 under pro-inflammatory conditions. It will be important to identify any potential non-specific activation of other death pathways as any translational studies involving regulation of cell death pathways must take into account unintended programmed cell death that may lead to graft dysfunction. Our observations in these experiments will not only expand our knowledge of the mechanisms that regulate necroptotic death but can potentially provide more targets for therapeutics against the mosaic of cell death that occurs during transplant injury.

Although we have demonstrated that renal TEC and other parenchyma are sensitive to necroptosis, other studies have also demonstrated that T cells can also undergo necroptosis under caspase-8 inhibition[124]. Further studies will be required to clarify how blocking necroptosis affects the adaptive immune system as systemic inhibition of necroptosis in the recipient would have effects on B and T cells as well any resident immune cells in the donor graft. Studies in T cell death pathways have revealed that loss of both caspase-8 and RIPK3 leads to progression of lymphoproliferative disease and accumulation of $CD3^{+}CD4^{-}CD8^{-}$ T cells[66]. In addition, RIPK1 inhibition by Nec-1 in T cells has been found to not only prevent necroptosis but also interferes with TCR signal transduction pathways preventing proliferation[63]. Our future studies will characterize isolated lymphocytes, including B, T, and NK cells, from both RIPK3$^{-/-}$ and caspase-8$^{-/-}$/RIPK3$^{-/-}$ mice to determine if inhibition of apoptosis and/or necroptosis results in activation or proliferation under pro-inflammatory conditions *in vitro*. Their cytotoxic function will also be tested against allogeneic lymphocytes and renal TEC to determine if blocking cell death pathways affects their ability to kill targeted cells *in vitro*. Additionally, to test effects of cell death inhibition on recipient alloimmune responses that propagate inflammatory injury *in vivo*, murine renal transplants using wild type allografts into RIPK3$^{-/-}$ or caspase-8$^{-/-}$/RIPK3$^{-/-}$ recipients will be assessed for graft function and survival. These studies will provide greater insight into the effects of

necroptosis inhibition on adaptive immunity before being applied to a clinical transplantation setting.

Post-translational modifications of HMGB1 and its effects on alloimmune responses

Our study on the propagation of inflammation during renal IRI by HMGB1 released from necrotic parenchymal cells, including TEC, suggests that HMGB1 could also have a similar effect in transplant injury. The use of GZA as a small molecule inhibitor of extracellular HMGB1 both *in vitro* and *in vivo* was effective in the reduction of inflammation and injury after renal IRI. Future studies would include the use of GZA as a therapeutic during murine renal transplantation either as a component of perfusion solution during cold preservation and/or as a form of immunosuppression in the recipient post-transplant. We expect that the benefits that were observed in our model of AKI to translate into renal transplantation as we have demonstrated that HMGB1 is upregulated in renal allografts post-transplantation and may contribute to chronic inflammation and rejection. Inhibition of HMGB1 may aid with ameliorating rejection and tissue injury as infiltrating immune cells were prominent in the allograft. In addition, use of GZA may provide a more feasible translational application of HMGB1 inhibition as use of anti-HMGB1 antibodies is not readily available commercially and as an antibody, is inherently difficult to use therapeutically as they may not specifically localize in the target tissue. Further investigation will also be conducted on the effects of HMGB1 inhibition on other immune cell types relevant to transplantation including B and T cells as they also express TLR2/4 and thus may be susceptible to HMGB1 activation. This would also include functional assays that will determine the effects of HMGB1 inhibition on their ability to recognize allo-antigen, induce cytotoxicity, and/or induce pro-inflammatory responses. Further understanding of the role of HMGB1 in a transplant setting may lead to the development of a new class of therapeutics that target DAMPs as well as reveal connections between innate and adaptive immune pathways during rejection. As HMGB1 is consistently present during IRI and persists in the allograft, HMGB1 along with other DAMPs may have a role in the development of chronic rejection due to their ability to perpetuate inflammation.

We have observed that the inhibition of extracellular HMGB1 after release from necrosis by GZA attenuated cell death in the surrounding TEC *in vitro* and *in vivo*. HMGB1 and TLR

mediated death has also been observed in other studies[142] and it has also been demonstrated that TRIF, an adaptor molecule of TLR, contains a RHIM motif that would allow for interaction with RIPK3, potentially allowing for TLR-induced necroptosis[131]. Future studies would elucidate any potential mechanisms behind HMGB1-mediated necroptosis in TEC and determine if blocking by GZA, TLR inhibitors, or necroptosis inhibitors can block this pathway. As we have shown that extracellular HMGB1 persists in the renal allograft and may be responsible for driving prolonged intra-graft inflammation leading to decreased allograft survival, inhibition of HMGB1-mediated cell death may provide a way to break the cycle of cell death derived inflammatory injury. Interestingly, studies in the effects of cell death on post-translational modification of HMGB1 have indicated that HMGB1 resulting from ROS produced during apoptosis death were unable to activate dendritic cells suggesting an anti-inflammatory effect[92]. Further studies are required to delineate the forms of HMGB1 that are present during allograft rejection to determine whether a greater proportion of immunostimulatory HMGB1 is present in grafts undergoing rejection and whether blocking necroptosis can modify the HMGB1 profile to reduce inflammation. Elucidation of differential effects of reduced and oxidized forms of HMGB1 on renal TEC *in vitro* would provide insight into how tissue injury may regulate inflammation through innate immunity. Additionally, as oxidation of extracellular HMGB1 reduces its immunostimulatory function, translational studies using oxidized HMGB1 or anti-HMGB1 antibodies targeted against its reduced form specifically as a form of immunosuppression would be instructive on its potential as a therapeutic. Greater understanding of HMGB1 participation in the context of cell death and inflammation during renal transplantation could provide insights into the mechanisms of chronic rejection and transplant injury.

