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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology and Immunology

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CONTRIBUTION OF ENHANCED INTRINSIC APOPTOSIS TO RENAL
ISCHEMIA REPERFUSION INJURY IN THE ABSENCE OF RIPK3 AND
CASPASE-8 DEPENDENT REGULATED CELL DEATH

(Thesis format: Monograph)

by

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Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

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Abstract

Ischemia-reperfusion injury (IRI) contributes to reduced long term organ transplant survival. IRI is a complex process related to multiple mechanisms including regulated forms of cell death such as apoptosis and necroptosis. We have previously shown that preventing necroptosis by genetic elimination of the key molecule (RIPK3) or apoptosis inhibition through caspase-8 silencing are of benefit in kidney IRI. As these pathways of regulated cell death are unique, we tested the hypothesis that deletion of both RIPK3 and caspase-8 activity in kidneys would synergistically improve renal IRI, compared to inhibition of individual pathways. Interestingly we found that IRI was not improved *in vivo* using RIPK3/caspase-8 null (DKO) mice. *In vitro* testing of renal tubular epithelial cells (TEC) from DKO kidneys showed enhanced death following exposure to IFN- γ + IL-1 β suggesting enhanced activation of an intrinsic apoptosis pathway. Indeed enhanced caspase-9 and -3 activation was observed, as well as death of DKO TEC was inhibited by the BAX inhibiting peptide V5 (BIP). Finally, murine cytomegalovirus (MCMV) infection, which can block intrinsic apoptosis, was able to abrogate death in DKO TEC. In the absence of cell death, cytokine exposed DKO TEC showed increased expression of pro-inflammatory chemokines. Collectively these results indicate that cell death pathways are complex and counterbalanced. In the absence of RIPK3 and caspase-8, intrinsic apoptosis and chemokine expression is up-regulated in TEC which may account for the absence of the expected benefit from the loss of both apoptosis and necroptosis. These results highlight the complex biology that results from targeted perturbations of death pathways in TEC and suggest that the inhibition of multiple forms of cell death will likely be required to maximize clinical benefit in IRI and transplants.

Keywords

Intrinsic apoptosis, RIPK3, Caspase-8, necroptosis, renal tubular epithelial cell, ischemia reperfusion injury, mouse

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List of Abbreviations

| | |
|--------|--|
| AIF | Apoptosis inducing factor |
| AKI | Acute kidney Injury |
| ANT | Adenine nucleotide translocator |
| APAF-1 | Apoptosis protease activating factor 1 |
| ARF | Acute renal failure |
| ATN | Acute tubular necrosis |
| ATP | Adenosine triphosphate |
| BAD | BCL-2-associated death promoter |
| BAK | BCL-2 homologous antagonist killer |
| BAX | BCL-2-associated X protein |
| BCL-xL | B-cell lymphoma-extra large |
| BCL-2 | B-cell lymphoma 2 |
| BID | BH3 interacting-domain death agonist |
| BIM | BCL-2-interacting mediator of cell death |
| BIP | BAX-inhibiting peptide v5 |
| C-TGF | Connective tissue growth factor |
| CAD | Caspase activated DNase |
| CAR | Chronic allograft rejection |

| | |
|-------|---|
| CARD | Caspase activation and recruitment domain |
| CHAK | Chemokine-activated killer |
| CHX | Cyclohexamide |
| CKD | Chronic kidney disease |
| CMF | Cell mediated response |
| CYLD | Cyclindromatosis |
| DAMP | Danger-associated molecular pattern |
| DNA | Deoxyribonucleic acid |
| DR 5 | Death receptor 5 |
| EMT | Epithelial-to-mesenchymal transition |
| EPO | Erythropoietin |
| ESRD | End-stage renal disease |
| FADD | Fas-associated protein with death domain |
| FASL | Fas ligand |
| FLIP | FLICE-like inhibitory protein |
| FSG | Focal segmental glomerulosclerosis |
| GFR | Glomerular filtration rate |
| GLUD1 | Glutamate dehydrogenase |
| GLUL | Glutamine synthase |
| GVHD | Graft-versus-host disease |

| | |
|---------------|---|
| HMGB-1 | High-mobility group box-1 |
| HSP | Heat shock protein |
| ICAD | Inhibitor of caspase activated DNase |
| hTNF α | Human tumour necrosis factor alpha |
| iAP | inhibitor of apoptosis protein |
| IFN- γ | Interferon-gamma |
| IGF-1 | Insulin-like growth factor-1 |
| IKK | I κ B kinase |
| IL-1 β | Interleukin-1 beta |
| IL-6 | Interleukin-6 |
| IL-10 | Interleukin-10 |
| IL-27 | Interleukin-27 |
| ILK | Integrin-linked kinase |
| IRI | Ischemia reperfusion injury |
| KIM-1 | Kidney injury molecule-1 |
| LPS | Lipopolysaccharide |
| MAC | Mitochondrial apoptosis-induced channel |
| MAPK | Mitogen-activated protein kinase |
| MCL-1 | Induced myeloid leukemia cell differentiation protein-1 |
| MCP-1 | Monocyte chemotactic protein-1 |

| | |
|----------------|--|
| MCMV | Murine cytomegalovirus |
| MIM | Mitochondrial inner membrane |
| MLKL | Mixed lineage kinase domain-like |
| MMP | Mitochondrial membrane potential |
| MOI | Multiplicity of infection |
| MOM | Mitochondrial outer membrane |
| MPTP | Mitochondrial permeability transition pore |
| Nec-1 | Necrostatin-1 |
| NEMO | NF-kappa B essential modulator |
| NET | Neutrophil extracellular trap |
| NF- κ B | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NK cell | Natural killer cell |
| NKT cell | Natural killer T cell |
| PAMP | Pathogen-associated molecular pattern |
| PI | Propidium iodide |
| PKB | Protein kinase B |
| PMN | Polymorphonuclear |
| PP1 α | Protein phosphatase 1 alpha |
| PP2A | Protein phosphatase 2A |
| PRR | Pattern recognition receptor |

| | |
|--------------|---|
| PS | Phosphatidylserine |
| PYGL | Glycogen phosphorylase |
| RAGE | Receptor for advanced glycan end-products |
| RANTES | Regulated on activation, normal T cell expressed and secreted |
| RHIM | RIP homotypic interaction motif |
| RIPK | Receptor interacting protein kinase |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RRT | Renal replacement therapy |
| TEC | Tubular epithelial cell |
| TGF- β | Transforming growth factor- β |
| TLR 2 | Toll like receptor 2 |
| TLR 4 | Toll like receptor 4 |
| TNF α | Tumour necrosis factor alpha |
| TNFR-1 | Tumour necrosis factor receptor 1 |
| TNFR-2 | Tumour necrosis factor receptor 2 |
| TRADD | TNF receptor-associated death domain |
| TRAF | TNF receptor-associated factor |
| TRAIL | TNF receptor-associated apoptosis-inducing ligand |
| TRIF | TIR-domain-containing adaptor-inducing interferon- β |

| | |
|--------|---|
| VDAC-1 | Voltage-dependent anion channel-1 |
| vCIA | Viral inhibitor of caspase-induced apoptosis |
| vIBO | Viral inhibitor of BAK oligomerization |
| vIRA | Viral M45-encoded inhibitor of RIP activation |
| vMIA | Viral inhibitor of mitochondrial apoptosis |

Chapter 1

1 Introduction

1.1 Current situation and challenges of kidney allograft transplantation

Kidney transplantation and dialysis, collectively known as renal replacement therapies (RRT), are the only two treatment options after end-stage kidney failure occurs and organ damage is irremediable. While dialysis remains a common treatment option for the majority of patients¹, kidney transplantation is both cost-effective and preferred therapy as it consistently improves survival in patients when compared to those on dialysis²⁻⁴. It is known since 2005 that high blood pressure, diabetes mellitus, glomerulonephritis, and hypertension are factors that contributed to the development of chronic kidney disease (CKD)⁵. Typical signs of CKD are related to loss of kidney function and include loss of water, acid-base, and electrolyte homeostasis, as well as loss of growth factors such as erythropoietin (EPO). CKD is categorized into five stages based on the level of kidney function, severity of the damage, symptoms, and glomerular filtration rate (GFR) (Figure 1). Normally, CKD gradually and steadily progresses over these stages, taking years at times. However, CKD can accelerate at times, leading to more acute loss of kidney function requiring emergency dialysis. Combination of proper diet and the use of medications to treat blood pressure and other factors that injure kidneys can significantly attenuate the progress of CKD during its early stages. Indeed not every patient fully goes through every stage of CKD. Once CKD reaches Stage 5, also known as end-stage renal disease (ESRD), kidneys are considered to have failed as their function drops below 15%. ESRD patients require RRTs, as onset of complications can ultimately cause death^{6,7}.

Kidney transplantation offers the best chance of survival in ESRD patients, but it poses variety of complications⁸⁻¹⁰, particularly with insufficient numbers of donor organ and the use of deceased donor organs. Storage of organs and use of more marginal donors with underlying medical conditions can lead to reduced organ viability and contribute to increased rejection. Graft survival depends on multiple factors, including the age and health conditions of both the donor and the recipient, use of live or deceased donors, cold storage and ischemic times, development of acute tubular necrosis (ATN), and amount of fibrosis^{11,12}. To counter rejection post-transplantation, immunosuppressive therapies are provided, which have side effects including predisposing recipients to some forms of cancer and opportunistic infections¹¹. Immunological complications include allo-rejection and in some transplant situations Graft vs. Host Disease (GVHD) further reduce graft and even recipient survival. The total injury of transplanted kidneys extends from early injury that invariably is associated with the removal and storage of kidneys from the donor, to the complex injury that follows re-establishing the blood supply, namely ischemia-reperfusion injury (IRI), to acute and chronic immunological rejection. Interventions at any of these levels may improve transplant organ survival but most current efforts have relied almost entirely on the prevention of immune rejection. Therefore, to maximize kidney graft survival, it is imperative to further study and understand the early mechanisms that underlie initial graft injury. This is even more important considering the irreconcilable disparities between the rapidly increasing demand of kidneys and growing shortage of available donor organs^{13,14}.

| Five Stages of Chronic Kidney Disease | | | | |
|---------------------------------------|-----------------|--|---|----------------------------|
| Stage | Kidney function | Description of damage | Symptoms | Glomerular filtration rate |
| Stage 1 | >90% | Minor kidney damage with normal function | Normal urea and creatinine level | 90mL/min |
| Stage 2 | 60~89% | Mild damage and decreased function | Slightly elevated urea and creatinine level | 60~89mL/min |
| Stage 3 | 30~59% | Mild to moderate kidney damage | Onset of fatigue and itching | 30~59mL/min |
| Stage 4 | 15~29% | Severe damage and poor function | Tiredness, loss of appetite | 15~29mL/min |
| Stage 5 | <15% | Kidney failure and onset of ESRD | High urea and creatinine level, insomnia, frequent vomiting | <15mL/min |

Figure 1. Characteristics of CKD by stages

CKD is grouped into five different stages, each categorized by level of kidney function, severity of damage, symptoms, and glomerular filtration rate. CKD starts off from Stage 1, and progressively worsens as the stage level increases. Once Stage 5 is reached, ESRD occurs and RRTs are needed to keep the patients alive.

1.2 Anatomy of the kidney and immunophysiology of tubular epithelial cells (TEC)

Kidneys are a pair of ‘bean-shaped’ organs with parenchymal cells encased by a renal capsule composed of fibrous tissue. The structure of kidneys maintains a concave and convex shape. Beside the concave surface on the medial border of the kidney, lies the renal pedicle, which consists of a structure containing blood vessels, nerves, and the ureter. The renal parenchyma can be divided into two distinct anatomical regions: the superficial renal cortex and the deep renal medulla. Spanning both the cortex and medulla are ‘nephrons’, the basic functional unit of the kidney. Nephrons jointly form into renal columns that display a pyramid-like appearance. Each nephron contains proximal and distal tubules that are aligned by individual tubular epithelial cells (TEC) that are the kidney’s most metabolically active and functional cells. TEC possess both apical and basolateral aspects as polarity (an ‘up’ and ‘down’ of the cell) which is a unique characteristic of epithelial cells (Figure 2). These cells are also key participants during graft recognition by host immune cells and kidney rejection. TEC comprise more than 75% of the renal parenchyma, and are particularly susceptible to damage caused by reactive oxygen species (ROS), nitric oxide, pro-inflammatory cytokines, and cell-mediated killing that are the primary mechanisms by which ischemia reperfusion injury (IRI) occurs, as well as later by immune rejection¹⁵⁻¹⁷. With the ability to repair injury during the IRI¹⁸, the viability of TEC and other parenchymal cells primarily influence the outcome of both short- and long-term kidney allograft survival¹⁹. As a result, TEC play a pivotal role in modulating alloimmune responses post-renal transplantation since they act as the first barrier between the recipient and the graft survival.