References

1. Canadian Organ Replacement Register. Canadian Organ Replacement Register Annual Report: Treatment of End-Stage Organ Failure in Canada, 2003 to 2012. (2013).

2. Matas, A. J. *et al.* 2202 kidney transplant recipients with 10 years of graft function: what happens next? *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* **8,** 2410–2419 (2008).

3. Mannon, R. B. *et al.* Altered intragraft immune responses and improved renal function in MHC class II-deficient mouse kidney allografts. *Transplantation* **69,** 2137–2143 (2000).

4. Sayegh, M. H. & Krensky, A. M. Novel immunotherapeutic strategies using MHC derived peptides. *Kidney Int. Suppl.* **53,** S13–20 (1996).

5. Shoskes, D. A. & Halloran, P. F. Delayed graft function in renal transplantation: etiology, management and long-term significance. *J. Urol.* **155,** 1831–1840 (1996).

6. Kouwenhoven, E. A., de Bruin, R. W., Bajema, I. M., Marquet, R. L. & Ijzermans, J. N. Cold ischemia augments allogeneic-mediated injury in rat kidney allografts. *Kidney Int.* **59,** 1142–1148 (2001).

7. Zhang, Z.-X. *et al.* NK cells induce apoptosis in tubular epithelial cells and contribute to renal ischemia-reperfusion injury. *J. Immunol. Baltim. Md 1950* **181,** 7489–7498 (2008).

8. Du, C. *et al.* Renal tubular epithelial cell apoptosis by Fas-FasL-dependent self-injury can augment renal allograft injury. *Transplant. Proc.* **35,** 2481–2482 (2003).

9. Du, C. *et al.* Increasing resistance of tubular epithelial cells to apoptosis by shRNA therapy ameliorates renal ischemia-reperfusion injury. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* **6,** 2256–2267 (2006).

10. Mohib, K. *et al.* Indoleamine 2,3-dioxygenase expression promotes renal ischemia-reperfusion injury. *Am. J. Physiol. Renal Physiol.* **295,** F226–234 (2008).

11. Bonventre, J. V. Dedifferentiation and proliferation of surviving epithelial cells in acute renal failure. *J. Am. Soc. Nephrol. JASN* **14 Suppl 1,** S55–61 (2003).

12. Jang, H. R. & Rabb, H. The innate immune response in ischemic acute kidney injury. *Clin. Immunol. Orlando Fla* **130,** 41–50 (2009).

13. Jang, H. R., Ko, G. J., Wasowska, B. A. & Rabb, H. The interaction between ischemia-reperfusion and immune responses in the kidney. *J. Mol. Med. Berl. Ger.* **87,** 859–864 (2009).

14. Boros, P. & Bromberg, J. S. New cellular and molecular immune pathways in ischemia/reperfusion injury. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* **6,** 652–658 (2006).

15. Hernandez, L. A. *et al.* Role of neutrophils in ischemia-reperfusion-induced microvascular injury. *Am. J. Physiol.* **253,** H699–703 (1987).

16. Wu, H. *et al.* TLR4 activation mediates kidney ischemia/reperfusion injury. *J. Clin. Invest.* **117,** 2847–2859 (2007).

17. Tsuboi, N. *et al.* Roles of Toll-Like Receptors in C-C Chemokine Production by Renal Tubular Epithelial Cells. *J Immunol* **169,** 2026–2033 (2002).

18. Prodjosudjadi, W. *et al.* Production and cytokine-mediated regulation of monocyte chemoattractant protein-1 by human proximal tubular epithelial cells. *Kidney Int.* **48,** 1477–1486 (1995).

19. Clayberger, C. Cytolytic molecules in rejection. *Curr. Opin. Organ Transplant.* **14,** 30–33 (2009).

20. Grimbert, P. *et al.* The regulatory/cytotoxic graft-infiltrating T cells differentiate renal allograft borderline change from acute rejection. *Transplantation* **83,** 341–346 (2007).

21. Linkermann, A. *et al.* Renal tubular Fas ligand mediates fratricide in cisplatin-induced acute kidney failure. *Kidney Int.* **79,** 169–178 (2011).

22. Lau, A. *et al.* RIPK3-mediated necroptosis promotes donor kidney inflammatory injury and reduces allograft survival. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* **13,** 2805–2818 (2013).

23. Mohib, K., Guan, Q., Diao, H., Du, C. & Jevnikar, A. M. Proapoptotic activity of indoleamine 2,3-dioxygenase expressed in renal tubular epithelial cells. *Am. J. Physiol. Renal Physiol.* **293,** F801–812 (2007).

24. Misseri, R. *et al.* TNF-alpha mediates obstruction-induced renal tubular cell apoptosis and proapoptotic signaling. *Am. J. Physiol. Renal Physiol.* **288,** F406–411 (2005).

25. Glynne & Evans. Inflammatory cytokines induce apoptotic and necrotic cell shedding from human proximal tubular epithelial cell monolayers. *Kidney Int.* **55,** 2573–2597 (1999).

26. Linkermann, A., De Zen, F., Weinberg, J., Kunzendorf, U. & Krautwald, S. Programmed necrosis in acute kidney injury. *Nephrol. Dial. Transplant. Off. Publ. Eur. Dial. Transpl. Assoc. - Eur. Ren. Assoc.* **27,** 3412–3419 (2012).

27. Linkermann, A. *et al.* Necroptosis in immunity and ischemia-reperfusion injury. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* **13,** 2797–2804 (2013).

28. Moers, C. *et al.* Machine perfusion or cold storage in deceased-donor kidney transplantation. *N. Engl. J. Med.* **360,** 7–19 (2009).

29. Kosieradzki, M. & Rowiński, W. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. *Transplant. Proc.* **40,** 3279–3288 (2008).

30. Mühlbacher, F. Preservation solutions for transplantation. *Transplant. Proc.* **31,** 2069–2070

31. Coulson, M. T., Jablonski, P., Howden, B. O., Thomson, N. M. & Stein, A. N. Beyond operational tolerance: effect of ischemic injury on development of chronic damage in renal grafts. *Transplantation* **80,** 353–361 (2005).

32. Chapman, J. R., O'Connell, P. J. & Nankivell, B. J. Chronic renal allograft dysfunction. *J. Am. Soc. Nephrol. JASN* **16,** 3015–3026 (2005).

33. Hariharan, S., McBride, M. A. & Cohen, E. P. Evolution of endpoints for renal transplant outcome. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* **3,** 933–941 (2003).

34. Joosten, S. A., Sijpkens, Y. W. J., Van Kooten, C. & Paul, L. C. Chronic renal allograft rejection: Pathophysiologic considerations. *Kidney Int.* **68,** 1–13 (2005).

35. Cornell, L. D., Smith, R. N. & Colvin, R. B. Kidney transplantation: mechanisms of rejection and acceptance. *Annu. Rev. Pathol.* **3,** 189–220 (2008).

36. Hawksworth, J. S. *et al.* New directions for induction immunosuppression strategy in solid organ transplantation. *Am. J. Surg.* **197,** 515–524 (2009).

37. Colvin, R. B. *et al.* Evaluation of pathologic criteria for acute renal allograft rejection: reproducibility, sensitivity, and clinical correlation. *J. Am. Soc. Nephrol. JASN* **8,** 1930–1941 (1997).

38. Porter, C. J. 2, Ronan, J. E. & Cassidy, M. J. D. Fas-Fas-Ligand Antigen Expression and Its Relationship to Increased Apoptosis in Acute Renal Transplant Rejection1. *Transplant. March 27 2000* **69,** 1091–1094 (2000).

39. Zeisberg, M. & Kalluri, R. The role of epithelial-to-mesenchymal transition in renal fibrosis. *J. Mol. Med. Berl. Ger.* **82,** 175–181 (2004).

40. Galluzzi, L. *et al.* Programmed necrosis from molecules to health and disease. *Int. Rev. Cell Mol. Biol.* **289,** 1–35 (2011).

41. Vanlangenakker, N., Vanden Berghe, T. & Vandenabeele, P. Many stimuli pull the necrotic trigger, an overview. *Cell Death Differ.* **19,** 75–86 (2012).

42. Dixon, S. J. *et al.* Ferroptosis: An Iron-Dependent Form of Nonapoptotic Cell Death. *Cell* **149,** 1060–1072 (2012).

43. Kang, Y., Tiziani, S., Park, G., Kaul, M. & Paternostro, G. Cellular protection using Flt3 and PI3Kα inhibitors demonstrates multiple mechanisms of oxidative glutamate toxicity. *Nat. Commun.* **5,** (2014).

44. Di Paola, R. *et al.* The renal injury and inflammation caused by ischemia-reperfusion are reduced by genetic inhibition of TNF-αR1: a comparison with infliximab treatment. *Eur. J. Pharmacol.* **700,** 134–146 (2013).

45. Al-Lamki, R. S. *et al.* Expression of tumor necrosis factor receptors in normal kidney and rejecting renal transplants. *Lab. Investig. J. Tech. Methods Pathol.* **81,** 1503–1515 (2001).

46. Carstens, J., Markussen, N. & Madsen, M. The granule exocytosis and Fas/FasLigand pathways at the time of transplantation and during borderline and acute rejection of human renal allografts. *Transplant. Proc.* **37,** 3294–3297 (2005).

47. Linkermann, A. *et al.* Rip1 (Receptor-interacting protein kinase 1) mediates necroptosis and contributes to renal ischemia/reperfusion injury. *Kidney Int.* **81,** 751–761 (2012).

48. Linkermann, A. *et al.* Regulated Cell Death in AKI. *J. Am. Soc. Nephrol. JASN* (2014). doi:10.1681/ASN.2014030262

49. Laforge, M. *et al.* Apoptotic death concurrent with CD3 stimulation in primary human CD8+ T lymphocytes: a role for endogenous granzyme B. *J. Immunol. Baltim. Md 1950* **176,** 3966–3977 (2006).

50. Hirst, C. E. *et al.* The intracellular granzyme B inhibitor, proteinase inhibitor 9, is up-regulated during accessory cell maturation and effector cell degranulation, and its overexpression enhances CTL potency. *J. Immunol. Baltim. Md 1950* **170,** 805–815 (2003).

51. Bladergroen, B. A. *et al.* The granzyme B inhibitor, protease inhibitor 9, is mainly expressed by dendritic cells and at immune-privileged sites. *J. Immunol. Baltim. Md 1950* **166,** 3218–3225 (2001).

52. Zhang, M. *et al.* Serine protease inhibitor 6 protects cytotoxic T cells from self-inflicted injury by ensuring the integrity of cytotoxic granules. *Immunity* **24,** 451–461 (2006).

53. Sun, J. *et al.* Importance of the P4' residue in human granzyme B inhibitors and substrates revealed by scanning mutagenesis of the proteinase inhibitor 9 reactive center loop. *J. Biol. Chem.* **276,** 15177–15184 (2001).

54. Rowshani, A. T. *et al.* Hyperexpression of the granzyme B inhibitor PI-9 in human renal allografts: a potential mechanism for stable renal function in patients with subclinical rejection. *Kidney Int.* **66,** 1417–1422 (2004).

55. Muthukumar, T. *et al.* Serine proteinase inhibitor-9, an endogenous blocker of granzyme B/perforin lytic pathway, is hyperexpressed during acute rejection of renal allografts. *Transplantation* **75,** 1565–1570 (2003).

56. Schmitz, I., Kirchhoff, S. & Krammer, P. H. Regulation of death receptor-mediated apoptosis pathways. *Int. J. Biochem. Cell Biol.* **32,** 1123–1136 (2000).