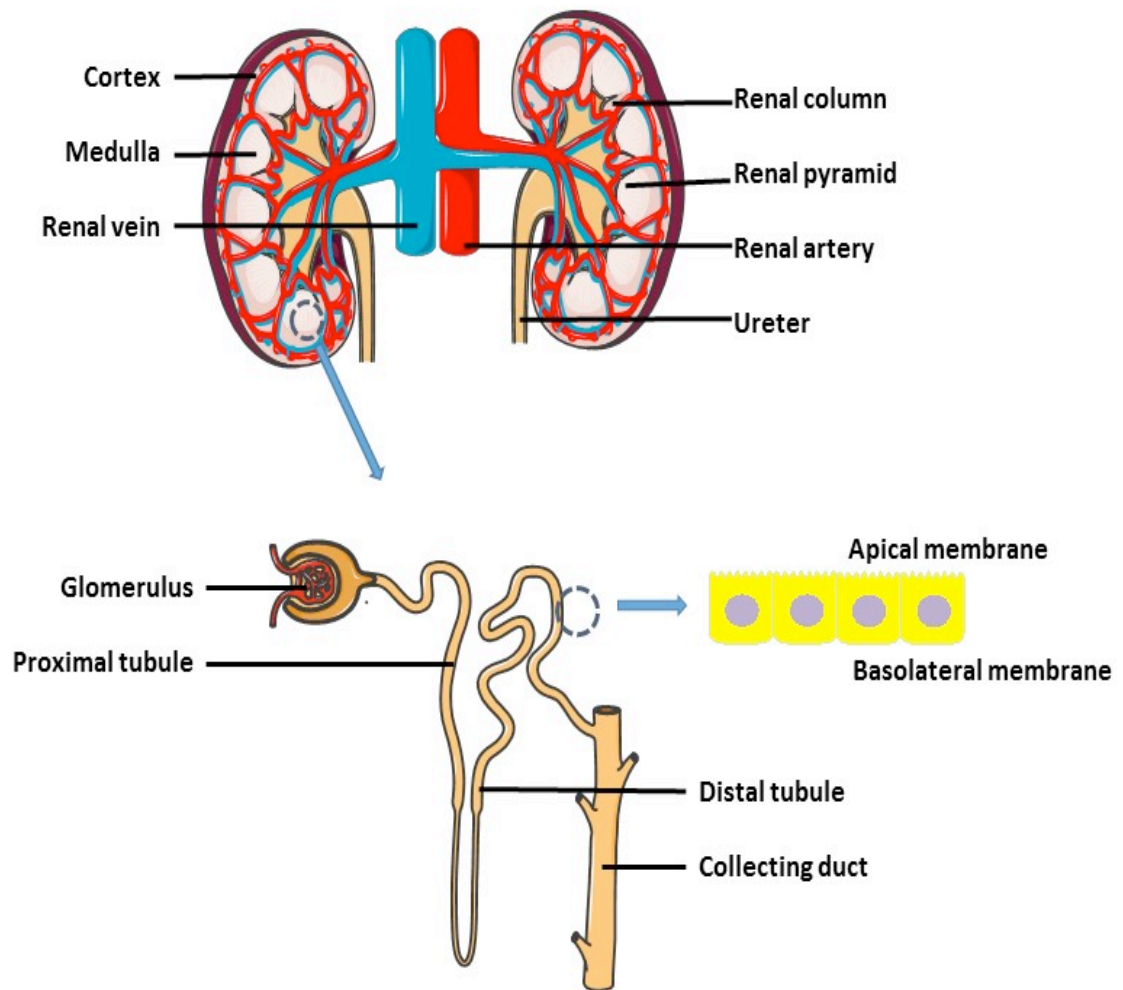


Figure 2. Anatomy and morphology of kidney and TEC

The kidney possesses a concave and convex shape. The kidney is divided into two main sections, namely the outer cortical region and the inner medulla region. The outer cortical region consists of renal columns and renal pyramids composed of nephrons, which are the fundamental functional units of the kidney. TEC reside in proximal and distal tubules of the nephrons, where they actively perform filtration and reabsorption. After transplantation, the initial severe damage to TEC during IRI leads to inflammation, dysfunction of cells, and organ failure.

1.3 Mechanisms of IRI and regulation of cell death in acute renal allograft rejection

Renal allografts undergo injury and damage induced by many different factors^{20,21}, but the earliest is IRI. IRI consists of inevitable and diverse inflammatory responses elicited by the interaction of recipient cells with factors and cells within the donor organ, and occurs during the early phase of renal transplantation²². IRI is responsible for causing graft dysfunction and promoting rejection²³, but it is important that there are diverse forms of IRI related kidney injury including drugs, sepsis, cardiac injury and others that lead to acute renal failure (ARF), as these can have mortality rates that reach 50% or higher²⁴.

During renal ischemia, the blood supply to the kidney tissues becomes severely restricted, or nonexistent. This results in depletion of cellular oxygen and glucose storage required for cell survival and metabolism²⁵. Absence of oxygen and nutrients during the ischemic period pre-disposes the renal tissues to reperfusion damage²⁶, which occurs after the blood supply returns to the organ. In the case of transplantation, this is with the establishment of the anastomoses. The majority of damage actually results from the restoration of circulation, as it results in a rapid and sudden increase in organ source generation of reactive oxygen species (ROS)²⁷, up-regulation of inflammatory cytokines and chemokines^{28,29}, and recruitment of immune cells^{30,31}. During renal IRI, complex interactions of innate^{32,33}, humoral³⁴⁻³⁶ and cell-mediated³⁷⁻³⁹ immune responses accelerate and amplify the widespread injury of the donor organ and this contributes to rejection by the signaling of adaptive immune responses by innate inflammation. Both innate and adaptive immune responses are capable of inflicting heavy damages on the graft independently and collectively through cross-talk and activation.

The innate immune system usually contributes first to IRI. Neutrophil accumulation^{40,41}, Toll like receptor (TLR) signaling⁴², natural killer (NK) cell activation⁴³, and complement pathways⁴⁴ represent the most common innate immune responses during IRI⁴⁵. These are often cross-linked in nature⁴⁶. The influx of polymorphonuclear (PMN) neutrophils takes place during IRI and these early cells mostly localize into peritubular capillaries, tubular lumens, vascular endothelium, and the interstitium⁴⁷. Upon entering the renal graft, PMN migrate into the microvasculature⁴⁸ and release oxygen-free radicals and proteases⁴⁹. They also contain high levels of three pro-inflammatory cytokines in TNF α , IFN- γ , and IL-6⁴⁷, consistent with their highly inflammatory capacity. Release of these cytokines as well as neutrophil extracellular traps (NET) composed of toxic intracellular contents triggers a ‘cascade’ of inflammation resulting in the death of TEC and other parenchymal cells⁵⁰, a process known as NETosis⁵¹. Dying renal cells often undergo necrosis²⁶ and consequently leak intracellular contents that act as danger-associated molecular patterns (DAMP), namely histone proteins⁵² and high-mobility group box-1 (HMGB-1)⁵³. These DAMPs can bind TLRs of neighbouring cells. TLR 2^{52,54}, 4^{55,56}, and receptor for advanced glycan end-products (RAGE)⁵⁷ play significant roles in triggering pro-inflammatory pathways such as NF- κ B in activated cells. HMGB-1 can also up-regulate IFN- γ production from macrophage-activated NK cells⁵⁸, further driving inflammatory response. DAMPs binding to TLRs and other receptors of the innate immune system activates NF- κ B signalling and results in increased production and release of cytokines^{59,60} in many cells including monocytes which stimulates of NADPH oxidase and consequent release of ROS from neutrophils^{61,62}.

DAMPs are also capable of activating complement systems through pattern recognition receptors (PRR). Alternative and mannose-lectin pathways are mainly associated with renal IRI. Several murine renal IRI models have shown alternative pathway activation leads to massive C3 production from activated TEC after reperfusion⁶³. The resultant up-regulation of C3a subunit plays a crucial role in CXC chemokine production⁶⁴. C3 also mediates T cell response⁶⁵ and silencing C3 gene attenuates IRI⁶⁶, further displaying the detrimental effect of alternative complement pathway activation in IRI. The Mannose-lectin pathway is also involved in IRI⁶⁷, and is activated via recognition of endogenous DAMPs presented on receptors of TEC⁶⁸. Both C5a⁴⁴ and C5b⁶⁹ are key players in this injury. Accumulation of the C5b-9 membrane attack complexes on TEC stimulates the production of TNF α and IL-6, and demonstrates the role of complement pathways via endogenous danger molecules in outcome of renal grafts following ischemia.

‘Humoral’ immune responses during IRI have not been studied extensively, but limited knowledge points to their involvement in IRI. Naive B cells that infiltrate kidneys during IRI become activated and differentiate into antibody producing plasma B cells⁷⁰, hindering injury repair and augmenting tubular atrophy⁷¹. Importantly, B cell deficiency in a murine model exhibited better renal function and reduced acute tubular necrosis in IRI, highlighting the poorly understood contribution of B cells to IRI⁷². Humoral immunity appears to have a larger role in ‘chronic rejection’, as alloantibody can be elicited in the graft in late stages of injury⁷³.

Cell-mediated responses (CMR) in acute injury following ischemia is highlighted by the presence of infiltrating leukocytes⁷⁴ which can induce death⁴³ in renal parenchymal cells and promote expressions of diverse pro-inflammatory chemokines and cytokines^{29,30,75,76}.

Cells from both innate and adaptive immune compartments participate in CMR associated with IRI, as activated CD4⁺ Helper T cells^{77,78}, CD8⁺ Cytotoxic T cells³⁸, NK cells^{43,79}, NKT cells⁸⁰, macrophages^{81,82}, and antibody-secreting plasma B cells⁷² have all been shown to participate. These effector cells infiltrate into the renal parenchyma and attack the graft in numerous ways. In the current model of CMR and IRI, there are two main aspects by which immune cells injure the graft, namely by direct or indirect killing (Figure 3).

In the direct killing model, mature and fully allo-activated T cells bind to TEC and induce their death. During ARF, CD4⁺ T helper cells are considered to be one of the main effectors due to their ability to infiltrate into the kidney quickly i.e. within 4 hours^{49,83} and induce apoptosis of TEC through the FAS-FASL pathway^{84,85}. This has been characterized as the “hit-and-run” phenomenon⁸⁶. Consistent with this, blocking FASL on leukocytes significantly attenuates IRI⁸⁴, thus supporting the importance of FAS-FASL interactions in ARF. Cytotoxic CD 8⁺ T cells also damage the graft by physically adhering to kidney cells and secreting perforin and granzyme⁸⁷, inducing death by apoptosis⁴³. In ARF, FAS-FASL and perforin/granzyme pathways are the two best characterized TEC death inducers^{88,89}. It should be noted that FAS and FASL are also expressed on the surface of TEC^{88,90}, and not restricted to infiltrating immune cells (Figure 3). This allows TEC to induce self-injury and apoptosis to contribute to IRI⁹¹, a phenomenon known as “fratricide,” or “self-tubulocide” (Figure 3). Further study into the role of FAS-FASL in TEC has shown that polymorphisms in Fas are important in long-term graft survival⁹².