57. Justo, P., Lorz, C., Sanz, A., Egido, J. & Ortiz, A. Intracellular mechanisms of cyclosporin A-induced tubular cell apoptosis. *J. Am. Soc. Nephrol. JASN* **14,** 3072–3080 (2003).

58. Green, D. R., Oberst, A., Dillon, C. P., Weinlich, R. & Salvesen, G. S. RIPK-dependent necrosis and its regulation by caspases: a mystery in five acts. *Mol. Cell* **44,** 9–16 (2011).

59. Varfolomeev, E. E. *et al.* Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity* **9,** 267–276 (1998).

60. Elstein, K. H. & Zucker, R. M. Comparison of cellular and nuclear flow cytometric techniques for discriminating apoptotic subpopulations. *Exp. Cell Res.* **211,** 322–331 (1994).

61. Zhang, X. *et al.* Prevention of renal ischemic injury by silencing the expression of renal caspase 3 and caspase 8. *Transplantation* **82,** 1728–1732 (2006).

62. Zheng, X. *et al.* Protection of renal ischemia injury using combination gene silencing of complement 3 and caspase 3 genes. *Transplantation* **82,** 1781–1786 (2006).

63. Cho, Y., McQuade, T., Zhang, H., Zhang, J. & Chan, F. K.-M. RIP1-Dependent and Independent Effects of Necrostatin-1 in Necrosis and T Cell Activation. *PLoS ONE* **6,** e23209 (2011).

64. Lau, A. *et al.* Serine Protease Inhibitor-6 Inhibits Granzyme B-Mediated Injury of Renal Tubular Cells and Promotes Renal Allograft Survival. *Transplantation* (2014). doi:10.1097/TP.0000000000000237

65. Noronha, I. L. *et al.* Apoptosis in Kidney and Pancreas Allograft Biopsies. *Transplant. May 15 2005* **79,** 1231–1235 (2005).

66. Oberst, A. *et al.* Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature* **471,** 363–367 (2011).

67. Günther, C. *et al.* Caspase-8 regulates TNF-α-induced epithelial necroptosis and terminal ileitis. *Nature* **477,** 335–339 (2011).

68. Declercq, W., Vanden Berghe, T. & Vandenabeele, P. RIP kinases at the crossroads of cell death and survival. *Cell* **138,** 229–232 (2009).

69. Zhang, D.-W. *et al.* Multiple death pathways in TNF-treated fibroblasts: RIP3- and RIP1-dependent and independent routes. *Cell Res.* **21,** 368–371 (2011).

70. Rebsamen, M. *et al.* DAI/ZBP1 recruits RIP1 and RIP3 through RIP homotypic interaction motifs to activate NF-kappaB. *EMBO Rep.* **10,** 916–922 (2009).

71. Kaiser, W. J. *et al.* RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature* **471,** 368–372 (2011).

72. Vanden Berghe, T. *et al.* Determination of apoptotic and necrotic cell death in vitro and in vivo. *Methods San Diego Calif* (2013). doi:10.1016/j.ymeth.2013.02.011

73. Cai, Z. *et al.* Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. *Nat. Cell Biol.* **16,** 55–65 (2014).

74. Green, D. R. Pseudokiller, qu'est-ce que c'est? *Immunity* **39,** 421–422 (2013).

75. Sun, L. *et al.* Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* **148,** 213–227 (2012).

76. Cho, Y. S. *et al.* Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* **137,** 1112–1123 (2009).

77. Mocarski, E. S., Kaiser, W. J., Livingston-Rosanoff, D., Upton, J. W. & Daley-Bauer, L. P. True grit: programmed necrosis in antiviral host defense, inflammation, and immunogenicity. *J. Immunol. Baltim. Md 1950* **192,** 2019–2026 (2014).

78. Kaczmarek, A., Vandenabeele, P. & Krysko, D. V. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity* **38,** 209–223 (2013).

79. Upton, J. W., Kaiser, W. J. & Mocarski, E. S. Virus inhibition of RIP3-dependent necrosis. *Cell Host Microbe* **7,** 302–313 (2010).

80. Chan, F. K.-M. Fueling the flames: Mammalian programmed necrosis in inflammatory diseases. *Cold Spring Harb. Perspect. Biol.* **4,** (2012).

81. He, S. *et al.* Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell* **137,** 1100–1111 (2009).

82. Takahashi, N. *et al.* Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models. *Cell Death Dis.* **3,** e437 (2012).

83. Linkermann, A. *et al.* Dichotomy between RIP1- and RIP3-mediated necroptosis in tumor necrosis factor-α-induced shock. *Mol. Med. Camb. Mass* **18,** 577–586 (2012).

84. Welz, P.-S. *et al.* FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation. *Nature* **477,** 330–334 (2011).

85. Makarov, R., Geserick, P., Feoktistova, M. & Leverkus, M. in *Mol. Dermatol.* (eds. Has, C. & Sitaru, C.) 201–218 (Humana Press, 2013). at <http://link.springer.com.proxy1.lib.uwo.ca/protocol/10.1007/978-1-62703-227-8_12>

86. Scaffidi, P., Misteli, T. & Bianchi, M. E. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* **418,** 191–195 (2002).

87. Degterev, A. *et al.* Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat. Chem. Biol.* **4,** 313–321 (2008).

88. Edwards, J. R., Diamantakos, E. A., Peuler, J. D., Lamar, P. C. & Prozialeck, W. C. A novel method for the evaluation of proximal tubule epithelial cellular necrosis in the intact rat kidney using ethidium homodimer. *BMC Physiol.* **7,** 1 (2007).

89. Matzinger, P. The danger model: a renewed sense of self. *Science* **296,** 301–305 (2002).

90. Pisetsky, D. Cell death in the pathogenesis of immune-mediated diseases: the role of HMGB1 and DAMP-PAMP complexes. *Swiss Med. Wkly.* **141,** w13256 (2011).

91. Challa, S. & Chan, F. K.-M. Going up in flames: necrotic cell injury and inflammatory diseases. *Cell. Mol. Life Sci. CMLS* **67,** 3241–3253 (2010).

92. Kazama, H. *et al.* Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity* **29,** 21–32 (2008).

93. Czura, C. J., Wang, H. & Tracey, K. J. Dual roles for HMGB1: DNA binding and cytokine. *J. Endotoxin Res.* **7,** 315–321 (2001).

94. Ugrinova, I., Pashev, I. G. & Pasheva, E. A. Nucleosome binding properties and Co-remodeling activities of native and in vivo acetylated HMGB-1 and HMGB-2 proteins. *Biochemistry (Mosc.)* **48,** 6502–6507 (2009).

95. Pisetsky, D. S. & Jiang, W. Role of Toll-like receptors in HMGB1 release from macrophages. *Ann. N. Y. Acad. Sci.* **1109,** 58–65 (2007).

96. Dumitriu, I. E. *et al.* Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products. *J. Immunol. Baltim. Md 1950* **174,** 7506–7515 (2005).

97. Lotze, M. T. & Tracey, K. J. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat. Rev. Immunol.* **5,** 331–342 (2005).

98. Van Beijnum, J. R., Buurman, W. A. & Griffioen, A. W. Convergence and amplification of toll-like receptor (TLR) and receptor for advanced glycation end products (RAGE) signaling pathways via high mobility group B1 (HMGB1). *Angiogenesis* **11,** 91–99 (2008).

99. Yu, M. *et al.* HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock Augusta Ga* **26,** 174–179 (2006).

100. Tian, J. *et al.* Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat. Immunol.* **8,** 487–496 (2007).

101. Wu, H. *et al.* HMGB1 contributes to kidney ischemia reperfusion injury. *J. Am. Soc. Nephrol. JASN* **21,** 1878–1890 (2010).

102. Quintana, F. J. & Cohen, I. R. Heat shock proteins as endogenous adjuvants in sterile and septic inflammation. *J. Immunol. Baltim. Md 1950* **175,** 2777–2782 (2005).

103. Ohashi, K., Burkart, V., Flohe, S. & Kolb, H. Cutting Edge: Heat Shock Protein 60 Is a Putative Endogenous Ligand of the Toll-Like Receptor-4 Complex. *J Immunol* **164,** 558–561 (2000).

104. Elsner, L. *et al.* The endogenous danger signals HSP70 and MICA cooperate in the activation of cytotoxic effector functions of NK cells. *J. Cell. Mol. Med.* (2009). doi:10.1111/j.1582-4934.2008.00677.x

105. Basu, S., Binder, R. J., Ramalingam, T. & Srivastava, P. K. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity* **14,** 303–313 (2001).

106. Chen, G. Y. & Nuñez, G. Sterile inflammation: sensing and reacting to damage. *Nat. Rev. Immunol.* **10,** 826–837 (2010).

107. Zhou, Y. *et al.* Uric Acid Induces Renal Inflammation via Activating Tubular NF-κB Signaling Pathway. *PLoS ONE* **7,** e39738 (2012).

108. Mariathasan, S. *et al.* Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* **440,** 228–232 (2006).

109. Du, C., Guan, Q., Diao, H., Yin, Z. & Jevnikar, A. M. Nitric oxide induces apoptosis in renal tubular epithelial cells through activation of caspase-8. *Am. J. Physiol. Renal Physiol.* **290,** F1044–1054 (2006).

110. Du, C., Guan, Q., Yin, Z., Zhong, R. & Jevnikar, A. M. IL-2-mediated apoptosis of kidney tubular epithelial cells is regulated by the caspase-8 inhibitor c-FLIP. *Kidney Int.* **67,** 1397–1409 (2005).

111. Mollica, L. *et al.* Glycyrrhizin binds to high-mobility group box 1 protein and inhibits its cytokine activities. *Chem. Biol.* **14,** 431–441 (2007).

112. Halloran, P. F. *et al.* Lesions of T-cell-mediated kidney allograft rejection in mice do not require perforin or granzymes A and B. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* **4,** 705–712 (2004).

113. Daemen, M. A., van de Ven, M. W., Heineman, E. & Buurman, W. A. Involvement of endogenous interleukin-10 and tumor necrosis factor-alpha in renal ischemia-reperfusion injury. *Transplantation* **67,** 792–800 (1999).

114. Wang, S. *et al.* Reduction of Foxp3-expressing regulatory T cell infiltrates during the progression of renal allograft rejection in a mouse model. *Transpl. Immunol.* **19,** 93–102 (2008).

115. Jiang, X. *et al.* Low concentrations of the soy phytoestrogen genistein induce proteinase inhibitor 9 and block killing of breast cancer cells by immune cells. *Endocrinology* **149,** 5366–5373 (2008).

116. Sirois, I. *et al.* Caspase-3-dependent export of TCTP: a novel pathway for antiapoptotic intercellular communication. *Cell Death Differ.* **18,** 549–562 (2011).

117. Soulez, M. *et al.* Epidermal growth factor and perlecan fragments produced by apoptotic endothelial cells co-ordinately activate ERK1/2-dependent antiapoptotic pathways in mesenchymal stem cells. *Stem Cells Dayt. Ohio* **28,** 810–820 (2010).

118. Brissette, M.-J. *et al.* MFG-E8 released by apoptotic endothelial cells triggers anti-inflammatory macrophage reprogramming. *PloS One* **7,** e36368 (2012).

119. Cailhier, J.-F. *et al.* Caspase-3 activation triggers extracellular cathepsin L release and endorepellin proteolysis. *J. Biol. Chem.* **283,** 27220–27229 (2008).