In indirect killing, renal cells are exposed to pro-inflammatory chemokines and cytokines secreted by infiltrating immune cells during IRI. Two of the best-characterized chemokines involved in renal IRI are monocyte chemoattractant protein-1 (MCP-1) and Regulated on Activation Normal T cell Expressed and Secreted (RANTES). MCP-1 acts as a chemotactic protein during IRI as it recruits monocytes³⁰, macrophages⁹³, dendritic cells³¹ to the graft. Once recruited, these immune effectors secrete other pro-inflammatory chemokines⁹⁴ and cytokines³¹, resulting in amplified activation of inflammation and infiltration by immune cells. Studies have shown that truncation of secreted MCP-1 significantly attenuated immune cell infiltration and tubular necrosis⁹⁵, thus supporting the involvement of MCP-1 in IRI. RANTES is secreted by TEC as well as infiltrating macrophages^{96,97} and contribute to inflammation and TEC death in renal IRI⁹⁸. RANTES recruits T cells⁹⁹, basophils¹⁰⁰, and eosinophils¹⁰¹ into the injury site of the graft. With the combination of IL-2 and IFN- γ secreted from surrounding cells, RANTES induces proliferation and activation of chemokine-activated killer (CHAK) cells from the NK cell population^{102,103}, which enhances apoptosis in TEC and compromises the graft survival. Release of early-phase pro-inflammatory cytokines and chemokines from both immune cells and TEC further augments acute kidney damage. TEC damaged by ROS production secrete DAMPs which in turn activate nearby infiltrating macrophages and triggers the secretion of IL-6¹⁰⁴. IL-6 is a pro-inflammatory acute phase response cytokine. Its maximal expression and secretion occurs as early as 4 hours after IRI^{29,105}. IL-6 secretion results in stimulation of production and infiltration of neutrophils into peritubular capillaries and the inner cortex of kidney grafts¹⁰⁶. Genetic deletion of IL-6 and its receptor results in significant improvement of renal function and reduction in

inflammation^{105,106}, which signifies the potential maladaptive and harmful role of IL-6 during IRI¹⁰⁷. Thus, during ARF, the combination of diverse recipient immune responses mediates TEC death and inflammation, which result in significant organ injury and promotes rejection. Since IRI-mediated ARF coordinates this complex set of responses, further studies may determine potential pathways that can be targeted to limit injury and promote long-term organ survival.

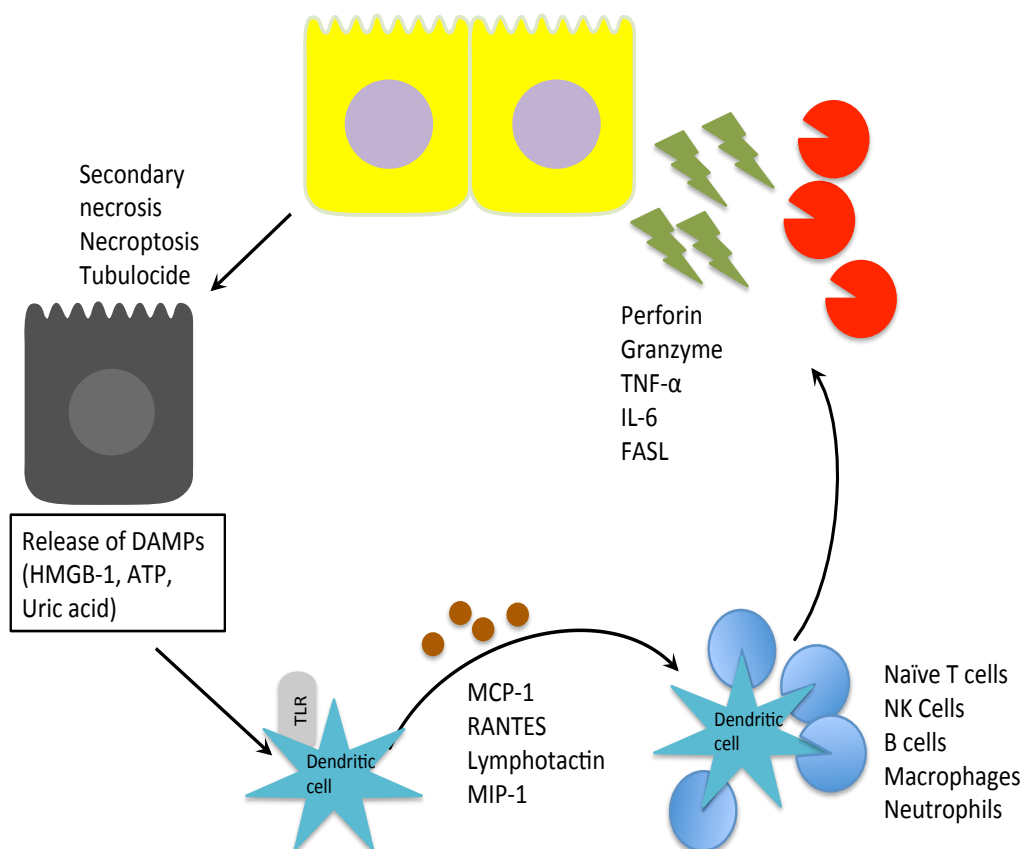


Figure 3. Characteristics of CMR during IRI

During IRI, TEC are invariably injured and potentially killed as a result of increased stresses and host immune responses. This leads to DAMP release from the TEC and the subsequent activation of immune cells effectors including dendritic cells, T cells, B cells, macrophages and NK cells. Once activated, these effectors cells contribute to further injury of TEC via the up-regulation of pro-inflammatory cytokines and chemokines as well as direct killing.

1.4 Recurrent problems in chronic renal allograft survival

Apart from acute graft rejection, chronic allograft rejection (CAR) presents a persistent and greater clinical obstacle, as it occurs in up to 60% of renal allografts. The onset of CAR varies among patients, with injury being apparent as early as months or as late as several years post-transplantation¹¹. The most significant components of CAR are tubular atrophy¹⁰⁸, tubulointerstitial inflammation¹⁰⁹, and fibrosis¹¹⁰, which can also result from recurrence of the original disease such as Focal Segmental Glomerulosclerosis (FSGS)^{11,111}. These result in a gradual but steady decline in the allograft function. Among the several pathophysiological aspects of CAR, renal fibrosis is currently considered as the most important and difficult issue. It has been speculated that TEC injury and death during the acute phase IRI causes a phenomenon known as epithelial-to-mesenchymal transition (EMT). Onset of EMT results in fibroblast expansion which outcompetes the regeneration of dead TEC and parenchymal cells within the graft¹¹². While EMT has been linked to other chronic kidney diseases as well^{113,114}, there have been reports that EMT may not be a major factor in the renal fibrosis observed¹¹⁵. Key players involved in fibrogenesis of renal allograft are transforming growth factor- β (TGF- β)^{116,117}, connective tissue growth factor (C-TGF)¹¹⁸, and PINCH-1^{119,120}. TGF- β signaling during renal IRI promotes fibrogenesis and also activates p38 mitogen-activated protein (MAP) kinase activity, which, interestingly accelerates the cleavage of pro-caspase-9, which is central to intrinsic apoptosis¹²¹. TGF- β signaling also results in downstream activation of C-TGF. C-TGF has been implicated in the profibrotic EMT that characterizes TGF- β signaling¹²², and is currently a leading target for antifibrotic drugs strategies¹²³. PINCH-1 is an adaptor

protein that binds and stimulates integrin-linked kinase (ILK), and this interaction is essential for TGF- β -mediated fibrosis¹¹⁹.

Currently, there are no effective immunosuppressive therapeutic strategies that can attenuate or ameliorate CAR. Although a few studies have suggested possible roles of acute phase IRI in permitting allograft tolerance¹²⁴⁻¹²⁷, there is a lack of convincing evidence to support this. Understanding the complex underlying mechanisms of CAR remains imperative for establishing clinically effective strategies to improve long-term graft survival.

1.5 Receptor interacting protein kinase 3 (RIPK3)-mediated necroptosis and caspase-8 dependent extrinsic apoptosis in renal IRI

Modalities and involvement of TEC death in renal IRI has been well documented, as many distinctive, unique forms of cell death exist and contribute to the graft injury. TEC can undergo various forms of cell death including but not limited to FAS-mediated fratricide^{17,91}, caspase-dependent extrinsic¹²⁸ and intrinsic apoptosis^{129,130}, necrosis^{131,132}, secondary necrosis^{133,134}, necroptosis^{135,136}, ferroptosis¹³⁷, pyroptosis^{138,139}, autophagy¹⁴⁰⁻¹⁴², and mitotic catastrophe¹⁴³. Recent studies have shown necroptosis, a regulated form of necrosis, plays a major role in exacerbating renal IRI¹⁴⁴. Morphological and biochemical characteristics of necroptotic death are indistinguishable from that of necrosis^{145,146}, as both show ATP depletion-induced swelling of the mitochondria and cytoplasm, plasma membrane rupture, and lysis of the cell^{147,148}. For a long time, necrosis has been understood as an unregulated and “messy” form of cell death¹³¹. This perception

has been altered with the recent demonstration of regulated forms of necrosis including necroptosis^{149,150}. Necroptosis possesses characteristics of apoptosis in being programmed and sharing some components with necrosis; it not only depends on programmed and controlled endogenous cellular machinery as in apoptosis¹⁵¹, but also leads to cell lysis and leakage of intracellular contents that act as immunogenic DAMPs¹⁵². Necroptosis was in fact classified as a “failsafe” mechanism against infections from caspase-8 inhibiting viruses^{153,154}.

Interactions between various death receptors and ligands are capable of triggering necroptosis, as activation of TNF receptor-1 (TNFR-1)¹⁵⁵, FAS¹⁵⁶, TNF-related apoptosis inducing ligand (TRAIL)^{157,158}, and TLR 4¹⁵⁹ signaling can all result in necroptosis, although the downstream pathways differ^{160,161}. Most necroptotic pathways rely on the activation of RIPK1 and RIPK3, two serine/threonine kinase family members whose presence is essential for the formation of the necrosome complex¹⁶²⁻¹⁶⁴. Upon death-receptor activation, multiple adaptor proteins¹⁶⁵ including poly-ubiquitinated RIPK1¹⁶⁶ are recruited to the cytosolic domain of the receptors, forming Complex I¹⁶⁷. The modification of the RIPK1 ubiquitin tail determines the fate of the cell at this point¹⁶⁸. If NF- κ B essential modulator (NEMO) binds to the M1-linked chain of the ubiquitin tail, the subsequent result is an increased stability of Complex I. Furthermore, NEMO-ubiquitin tail interaction causes activation of I κ B kinase (IKK) by inducing its conformational change and promoting NF- κ B signal transduction cascade, which is pro-inflammatory and pro-survival.

On the other hand, the ubiquitin tail can be cleaved off by enzymatic molecules such as A20¹⁶⁹ or cylindromatosis (CYLD)^{170,171}. With cleavage of the polyubiquitin tail,

Complex I proceeds to formation of Complex II, with both RIPK1 and RIPK3 attached to it^{145,172}. As in Complex I, Complex II faces two potentially different outcomes. If caspase-8 is recruited, it cleaves the kinase domains of both RIPK1¹⁷³ and RIPK3¹⁷⁴, rendering the kinases nonfunctional and resulting in activation of downstream caspase-8 mediated apoptosis. This ends in prototypical, “clean,” non-inflammatory death of the cell¹⁷⁵. In the alternative situation where caspase-8 recruitment is absent, necroptosis occurs. Without the inhibitory action of caspase-8, RIPK3 can phosphorylate RIPK1¹⁷⁶, which then autophosphorylates itself¹⁵³. This results in necrosome formation, as RIPK1 and RIPK3 heterodimerize using their RIP homotypic interaction motif (RHIM) domains^{153,162,163}. Activated RIPK3 starts the activation of downstream molecules, and mixed lineage kinase domain-like (MLKL) is a primary RIPK3 target. MLKL lacks an active kinase domain¹⁷⁷, and it is phosphorylated by RIPK3¹⁷⁸. These phosphorylation events are indispensable for necroptosis, as depletion of either RIPK3 or MLKL has shown to shift cell death from necroptosis to RIPK1-mediated apoptosis¹⁷⁹.

Phosphorylated MLKL creates pore structures in the cell membrane to release DAMP. It also proceeds to the outer mitochondrial membrane and activates downstream mitochondrial phosphatase PGAM5¹⁸⁰. Currently, the downstream kinetics of PGAM5 is poorly understood, as there have been conflicting results as to the involvement of mitochondrial molecules before necroptosis occurs^{181,182}.

Necroptosis is an energy-dependent process, as highlighted by ATP depletion, ROS production, and subsequent mitochondrial swelling which all can be attributed to metabolic changes. Necroptosis however can proceed in the absence of mitochondria, so the total amount of ATP needed to proceed must be quite limited¹⁸³. Active RIPK3 also

acts as a metabolism regulator by increasing the activity of multiple enzymes¹⁶⁴. Glutamine synthase (GLUL), glutamate dehydrogenase (GLUD1), and glycogen phosphorylase (PYGL) are three metabolic enzymes that significantly contribute to accumulation of ROS in the mitochondria during necroptosis^{184,185}. These enzymes participate in catabolism of glutamine, glutamate, and glycogen, thus accelerating and intensifying the energy production within cells¹⁴⁵. Metabolic stress up-regulates the expenditure of endogenous ATP, ROS production, mitochondrial swelling, and lysis of the cell, fulfilling the characteristics of necroptosis^{163,186}. With the resulting release of intracellular immunogenic DAMPs, and in the case of sepsis-PAMPs, necroptosis poses a significant threat to TEC function and thus kidney transplant organ function.