120. Kaiser, W. J., Upton, J. W. & Mocarski, E. S. Viral modulation of programmed necrosis. *Curr. Opin. Virol.* **3,** 296–306 (2013).

121. Ryter, S. W., Mizumura, K. & Choi, A. M. K. The impact of autophagy on cell death modalities. *Int. J. Cell Biol.* **2014,** 502676 (2014).

122. Remijsen, Q. *et al.* Depletion of RIPK3 or MLKL blocks TNF-driven necroptosis and switches towards a delayed RIPK1 kinase-dependent apoptosis. *Cell Death Dis.* **5,** e1004 (2014).

123. Dillon, C. P. *et al.* RIPK1 blocks early postnatal lethality mediated by caspase-8 and RIPK3. *Cell* **157,** 1189–1202 (2014).

124. Ch'en, I. L., Tsau, J. S., Molkentin, J. D., Komatsu, M. & Hedrick, S. M. Mechanisms of necroptosis in T cells. *J. Exp. Med.* **208,** 633–641 (2011).

125. Turkmen, K. *et al.* Apoptosis and autophagy in cold preservation ischemia. *Transplantation* **91,** 1192–1197 (2011).

126. Cinà, D. P. *et al.* MTOR regulates autophagic flux in the glomerulus. *Autophagy* **8,** 696–698 (2012).

127. Kikuchi, M. *et al.* Protease activity of procaspase-8 is essential for cell survival by inhibiting both apoptotic and nonapoptotic cell death dependent on receptor-interacting protein kinase 1 (RIP1) and RIP3. *J. Biol. Chem.* **287,** 41165–41173 (2012).

128. Leemans, J. C., Kors, L., Anders, H.-J. & Florquin, S. Pattern recognition receptors and the inflammasome in kidney disease. *Nat. Rev. Nephrol.* **10,** 398–414 (2014).

129. Vilaysane, A. *et al.* The NLRP3 inflammasome promotes renal inflammation and contributes to CKD. *J. Am. Soc. Nephrol. JASN* **21,** 1732–1744 (2010).

130. Andrade-Oliveira, V. *et al.* TLR4 mRNA levels as tools to estimate risk for early posttransplantation kidney graft dysfunction. *Transplantation* **94,** 589–595 (2012).

131. Kaiser, W. J. *et al.* Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL. *J. Biol. Chem.* **288,** 31268–31279 (2013).

132. Shigeoka, A. A. *et al.* TLR2 is constitutively expressed within the kidney and participates in ischemic renal injury through both MyD88-dependent and -independent pathways. *J. Immunol. Baltim. Md 1950* **178,** 6252–6258 (2007).

133. Krüger, B. *et al.* Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 3390–3395 (2009).

134. Andrade, C. F., Waddell, T. K., Keshavjee, S. & Liu, M. Innate immunity and organ transplantation: the potential role of toll-like receptors. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* **5,** 969–975 (2005).

135. Anders, H.-J. & Schaefer, L. Beyond Tissue Injury—Damage-Associated Molecular Patterns, Toll-Like Receptors, and Inflammasomes Also Drive Regeneration and Fibrosis. *J. Am. Soc. Nephrol.* **25,** 1387–1400 (2014).

136. Théry, C., Ostrowski, M. & Segura, E. Membrane vesicles as conveyors of immune responses. *Nat. Rev. Immunol.* **9,** 581–593 (2009).

137. Fleissner, F., Goerzig, Y., Haverich, A. & Thum, T. Microvesicles as Novel Biomarkers and Therapeutic Targets in Transplantation Medicine. *Am. J. Transplant.* **12,** 289–297 (2012).

138. Zheng, X. *et al.* Novel small interfering RNA-containing solution protecting donor organs in heart transplantation. *Circulation* **120,** 1099–1107, 1 p following 1107 (2009).

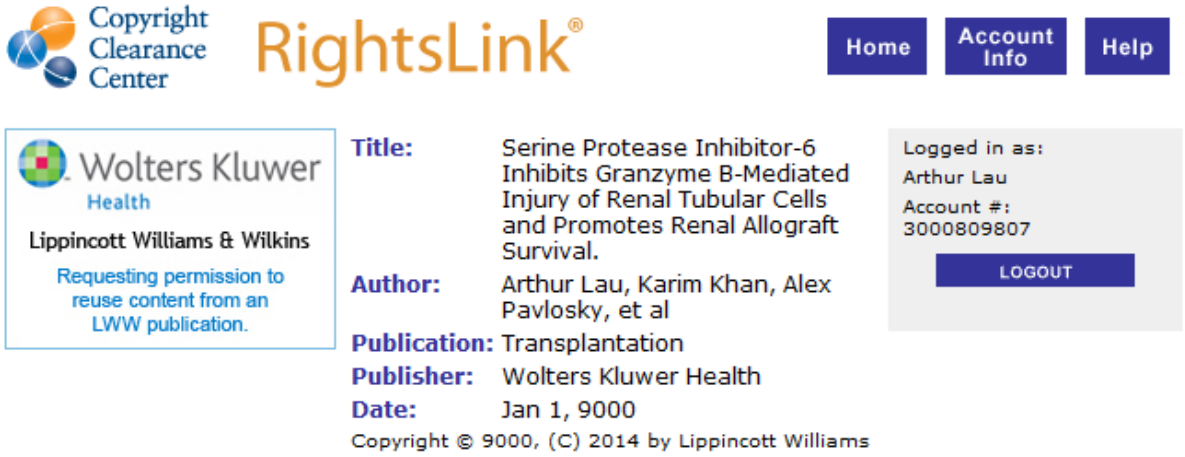
139. Pavlosky, A. *et al.* RIPK3-Mediated Necroptosis Regulates Cardiac Allograft Rejection. *Am. J. Transplant.* n/a–n/a (2014). doi:10.1111/ajt.12779

140. Kaiser, W. J. *et al.* RIP1 suppresses innate immune necrotic as well as apoptotic cell death during mammalian parturition. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 7753–7758 (2014).

141. Philip, N. H. *et al.* Caspase-8 mediates caspase-1 processing and innate immune defense in response to bacterial blockade of NF-κB and MAPK signaling. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 7385–7390 (2014).

142. Gdynia, G. *et al.* Danger signaling protein HMGB1 induces a distinct form of cell death accompanied by formation of giant mitochondria. *Cancer Res.* **70,** 8558–8568 (2010).

Appendix A – Copyright Release



Copyright Clearance Center RightsLink®

Home | Account Info | Help

Wolters Kluwer Health

Lippincott Williams & Wilkins

Requesting permission to reuse content from an LWW publication.

Title: Serine Protease Inhibitor-6 Inhibits Granzyme B-Mediated Injury of Renal Tubular Cells and Promotes Renal Allograft Survival.

Author: Arthur Lau, Karim Khan, Alex Pavlosky, et al

Publication: Transplantation

Publisher: Wolters Kluwer Health

Date: Jan 1, 9000

Copyright © 9000, (C) 2014 by Lippincott Williams

Logged in as:
Arthur Lau
Account #:
3000809807

LOGOUT

This reuse is free of charge. No permission letter is needed from Wolters Kluwer Health, Lippincott Williams & Wilkins. We require that all authors always include a full acknowledgement. Example: AIDS: 13 November 2013 - Volume 27 - Issue 17 - p 2679-2689. Wolters Kluwer Health Lippincott Williams & Wilkins© No modifications will be permitted.

BACK | CLOSE WINDOW

Copyright © 2014 Copyright Clearance Center, Inc. All Rights Reserved. Privacy statement.
Comments? We would like to hear from you. E-mail us at customercare@copyright.com

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Jul 09, 2014

This is a License Agreement between Arthur Lau ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3424891341068
License date	Jul 09, 2014
Licensed content publisher	John Wiley and Sons
Licensed content publication	American Journal of Transplantation
Licensed content title	RIPK3-Mediated Necroptosis Promotes Donor Kidney Inflammatory Injury and Reduces Allograft Survival
Licensed copyright line	© Copyright 2013 The American Society of Transplantation and the American Society of Transplant Surgeons
Licensed content author	A. Lau,S. Wang,J. Jiang,A. Haig,A. Pavlosky,A. Linkermann,Z.-X. Zhang,A. M. Jevnikar
Licensed content date	Sep 18, 2013
Start page	2805
End page	2818
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	Cell Death Regulates Injury and Inflammation During Renal Allograft Transplantation
Expected completion date	Sep 2014
Expected size (number of pages)	185
Total	0.00 USD

By e-mail communication:

Dear Mr Lau,

Thank you for your email. As to your request, I am pleased to inform you that permission is granted herewith to use your accepted paper

Lau A, Wang S, Liu W, Haig A, Zhang ZX, Jevnikar AM.: Glycyrrhizic acid (GZA) ameliorates HMGB1-mediated cell death and inflammation after renal ischemia reperfusion injury. Am J Nephrol;2014:in press

for publication in your dissertation, provided that proper credit will be given to the original source and that S. Karger AG, Basel will be mentioned.

Please note that this is a non-exclusive permission, hence any further use or distribution, either in print or electronically, requires written permission again as this permission is valid for the above mentioned purpose only.

This permission applies only to copyrighted content that S. Karger AG owns, and not to copyrighted content from other sources. If any material in our work appears with credit to another source, you must also obtain permission from the original source cited in our work. All content reproduced from copyrighted material owned by S. Karger AG remains the sole and exclusive property of S. Karger AG. The right to grant permission to a third party is reserved solely by S. Karger AG.

Thank you for your understanding and cooperation.

Hopefully, I have been of assistance to you with the above.

Best regards,

Silvia Meier
Rights Manager

S. Karger AG, Medical and Scientific

Publishers

Appendix B – Animal Ethics Approval

AUP Number: 2010-245
AUP Title: a) Regulation of Tissue Injury and Novel Approaches to Tolerance Induction to Promote Long Term Renal Transplant Survival and b) Limitation of Epithelial Cell and Renal Allograft Injury by Flice Inhibitory Protein (FLIP) and Endogenous Apoptosis Regulatory Proteins c) Regulation of renal tubular epithelial cell injury to promote kidney allograft survival d) The role of NK cell in kidney injury
Yearly Renewal Date: 11/01/2013

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2010-245 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.

2. Animals for other projects may not be ordered under this AUP number.

3. Purchases of animals other than through this system must be cleared through the ACVS office.
   Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

Arthur Lau

ACADEMIC BACKGROUND

2008- Present **Ph.D. Candidate**
Pathology
WESTERN UNIVERSITY

2004- 2008 **Honours Bachelor of Science (H.B.Sc.)**
Cell and Molecular Biology Specialist Program
UNIVERSITY OF TORONTO

PUBLICATIONS

Lau A, Wang S, Jiang J, Haig A, Pavlosky A, Linkermann A, Zhang Z-X, Jevnikar AM. RIPK3 mediated necroptosis promotes kidney inflammatory injury and reduces allograft survival. Am. J. Transplantation. 13(11): 2805-2818, 2013.

Lau A, Khan K, Pavlosky A, Yin Z-Q, Huang X-Y, Haig A, Liu W-H, Singh B, Zhang Z-X, Jevnikar AM. SPI-6 (Serine Protease Inhibitor-6) inhibits granzyme B mediated injury of renal tubular cells and promotes renal allograft survival. Transplantation. E-pub ahead of press.

Lau A, Wang S, Zhang Z-X, Jevnikar AM. Glycyrrhizic acid (GZA) ameliorates HMGB1 mediated inflammation after renal ischemia reperfusion injury. Am. J. Nephrology. In press.