Apoptosis is an innate mechanism required for cellular remodeling and repair, and is considered as a prototypical ‘programmed’ cell death. Apoptosis is a predominant and particularly intense form of death in kidneys, as TEC undergo rapid and frequent turnover¹⁸⁷. During renal IRI when apoptosis of TEC exceeds kidney’s regenerative capacity, it can lead to loss of function and graft failure. Caspase-8 is a cysteine-aspartic acid protease responsible for death receptor-mediated ‘extrinsic’ apoptosis, and its activation during IRI is triggered by cytokines such as TNF α and FASL^{188,189}, as well as by gasotransmitters such as nitric oxide¹⁹⁰. Caspase-8 promotes graft damage during IRI through TEC apoptosis, and its inhibition thus would be expected to confer survival benefits^{66,191}. Initial stages of extrinsic apoptosis cascade share identical features with necroptosis, such as assembly of Complex I, cleavage of polyubiquitin tail on RIPK1, and Complex II formation post-death receptor activation. Caspase-8 recruitment to Complex II results in cleavage of RIPK1 and RIPK3, inactivating necroptosis and diverting the fate

of TEC towards apoptosis¹⁹². Activated caspase-8 further proceeds to cleave downstream effector caspases-3, 6, and 7, all of which are also members of cysteine-aspartic acid protease family, into their activated forms¹⁹³⁻¹⁹⁵. The activated executioner caspases begins the demise of TEC, leading to DNA fragmentation, chromatin condensation, and membrane blebbing.

The dual-function of caspase-8 in promoting apoptosis and also inhibiting necroptosis has been clearly highlighted by embryonic lethality of caspase-8^{-/-} mice, which occurs around day 10. It has been demonstrated that RIPK3-mediated necroptosis was the cause of the embryonic lethality in the absence of caspase-8 and loss of suppression of necroptosis. The lethality of the caspase-8 loss can be recovered by the concurrent removal of suppressed necroptosis¹⁹⁶. These have been extremely important mouse models to study the effect of cell death on organ function. This also highlights important counter-balanced regulatory mechanisms that exist between apoptosis and necroptosis, as well as the role of caspase-8 to act as a master “switch” in skewing cell death to apoptosis or necroptosis. While there have been many genetic knockout models created to study the mechanisms of death pathways and interactions of proteins involved in them¹⁹⁷, it is noteworthy that many of these pathways can be studied and inhibited by murine cytomegalovirus (MCMV), which encodes inhibitors of cell death pathways and can simultaneously inhibit many pathways¹⁹⁸⁻²⁰⁰.

Strong evidence shows necroptosis and extrinsic apoptosis contribute to damage in renal IRI. Until recently, reagents suitable for clinical study have only been directed against apoptosis using caspase inhibitors. Recently, necrostatin-1 (Nec-1) has become the leading candidate for clinical trials in suppressing necroptosis. It prevents necroptosis

through allosteric inhibition of RIPK1 and has been shown to grant significant cytoprotective and survival benefits²⁰¹⁻²⁰³. Further studies are required in order to develop effective therapeutic and pharmacological strategies. Targeting important cell death mediators such as RIPK3 and apoptosis in combination should be considered, as it could lead to attenuation of renal IRI and survival of renal allografts. However, as these are counter balanced pathways with complex interactions, a detailed understanding of mechanisms is required to avoid potentiation of other forms of cell death as well as potential side-effects, as evidenced by the studies in this thesis.

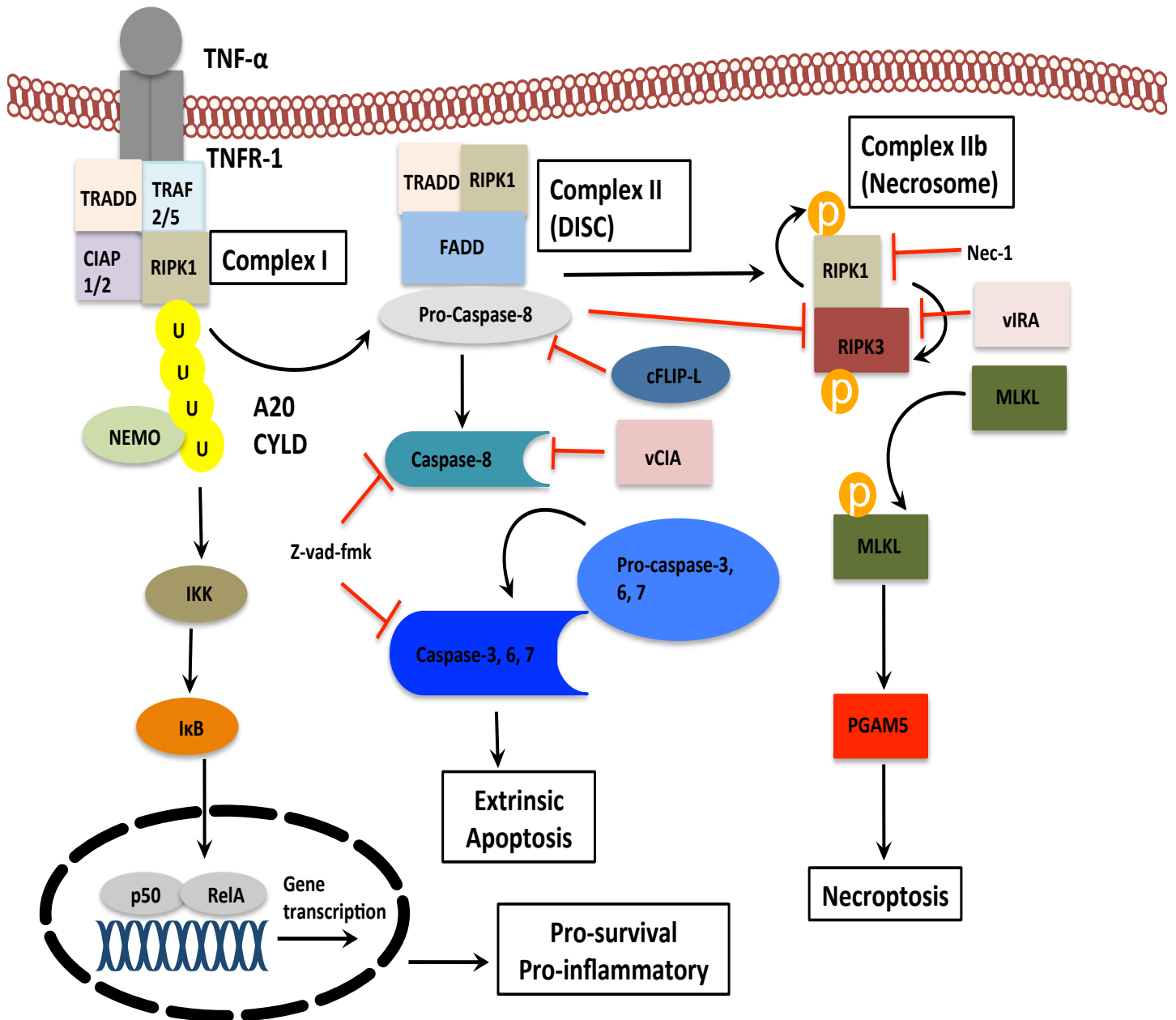


Figure 4. Mechanism of TNF α -induced necroptosis and apoptosis

After TNF α -TNFR-1 interaction, assembly of Complex I consisting of TRADD, ubiquitinated RIPK1, CIAPs, and TRAF2/5 occurs at the cytosolic tail domain of TNFR-1. NEMO binding to the ubiquitin tail of RIPK1 activates the pro-survival/pro-inflammatory NF- κ B signaling pathway, whereas removal of the ubiquitin tail by A20 or CYLD allows Complex I to proceed and form DISC/Complex II. If pro-caspase 8 is recruited by FADD and activated into active caspase-8, it will prevent necroptosis by cleaving RIPK1 and RIPK3, and initiate apoptosis through caspase-3, 6, and 7 activation. Caspase-8 mediated apoptosis can be inhibited by endogenous cFLIP-L, vCIA of MCMV, or pan-caspase inhibitor Z-vad-fmk to allow RIPK1-RIPK3 necrosome complex (Complex IIb) formation. Active necrosome complex further activates downstream MLKL and PGAM5 to initiate necroptosis. Necroptosis can be prevented by RIPK1 inhibition through Nec-1 or RIPK3 inhibition by vIRA of MCMV.

1.6 B-cell lymphoma-2 (BCL-2) family members and intrinsic apoptosis in renal IRI

Along with extrinsic apoptosis and necroptosis, intrinsic apoptosis is another form of death that cells can undergo during renal IRI. These three pathways could all relate to mitochondria in their downstream executioner mechanisms, as they may interact with each other at the mitochondrial level during renal IRI^{204,205}.

In order to maintain mitochondrial homeostasis, complex interactions amongst B-cell lymphoma 2 (BCL-2) protein family members are required²⁰⁶. BCL-2 family proteins have long been known as key players in mediating cell survival and death through mitochondria²⁰⁷ and can be classified into two subgroups of pro-apoptotic or anti-apoptotic proteins²⁰⁸. Pro-apoptotic members such as BCL-2 associated X protein (BAX), BCL-2 homologous antagonist killer (BAK), and BCL-2 associated death promoter (BAD), BH3 interacting-domain death agonist (BID), and BCL-2-interaction mediator of cell death (BIM) causes a cascade of interactions that results in mitochondrial/intrinsic apoptosis under stress stimuli²⁰⁹. Anti-apoptotic members BCL-2, B-cell lymphoma-extra large (BCL-xL), and induced myeloid leukemia cell differentiation protein (MCL-1) act as inhibitors of the intrinsic apoptosis by physically binding to their pro-apoptotic counterparts and inhibiting their functions through degradation and sequestration²¹⁰.

During renal IRI, TEC experience a variety of physiological stress, including acidic pH¹⁴² and ROS production²¹¹. Under stress, TEC begin to up-regulate mRNA expressions of the BCL-2 family²¹², with the largest difference seen in BAX levels which remain elevated up to 7 days post-surgery²¹³. Cytosolic BAX is activated and translocates to organelle membranes²¹⁴ primarily onto mitochondrial outer membrane (MOM)²¹⁵, where it

undergoes conformational change^{216,217}. BAX facilitates the opening of voltage-dependent anion channel-1 (VDAC-1) by directly binding to VDAC-1 and forming a BAX-VDAC-1 heterodimer²¹⁸. Transiently, VDAC-1 opening induces downstream mitochondrial permeability transition pore (MPTP) formation and mitochondrial inner membrane (MIM) permeability²¹⁹. Along with cyclophilin D in the intermembrane matrix and adenine nucleotide translocator (ANT) on the inner mitochondrial membrane, VDAC forms the MPTP complex^{220,221}, and promotes the loss of mitochondrial membrane potential (MMP) and swelling, MIM permeability, and secondary rupture of the MOM²²². This secondary rupture represents a ‘point of no return’ as massive release of cytochrome c from the intermembrane space follows²²³ and results in apoptosis²²⁴. However, recent studies have suggested that BAX may facilitate intrinsic apoptosis by forming mitochondrial apoptosis-induced channel (MAC) through which cytochrome c is released²²⁵. This MAC formation theory follows the same concept in BAX translocation onto MOM, but suggests that BAX opens up MAC via homodimerization and heterodimerization with BAK which is constitutively bound to MOM^{214,226}. Released cytochrome c binds to apoptotic protease activating factor 1 (APAF-1) and forms a holoenzymatic apoptosome complex in a dATP-dependent manner²²⁷. Apoptosome formation is accompanied by synchronized pro-caspase-9 recruitment to Caspase Activation and Recruitment Domain (CARD) present on the N-terminus of APAF-1²²⁸. The precise mechanism of caspase-9 activation remains unknown, as two conflicting theories exist. While apoptosome may directly mediate cleavage and activation of pro-caspase-9²²⁹, recent studies suggest that apoptosome indirectly induces caspase-9 activation by aiding pro-caspase-9 dimerization and

autocatalysis^{230,231}. Active caspase-9 proceeds to cleave the downstream executioner pro-caspases-3, -6, and -7, and completing the cytochrome c-mediated feedback loop^{232,233}. Active executioner caspases cleave Inhibitor of Caspase Activated DNase (ICAD), allowing localization of caspase activated DNase (CAD) into the nucleus and triggers the subsequent fragmentation of DNA, as well as degradation of numerous intracellular proteins¹⁹⁵. As noted previously, up-regulation of TGF- β in TEC during renal IRI further augments intrinsic apoptosis^{212,234} by potentiating pro-caspase-9 cleavage¹²¹, thus highlighting the importance of intrinsic apoptosis in graft injury.

However, the intrinsic apoptosis cascade is strictly regulated both upstream and downstream of mitochondria by anti-apoptotic BCL-2, BCL-xL, and MCL-1 proteins^{208,209}. There seems to be a benefit conferred by anti-apoptotic proteins as over expression and augmentation of BCL-xL results in the reduction of IRI-induced TEC apoptosis and autophagy²³⁵. BCL-2 and BCL-xL form heterodimers with cytosolic BAX to prevent its translocation to MOM²³⁶. In its dephosphorylated state, BAD heterodimerizes with BCL-2 and BCL-xL, rendering them inactive and allowing BAX translocation to MOM. Protein kinase B (PKB) and insulin-like growth factor-1 (IGF-1) are two examples of proteins that mediate BAD phosphorylation and consequent formation of 14-3-3- BAD heterodimers causing dissociation of BAD from BCL-2 and BCL-xL²³⁷⁻²³⁹. During apoptosis, BAD dephosphorylation is mediated by calcineurin²⁴⁰, protein phosphatase 1 (PP1 α)²⁴¹, and 2A (PP2A)²⁴². This leads to BAD heterodimerization with BCL-2, BCL-xL, or MCL-1, allowing dissociation of BAX and BAK and downstream apoptosis cascade²⁴³. BCL-xL is also capable of stopping the

intrinsic apoptosis after release of cytochrome c, as BCL-xL binds to APAF-1 and prevent pro-caspase-9 cleavage²⁴⁴.

Intrinsic apoptosis of TEC has been extensively studied, and considerable evidence supports its involvement in both renal IRI and allograft rejection²³⁹. BCL-2 expression greatly decreases in TEC during renal IRI²⁴⁵, and chemotherapeutic agents such as cisplatin have been shown to cause severe nephrotoxicity via DNA damage, which leads to p53-mediated Apoptosis Inducing Factor (AIF) activation and apoptosis of TEC^{246,247}. However, discoveries of alternatively spliced isoforms of BCL-2 family members whose roles are yet unclear suggest there are as yet unknown interactions and kinetics of intrinsic apoptosis mediators²⁴⁸. One example is the recent discovery of caspase-3's ability to cleave BCL-2 and converting it to pro-apoptotic BAX-like death effector protein²⁴⁹. The lack of understanding is reflected by four different proposed models of BCL-2 family interaction²⁵⁰. Since TEC can undergo cell death by intrinsic apoptosis in the absence of RIPK3 and caspase-8, due to intact mitochondria, it remains that better understanding of the dynamics of IRI-induced intrinsic apoptosis would benefit devising strategies to reduce IRI and prolong graft survival.

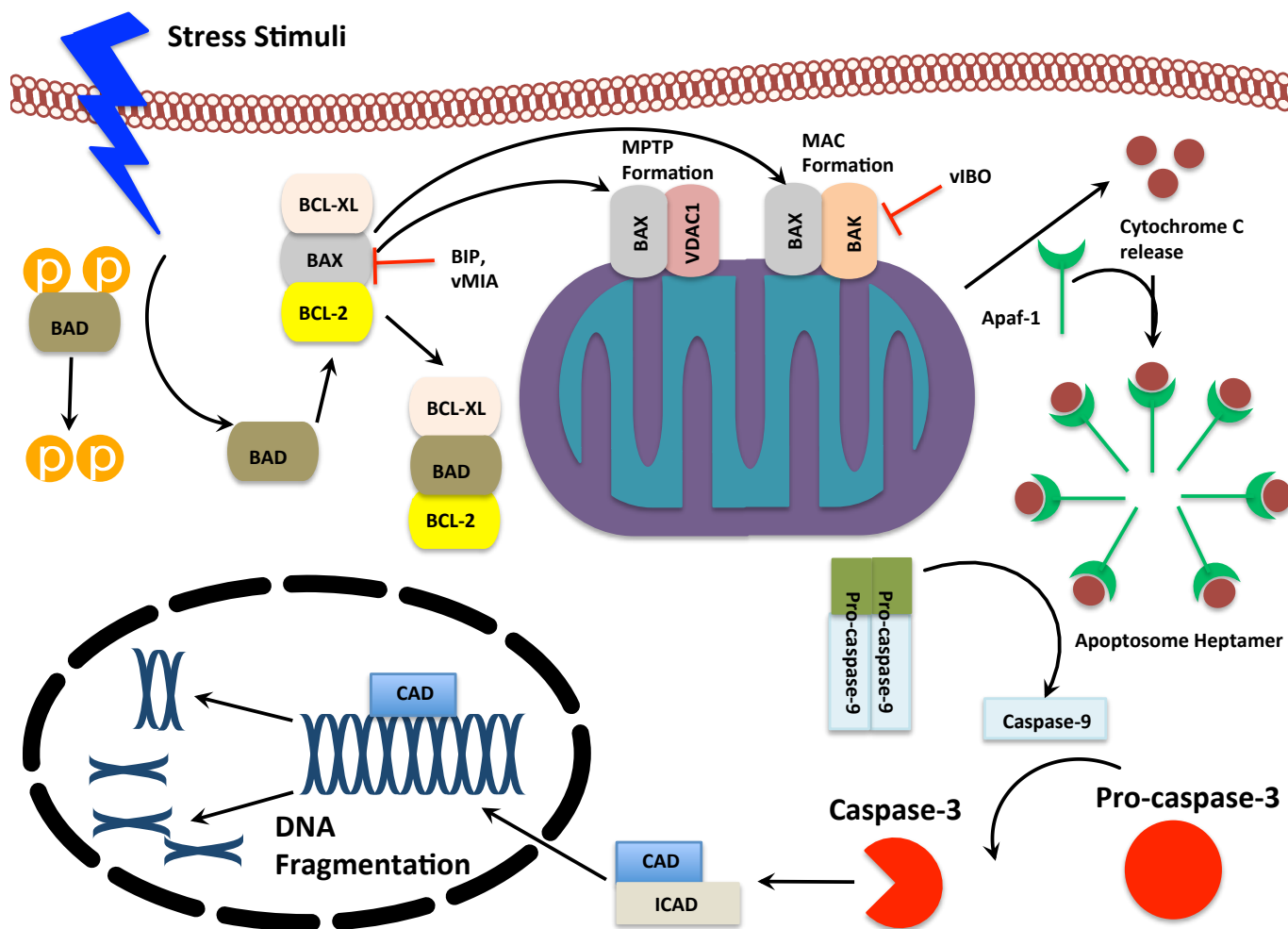


Figure 5. Mechanism of stress-induced intrinsic apoptosis

Stress stimuli including hypoxia and cytokines during IRI induce BAD dephosphorylation. BAD binds to anti-apoptotic BCL-2 and BCL-XL and inhibits their function, allowing dissociation of BAX. Free BAX translocates to mitochondria and starts the cascade of downstream effector molecule activation leading to mitochondrial depolarization, apoptosome formation, and caspase cleavage. Intrinsic apoptosis can be inhibited through BAX inhibition by BIP or vMIA protein of MCMV, or by BAK inhibition by vIBO protein of MCMV.

1.7 Hypothesis

We hypothesize that intrinsic apoptosis contributes to renal IRI but due to the counter-balanced nature of programmed cell death, it may be dysregulated in cells unable to undergo necroptosis or extrinsic apoptosis. Dysregulation or up-regulation of intrinsic apoptosis may counteract the benefits conferred by inhibition or loss of RIPK3. As cells lacking pathways of programmed cell death have intact NF- κ B and other pathways, we hypothesize that DKO TEC exposed to death ligand may demonstrate up-regulation of pro-inflammatory cytokine and chemokine production post-activation.

1.8 Specific aims

1. To show presence of remaining forms of cell death in TEC in the absence of necroptosis and caspase-8 dependent extrinsic apoptosis.
2. To investigate the effect of pro-inflammatory activation of TEC in the absence of RIPK3 and caspase-8 programs of cell death.
3. To explore the possibility of alleviating renal IRI by simultaneously inhibiting multiple cell death pathways including intrinsic apoptosis.

Chapter 2

2 Materials and Methods

2.1 Animals

C57BL/6 (H-2^b) mice were purchased from commercial sources (Jackson Laboratories, Bar Harbor, ME), the B6 RIPK3^{-/-} (H-2^b) were generously provided by Dr. Kim Newton (Genentech)²⁵¹, and the combined B6 RIPK3^{-/-} Caspase-8^{-/-} (H-2^b) (DKO) were provided by Dr. Douglas Green (St. Jude Children's Research Hospital, TN)¹⁹². Mice were maintained in the animal facility at the Western University and regularly screened for pathogens. RIPK3^{-/-} mice are phenotypically unremarkable, and have normal kidney function and breeding²⁵¹. RIPK3^{-/-} Caspase-8^{-/-} mice are normal phenotypically at a young age, but develop lymphadenopathy by four months of age due to accumulation of abnormal double negative T (DNT) cells in the periphery¹⁹⁶. Animal experiments were conducted in accordance with the Canadian Council on Animal Care guidelines under protocols approved by Western University.

2.2 Antibodies

Cell surface death receptor expressions were quantified with staining with PE-conjugated anti-TNFR-1 (eBiosciences), anti-DR5 (eBiosciences), anti-TLR 2 (eBiosciences), anti-TLR 4 (eBiosciences), and FITC-conjugated anti-TNFR-2 (Abcam) and anti-FAS (BD Pharmingen). All experiments were analyzed on a Cytomics FC500 flow cytometer (Beckman Coulter, Mississauga, ON).

2.3 Tubular epithelial cell (TEC) culture

Primary TEC cultures were obtained from the cortex region of kidneys from wild type, RIPK3^{-/-}, and RIPK3^{-/-} Caspase-8^{-/-} mice with a C57/BL6 background, by digestion with collagenase V at 1 mg/mL (Sigma) for 15 min in complete K1 media in a humidified incubator at 37 °C. The digested cells were then passed through a 40 µm cell strainer (BD Biosciences, San Jose, CA) and washed with PBS solution. The primary TEC were grown to a confluent monolayer inside 75 cm² vented flasks (BD Biosciences) supplemented with complete K1 culture medium (DMEM/Hams F12, 50:50; Invitrogen) supplemented with 5% bovine calf serum, hormone mix (5g/ml insulin, 1.25ng/ml prostaglandin E1, 34pg/ml triiodothyronine, 5g/ml transferrin, 1.73ng/ml sodium selenite, and 18ng/ml hydrocortisone), and 25ng/ml epidermal growth factor in a humidified incubator at 37 °C. The primary TEC were immortalized through lipofection of origin-defective SV40 using Lipofectamine 2000 reagent (Invitrogen) to be used for *in vitro* experiments. Phenotype of the TEC was confirmed using PE conjugated anti-CD 13 (Abcam), FITC conjugated anti-CD26 (BD Pharmingen), and FITC conjugated anti-E-cadherin (BioLegend)⁴³. 0.05% Trypsin-EDTA was used to passage the cells.

2.4 Fibroblast culture and MCMV propagation

NIH 3T3 fibroblasts were grown in 4.5g/ml glucose DMEM (Invitrogen) mixed with 10% fetal bovine serum, 2 mM L-glutamine, 100U/ml penicillin, and 100U/ml streptomycin (Invitrogen)²⁵². Stocks of wild-type K181 BAC MCMV strain were generously provided by Dr. Edward Mocarski (Emory University, GA)²⁵³. K181 BAC strain was grown and propagated on NIH 3T3 cells²⁵⁴, and crude stocks for *in vitro*

experiments were prepared as described²⁵⁵. TEC were infected with MCMV at MOI of 5 for 24 hours before treatment.

2.5 Kidney ischemia reperfusion injury (IRI)

A renal clamp was applied to the right kidney pedicle and removed after 45 minutes. The mouse was kept at 34°C and the left kidney was removed^{19,43,256}. Kidneys were collected at 48h post- IRI after being flushed with normal saline. Serum creatinine levels were tested by using an automated CX5 clinic analyzer (Beckman, Fullerton, CA).