Zhang Z-X, Shek K, Wang S, Huang X, **Lau A**, Yin Z., Sun H., Liu W., Garcia B, Rittling S., Jevnikar AM. OPN expressed in tubular epithelial cells regulates NK cell-mediated kidney ischemia reperfusion injury. J. Immunology. 185(2): 967-73, 2010.

Choi J, Diao H, Feng ZC, **Lau A**, Wang R, Jevnikar AM. A fusion protein derived from plants holds promising potential as a new oral therapy for type 2 diabetes. Plant Biotechnol. J. 2013.

Pavlosky A, **Lau A**, Su Y, Lian D-M, Huang X-Y, Yin Z-Q, Haig A, Jevnikar AM, Zhang Z-X. RIPK3-mediated necroptosis regulates cardiac allograft rejection. Am. J. Transplantation. E-pub ahead of press. 2014.

Zhang Z-X, Huang X, Jiang J, **Lau A**, Yin Z, Liu W, Haig A, Jevnikar AM. NK cells mediate chronic kidney allograft injury. Transplantation. Submitted. 2014.

AWARDS

2014 American Society of Transplantation – Young Innovator Award

2013, 2012 Canadian Society of Transplantation – Basic Science Award

2012 Dutkevich Memorial Foundation Award

2011 Canadian Society of Transplantation/American Society of Transplantation Distinguished Fellows Travel Award

2010 Canadian Society of Transplantation Travel Award

2009 Lawson Research Day – Sister Mary Doyle Award

SCHOLARSHIPS

2014-2016 Beverly Phillips Rising Star Postdoctoral Fellowship
UNIVERSITY OF CALGARY

2010-2012 Ontario Graduate Scholarship
ONTARIO MINISTRY OF TRAINING, COLLEGES, AND UNIVERSITIES

2008-2013 Western Graduate Research Scholarship
WESTERN UNIVERSITY

CONFERENCES

2014 World Transplant Congress, American Society of Transplantation/ The Transplantation Society
Endogenous expression of SPI-6 (Serine Protease Inhibitor-6) in renal tubular epithelial cells inhibits granzyme B mediated injury and promotes renal allograft survival - Poster

2014 CST Annual Scientific Conference, Canadian Society of Transplantation
SPI-6 (Serine Protease Inhibitor-6) inhibits granzyme B mediated injury of renal tubular epithelial cells and promotes renal allograft survival - Poster

2014 Cutting Edge of Transplantation (CEOT), American Society of Transplantation
Inhibition of Receptor Interacting Protein Kinase 3 (RIPK3) Promotes Long Term Renal Allograft Survival – Oral Presentation

2013 American Transplant Congress, American Society of Transplantation
Inhibition of Receptor Interacting Protein Kinase 3 (RIPK3) Promotes Long Term Renal Allograft Survival - Poster

2013 CST Annual Scientific Conference, Canadian Society of Transplantation
Inhibition of Receptor Interacting Protein Kinase 3 (RIPK3) Promotes Long Term Renal Allograft Survival - Oral Presentation

2012 American Transplant Congress, American Society of Transplantation
Receptor Interacting Protein 3 (RIP3) Regulates Tubular Epithelial Cell (TEC) Injury and Inflammation Following Renal IRI - Oral Presentation

2012 CST Annual Scientific Conference, Canadian Society of Transplantation
Receptor Interacting Protein 3 (RIP3) Regulates Tubular Epithelial Cell Injury (TEC) and Inflammation Following Renal IRI - Oral Presentation

2011 American Transplant Congress, American Society of Transplantation
Glycyrrhizic acid (GZA) can block HMGB1 mediated tubular epithelial cell (TEC) injury and NK cell activation following renal IRI - Poster

Expression of SPI-6 (Serine Protease Inhibitor-6) in renal tubular epithelial cell (TEC) is required for protection from Granzyme B mediated effector cell injury following transplantation - Oral Presentation

2011 CST Annual Scientific Conference, Canadian Society of Transplantation
Glycyrrhizic acid (GZA) can block HMGB1 mediated tubular epithelial cell (TEC) injury and NK cell activation following renal IRI - Oral Presentation

2010 International Congress of The Transplantation Society, The Transplantation Society
Hypoxia induced tubular cell (TEC) release of HMGB1 propagates renal injury by effects on TEC viability and NK cell activation - Oral Presentation

2010 CST Annual Scientific Conference, Canadian Society of Transplantation
Hypoxia induced tubular cell (TEC) release of HMGB1 propagates renal injury by effects on TEC viability and NK cell activation - Oral Presentation

2010 American Transplant Congress, American Society of Transplantation
HMGB1 Regulates Tubular Epithelial Cell (TEC) Expression of MCP-1 in Ischemic Kidney Injury - Poster

2009 American Society of Transplantation Annual Scientific Exchange, American Society of Transplantation
HMGB1 Regulates Tubular Epithelial Cell (TEC) Expression of MCP-1 in Ischemic Kidney Injury - Poster

TEACHING/SUPERVISORY EXPERIENCE

2012-2013 **Teaching Assistant, Medical Sciences Laboratory**
WESTERN UNIVERSITY

2009-2012 **Teaching Assistant, Introduction to Comparative Pathology**
WESTERN UNIVERSITY

2009 **Undergraduate Thesis Student Mentor**
WESTERN UNIVERSITY

ACADEMIC DEVELOPMENT

2012 **Department of Pathology Research Committee – Graduate Student Representative**
WESTERN UNIVERSITY

2011-2012 **Western Pathology Association, Co-Founder and Co-Chair**
WESTERN UNIVERSITY

2010-2011 **Department of Pathology Graduate Education Committee – Graduate Student Representative**
WESTERN UNIVERSITY

VOLUNTEER EXPERIENCE

2009-2010 **Community Outreach Volunteer – Let's Talk Science**
WESTERN UNIVERSITY