2.6 RNA isolation and real-time polymerase chain reaction (RT-PCR)

Total RNA extraction from cultured TEC were performed with Trizol (Invitrogen, Carlsbad, CA). cDNA was generated using Superscript II (Invitrogen) and quantified by real-time polymerase chain reaction (RT-PCR) MX3005 (Stratagene, Santa Clara, CA) using SYBR QPCR Master Mix kit (Bio-Rad, Hercules, CA). β -actin amplification was used as the endogenous control. RT-PCR was performed with standard amounts of cDNA using the SYBR qPCR Master Mix kit (BioRad) and the normalized delta threshold cycle (Ct) value and relative expression levels ($\Delta\Delta C_t$) were calculated according to the manufacturer's protocol. Primers used in the RT-PCR include: β -actin: 5'-CTGTGCTATGTTGCTCTA-3' and 5'-AGGATTCCATACCCAAGA-3', BAX: 5'-TTTGCTACAGGGTTTCAT-3' and 5'-GTCCAGTTCATCTCCAAT-3', BAK: 5'-CATGAATCCACTGATACCA-3' and 5'-GTCACTTGTCACCTGAAT-3', BAD: 5'-CGATGAGTTTGAGGGTTC-3' and 5'-CTTTGTCGCATCTGTGTT-3', BID: 5'-

GGTTAGAAACGAGATGGA-3' and 5'-AACTCTTCAGATACACTCA-3', BIM: 5'-TGACAGAATAAGAGGACTT-3' and 5'-CAGGCAGAACTAAGATTG-3', BCL-2: 5'-ACTGAATGTAGATAATGG-3' and 5'-TAACTGTAACTGATAAGG-3', BCL-XL: 5'-ATAACCTTATCTTGGCTTTGG-3' and 5'-AACACCTGCTCACTTACT-3', MCL-1: 5'-GATTGGCATTCTTGGATTG-3' and 5'-GAACACAGTCATACTTGG-3', MCP-1: 5'-AGCTGTAGTTTTTGTCA-3' and 5'-GGTCAACTTCACATTCAA-3', RANTES: 5'-ATATGGCTCGGACACCACTC-3' and 5'-TCCTTCGAGTGACAAACACG-3', and IL-6: 5'-GAGGATACCACTCCCAACAGACC-3' and 5'-AAGTGCATCATCGTTGTTCATACA-3'.

2.7 Cell viability and death assays

TEC were grown to confluent monolayers and treated with cyclohexamide (CHX) (Sigma), recombinant human TNF α (hTNF α) (Peprotech), and lipopolysaccharide (LPS) (Sigma). Cell death was detected with propidium iodide (PI) or Annexin-V labeling (BD Bioscience) and analyzed by flow cytometry (Beckman Coulter). TEC were also treated with murine interferon- γ (IFN- γ ; Peprotech, Rocky Hill, NJ), interleukin-1 β (IL-1 β ; R&D Systems, Minneapolis, MN), and Bax-inhibiting peptide V5 (BIP; Sigma, Oakville, ON, Canada) in serum-free media. After 24 hours, TEC were incubated with 12mM MTT (Life Technologies, Burlington, ON, Canada) for 4 hours before absorbance was measured at 490nm. Untreated TEC were set as 100% viable. Cell death was quantified using the Sytox Green reagent (Essen Bioscience, Ann Harbor, MI). 100nM of Sytox Green reagent was added to wells containing TEC. Incucyte ZOOM (Essen Bioscience)

live cell imaging incubator was used to scan for Sytox Green positivity at 30-45 min intervals over 24 hours.

2.8 Caspase-9 and caspase-3 assays

To quantify caspase-9 activity, luciferase activity dependent on caspase-9-mediated generation of luciferase substrate was measured. TEC were grown to confluent monolayers and treated with IFN- γ , IL-1 β , and BIP for 24 h. 70 μ L of Caspase-Glo-9 reagent (Caspase-Glo-9; Promega, Madison, WI) was added directly to the TEC cultures. Luminescence emission was detected after 1 hour using a VictorX Light (PerkinElmer). Cleaved caspase-3 activity was measured using CellPlayer™ Kinetic Caspase-3/7 Apoptosis Assay Reagent (Essen Bioscience, Ann Harbor, MI). 5 μ M of the CellPlayer™ Kinetic Caspase-3/7 Apoptosis Assay Reagent was added to wells containing TEC and Incucyte ZOOM (Essen Bioscience) live cell imaging incubator was used to scan for the reagent positivity at 30-45 min intervals over 24 hours.

2.9 Histology and immunochemistry

Kidney sections were stored in 5% formalin (Sigma) for at least 3 days and fixed in paraffin before being stained with hematoxylin and eosin (H&E). The slides were scored for acute tubular necrosis by a pathologist in a blinded fashion (0: no change, 1: <25% area change, 2: 25–50% area change, 3: 50–75% area change, 4: 75% area change) using a semiquantitative method as described^{43,257,258}.

2.10 Statistical analysis

For parametric data, One way- and Two way- Analysis of Variance (ANOVA) tests were used to compare multiple groups, and Student's t-test was used for unpaired values.

GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA) was used for all statistical analysis. Data are presented as mean \pm SEM using $p < 0.05$ for significance unless specified.

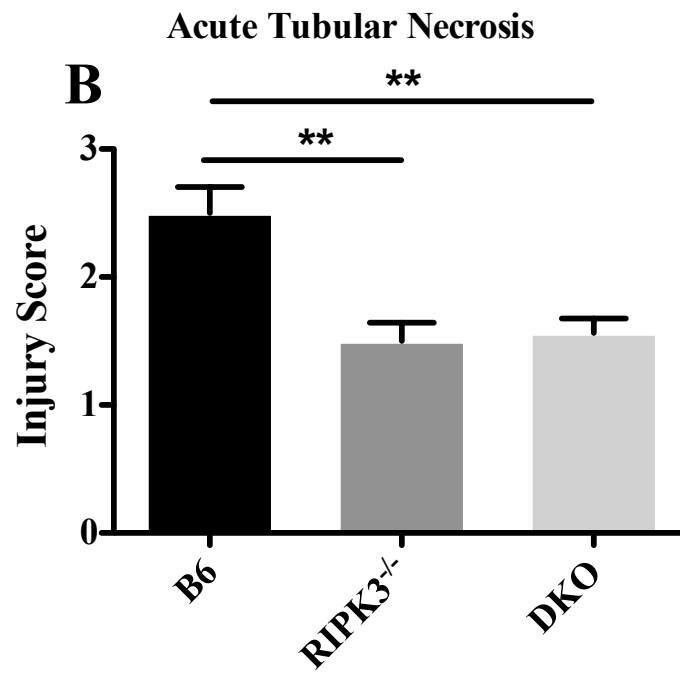
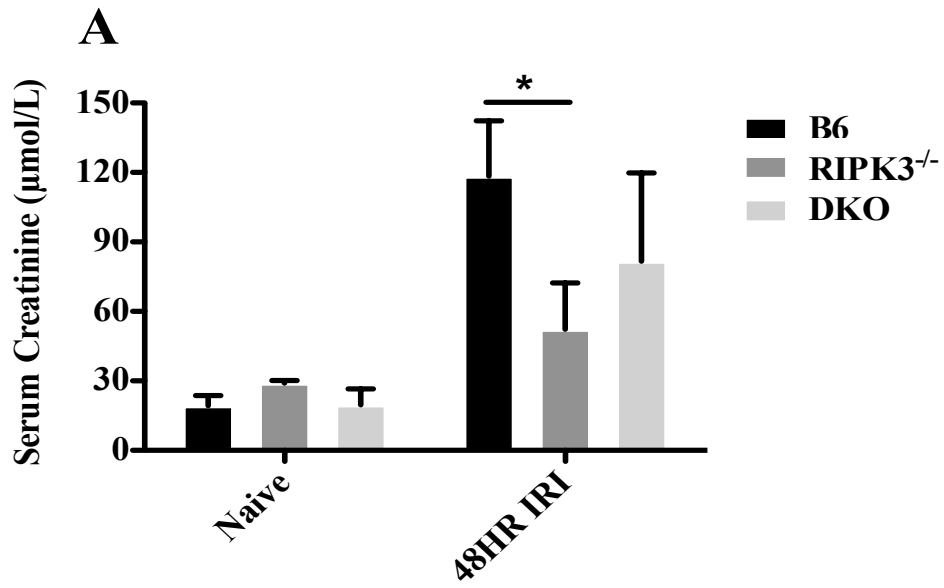
Chapter 3

3 Results

3.1 Loss of RIPK3 and caspase-8 does not show additional benefits over RIPK3 deletion alone in renal IRI

Blocking necroptosis with the RIPK1 inhibitor Nec-1 can significantly attenuate damage due to renal IRI¹³⁶. Similarly, RIPK3^{-/-} mice are more resistant to kidney damage post IRI¹³⁵. Silencing of caspase-8 as well as FAS (CD95) by siRNA and has shown to be of benefit in a mouse IRI model²⁵⁹. However, remaining residual injury in these models may reflect that total injury was related to the sum of individual pathways of cell injury and death. Recently DKO mice became available to study, as the loss of caspase-8 is embryonic lethal and is salvaged by the genetic deletion of RIPK3. We thus decided to investigate the potential benefit of inhibition of both RIPK3 and caspase-8 by subjecting wild type, RIPK3^{-/-}, and DKO mice to renal IRI as described¹⁹. Kidney and serum creatinine samples were collected at 48h post-IRI to assess ATN and kidney function, respectively. We confirmed that the absence of RIPK3 resulted in improved kidney function as shown by lower serum creatinine levels post-IRI (51 ± 20 $\mu\text{mol/L}$ in IRI-treated RIPK3^{-/-} vs. 118 ± 24 $\mu\text{mol/L}$ in IRI-treated wild type C57BL/6 mice, $n=9/\text{group}$, $p=0.04$, Figure 6A). In contrast, DKO mice did not show any further improvements compared to RIPK3 knockout alone, and indeed DKO mice showed slightly increased serum creatinine levels (82 ± 38 $\mu\text{mol/L}$ in IRI-treated DKO mice vs. 51 ± 20 $\mu\text{mol/L}$ in IRI-treated RIPK3^{-/-} mice, $p>0.05$, $n=6-9/\text{group}$). Histological assessment of ATN showed similar results, where wild-type mice showed higher injury scores compared to both RIPK3^{-/-} (2.5 ± 0.1 vs. 1.5 ± 0.2 , $n=4/\text{group}$, $p=0.007$, Figure 6B) and DKO mice

(2.5 ± 0.1 vs. 1.6 ± 0.1 , $p=0.0014$, $n=4-8/\text{group}$). Consistent with serum creatinine levels, DKO mice showed no improvement over $\text{RIPK3}^{-/-}$ mice (Injury score 1.6 ± 0.1 vs. 1.5 ± 0.2 , $p=0.75$, $n=4-8/\text{group}$). Therefore, in contrast to our original belief that combined loss of both caspase-8 apoptosis and RIPK3 necroptosis would improve IRI by eliminating independent pathways of injury within the kidney, our data clearly demonstrates that the absence of both RIPK3 and caspase-8 does not result in any further benefit compared to RIPK3 loss alone. As these pathways of cell death share components and are counter-balanced in some cases, such as caspase-8 control of necroptosis, these data suggest that there may be an interaction on remaining forms of cell death or a compensating mechanism that attenuated the expected benefit.



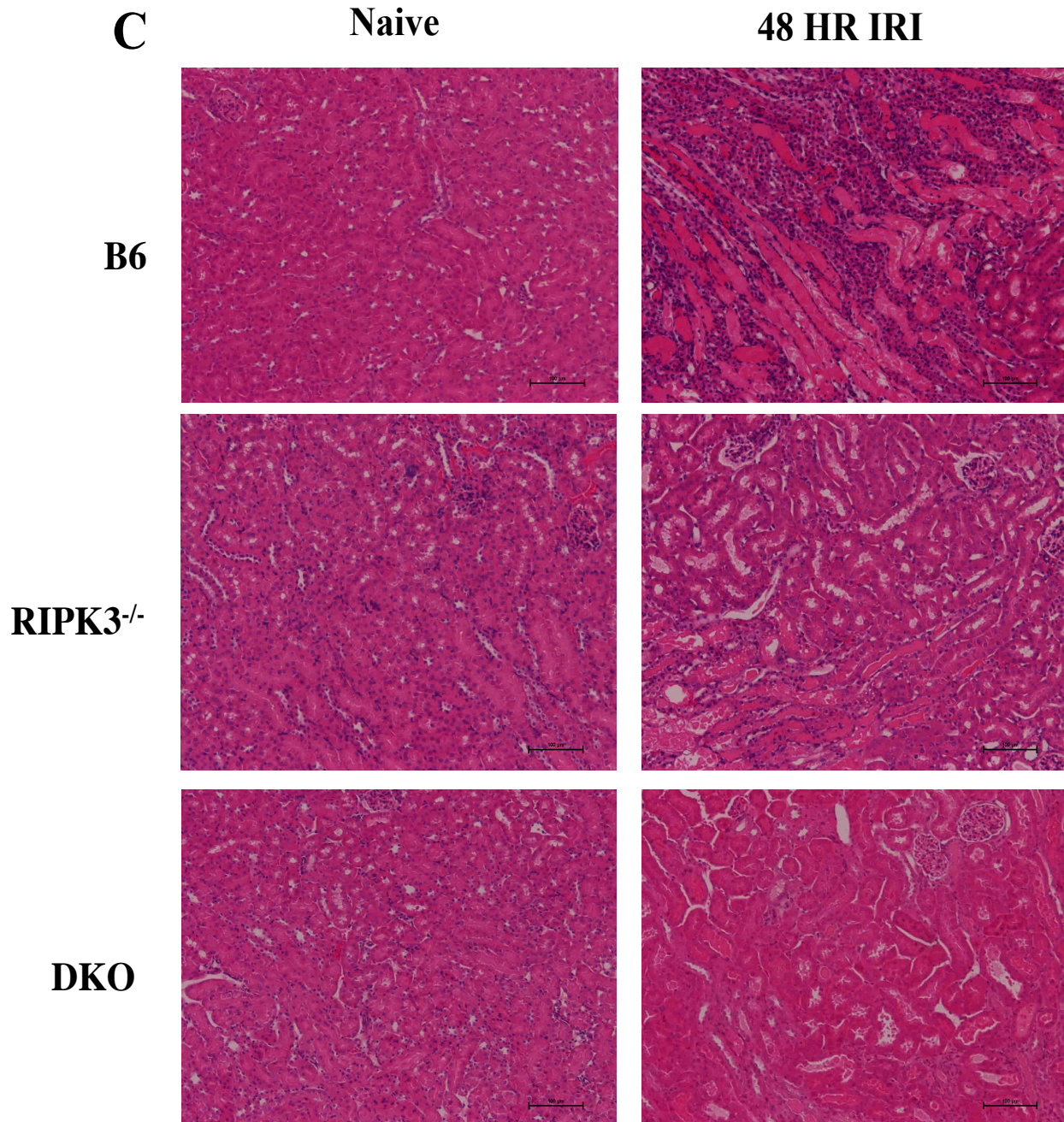


Figure 6. Loss of RIPK3 and caspase-8 does not show additional benefits over RIPK3 deletion alone in renal IRI

A renal clamp was applied to the right kidney pedicle of wild type C57BL/6, RIPK3^{-/-}, and RIPK3^{-/-} Caspase-8^{-/-} (H-2b), and was removed after 45 minutes at 34°C and the left kidney was collected. Kidneys and serum creatinine were collected at 48h post- IRI to measure acute tubular necrosis and kidney function, respectively. **A.** Serum creatinine levels were tested by using an automated CX5 clinic analyzer (n=6-9/group, *: p <0.05, Two-Way ANOVA). **B-C.** Obtained kidneys were fixed in formalin at the time of sacrifice and stained with H&E. Slides were scored for acute tubular necrosis by a pathologist in a blinded fashion (0: no change, 1: <25% area change, 2: 25–50% area change, 3: 50–75% area change, 4: 75% area change) using a semiquantitative method. (n=4-8/group, **: p <0.01, One-Way-Anova)

3.2 Establishment and characterization of the C57BL/6 tubular epithelial cell line

Immortalized wild type B6, RIPK3^{-/-}, and DKO TEC cell lines were generated from C57BL/6 kidneys as described in the methods for *in vitro* experiments. The cobblestone phenotype of TEC was confirmed by morphology (Figure 7A), and CD13, CD26, and E-cadherin positivity were analyzed by flow cytometry (Figure 7B).

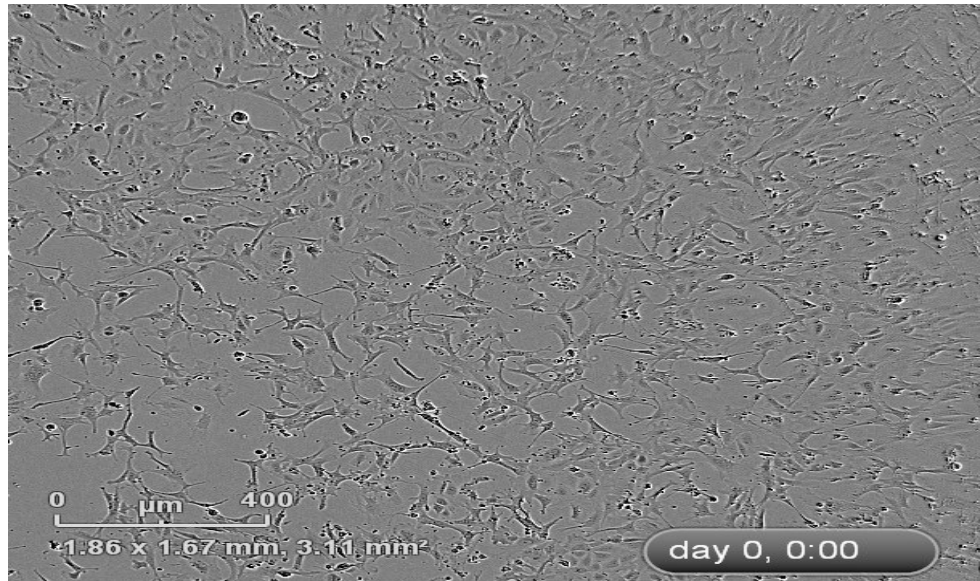
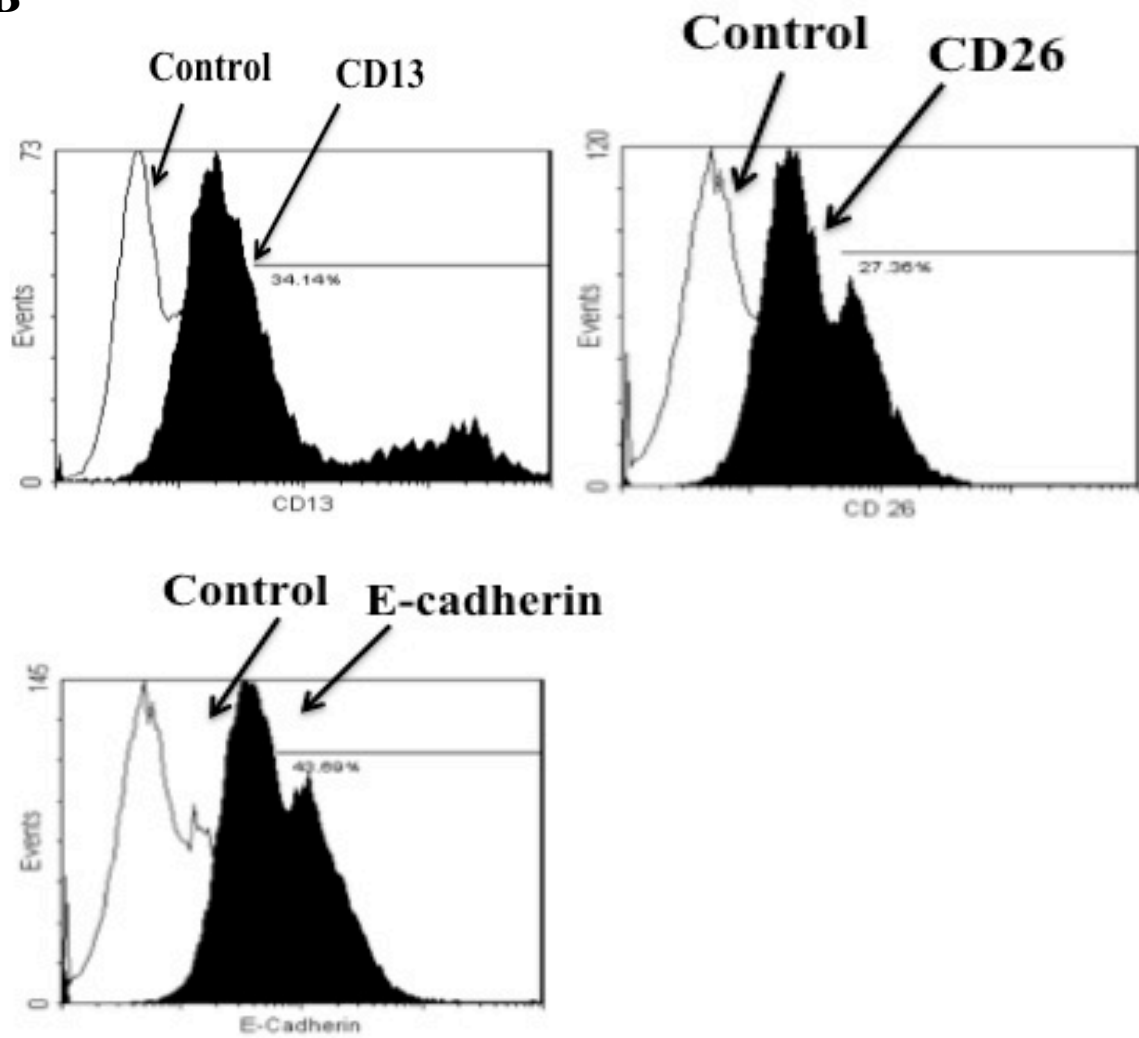
A**B**

Figure 7. Establishment and characterization of the C57BL/6 tubular epithelial cell line

TEC were isolated and immortalized with origin deficient SV40 DNA. **A.** Cells were grown as monolayers and the typical cobblestone phenotype of epithelial cells was confirmed using Incucyte Zoom live cell tracker at 20 X magnification. **B.** Cells were also analyzed for the presence of the epithelial markers CD13, CD26 and E-cadherin by flow cytometry.

3.3 Death receptor expressions in the TEC do not change with cytokine stimulation

The absence of caspase-8 or RIPK3 may have altered the expression of surface receptors, as for example; the loss of FAS increases the expression of FASL in other cells. We therefore investigated for expression level differences of various cell surface death receptors across the three cell lines, as a potential compensation for the loss of RIPK3 and caspase-8. As well during IRI, regulation of numerous genes that contribute to IRI are affected²³⁴, which includes the up-regulation of cell surface death receptors that contributes heavily in promoting inflammation and death of TEC²⁶⁰. The current knowledge of whether there are any regulational differences of the surface death receptors in the absence of RIPK3 and caspase-8 is limited. The expression levels of TNFR-1, TNFR-2, FAS, DR 5, TLR 2, and TLR 4 in the three cell lines were surveyed using FACS analysis, as all have been associated with IRI-induced damage. As shown in Figure 8, the basal expression levels of these death receptors on TEC was not found to be different across the cell lines. As well, treating the cells with the combination of 10ng/mL TNF α and 250U/mL IFN γ to activate the TEC did not up-regulate these receptors. Therefore, the absence of RIPK3 and caspase-8 does not change cell surface death receptor expression to account for any increased injury or death in DKO mice, or to account for absence of benefit with IRI. Similar results were obtained with primary TEC.

Wild type
RIPK3^{-/-}
DKO

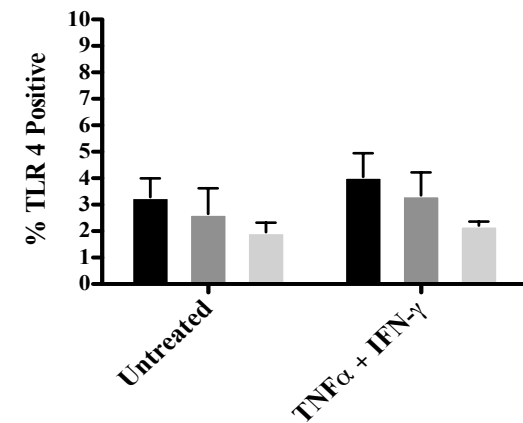
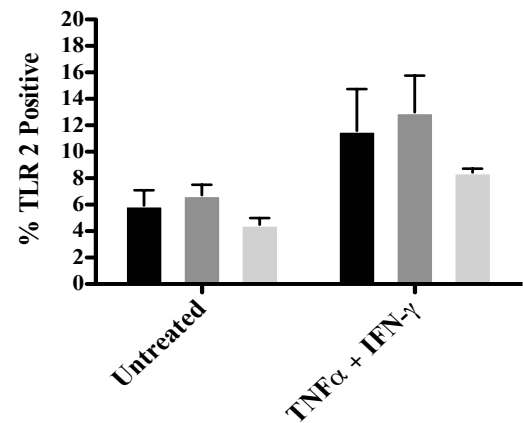
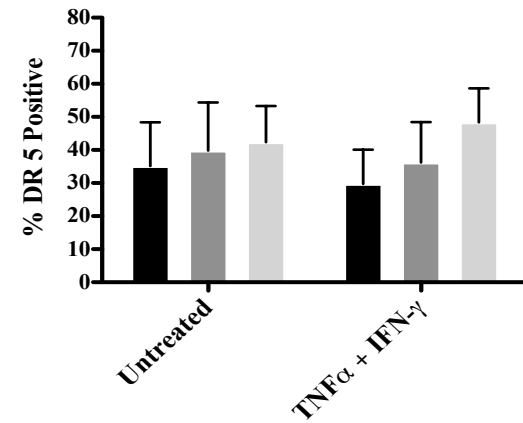
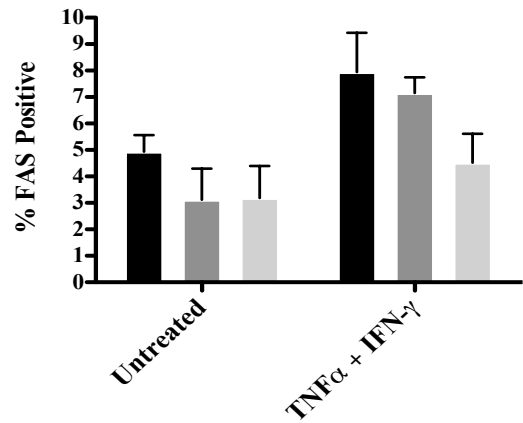
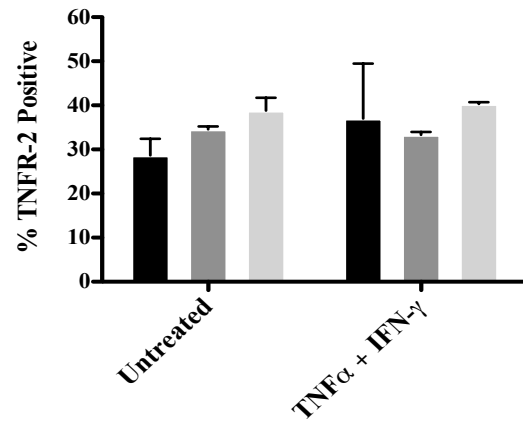
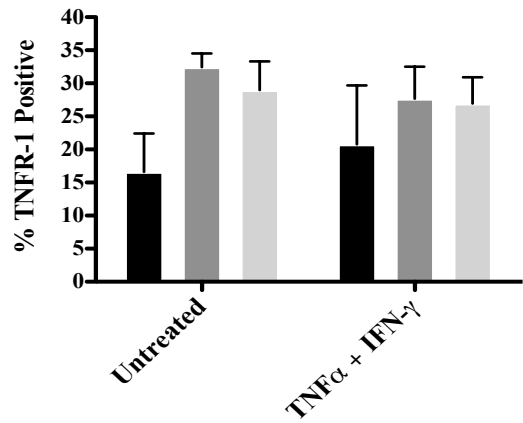


Figure 8. Death receptor expressions across the cell lines do not change with cytokine-mediated activation

TEC were cultured as described in the methods. TEC were treated with 10ng/mL TNF α and 250U/mL IFN- γ for 24 hours in serum-free media. TEC were analyzed for surface expression levels of TNFR-1, TNF-R2, FAS, DR5, TLR 2, and TLR 4 by flow cytometry. Figure values represent the mean \pm SEM of three independent experiments (n=3, Two-Way-ANOVA).

3.4 TEC have endogenous resistance to cell death

After observing no differences in cell surface death receptor expression, we next attempted to induce cell death in TEC *in vitro* and assess for potential differences in cell death using the combination of CHX + TNF α and LPS, as they are classic activators of receptor-mediated death pathways. In previous work, we have used additional exposure to IFN γ to promote cell death. TEC were treated with combination of 1 ug/mL of CHX, 300 ng/mL of hTNF α , and 1 ug/mL LPS for 24 hours and cell death was determined by Annexin-V and PI staining using flow cytometry. Since murine TNF α signals through both TNFR-1 and TNFR-2 and promotes both cell death and survival pathways respectively^{261,262}, hTNF α was used to maximize TNFR-1-mediated cell death due to its preferential binding and activation of TNFR1-mediated death pathways²⁶³. The addition of CHX further enhances this process^{136,264}. LPS binds and activates TLR 2^{265,266}, as well as TLR 4^{267,268}. TLR activation leads to recruitment of adaptor protein TIR-domain-containing adaptor-inducing interferon- β (TRIF)^{269,270}, which interacts with RIPK1 and RIPK3 complexes using RHIM domain and thus activates necroptosis²⁷¹. As shown in Figure 9, CHX + hTNF α increased Annexin-V in the wild type cells corresponding to increased apoptosis. LPS treatment had no additional effect. These treatments had no effect on either the RIPK3^{-/-} TEC or the DKO TEC and death in the TEC after 24 hours was similar to untreated controls. We observed similar results with primary TEC. This demonstrates that TEC are generally resistant to receptor-induced death but, importantly, there were no differences between DKO and other TEC to account for our *in vivo* results in IRI.

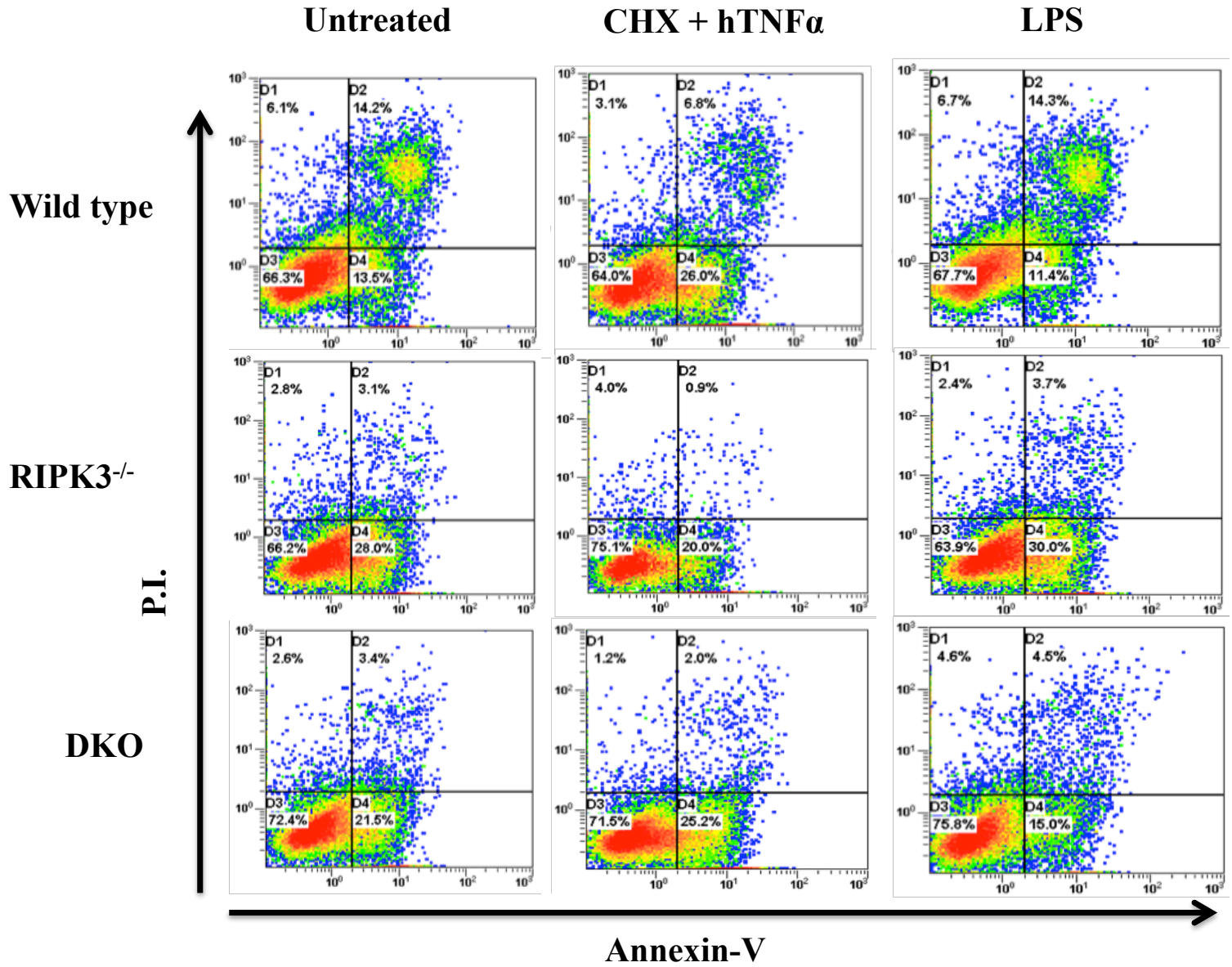


Figure 9. DKO TEC are not more sensitive to death induced by death receptors than RIPK3^{-/-} or wild type TEC

Wild type, RIPK3^{-/-}, and DKO TEC were each plated in equal numbers (2.5×10^5) and treated with CHX (1 $\mu\text{g/mL}$) + hTNF α (300 ng/mL) and LPS (1 $\mu\text{g/mL}$) for 24 hours in serum-free media. Cell death was characterized with Annexin-V and PI staining using flow cytometry analysis. Figures here are representative of five independent experiments in which the pattern of response was similar.

3.5 Absence of RIPK3 and caspase-8 sensitizes TEC to death induced by the combination of IL-1 β and IFN- γ

Alternate forms of cell death can be enhanced with perturbations of regulated cell death pathways, and TEC are clearly capable of undergoing other forms of cell death independent of RIPK3 and caspase-8. Since no additional benefits in renal IRI were observed from *in vivo* IRI experiments (Figure 6), we hypothesized that loss of both RIPK3 and caspase-8 might be sensitizing TEC to another form of cell death. Caspase-9 dependent intrinsic pathways of apoptosis should be intact in TEC even with loss of caspase-8 or RIPK3, but has not been tested before. Since IL-1 β and IFN- γ combination is known to induce BAX-dependent intrinsic apoptosis in other cell types²⁷², we performed repeated concentration finding assays by treating TEC with various amounts of IL-1 β and IFN- γ in combination. We determined that 4 ng/ml of IL-1 β and 120 ng/mL of IFN- γ most effectively induced TEC death (viability 68.7 ± 0.9 % in IL-1 β + IFN- γ treated wild type vs. 100 % in untreated wild type, n=4, p<0.0001; 79.2 ± 2.8 % in IL-1 β + IFN- γ treated RIPK3^{-/-} vs. 100% in untreated RIPK3^{-/-}, n=4, p=0.0018; 32.5 ± 10.1 % in IL-1 β + IFN- γ treated DKO vs. 100 % in untreated DKO, n=4, p=0.0026, Figure 10). Interestingly, DKO TEC treated with IL-1 β + IFN- γ showed greatest decrease in survival compared to wild type (viability 32.5 ± 10.1 % vs. 68.7 ± 0.1 %, n=4, p=0.024) and RIPK3^{-/-} TEC (viability 32.5 ± 10.1 % vs. 79.2 ± 2.8 %, n=4, p=0.011). Since MTT assays were used which measures total survival, it was not possible from these experiments to confirm the type of cell death induced by the IL-1 β + IFN- γ combination that accounted for the decreased cell viability we observed in DKO TEC. However, our results show that in the absence of necroptosis and extrinsic apoptosis, TEC appear to have an increased

sensitivity to IL-1 β + IFN- γ induced death. As this induces intrinsic apoptosis in other cells, it follows that DKO TEC may be more sensitive to induction of intrinsic apoptosis.

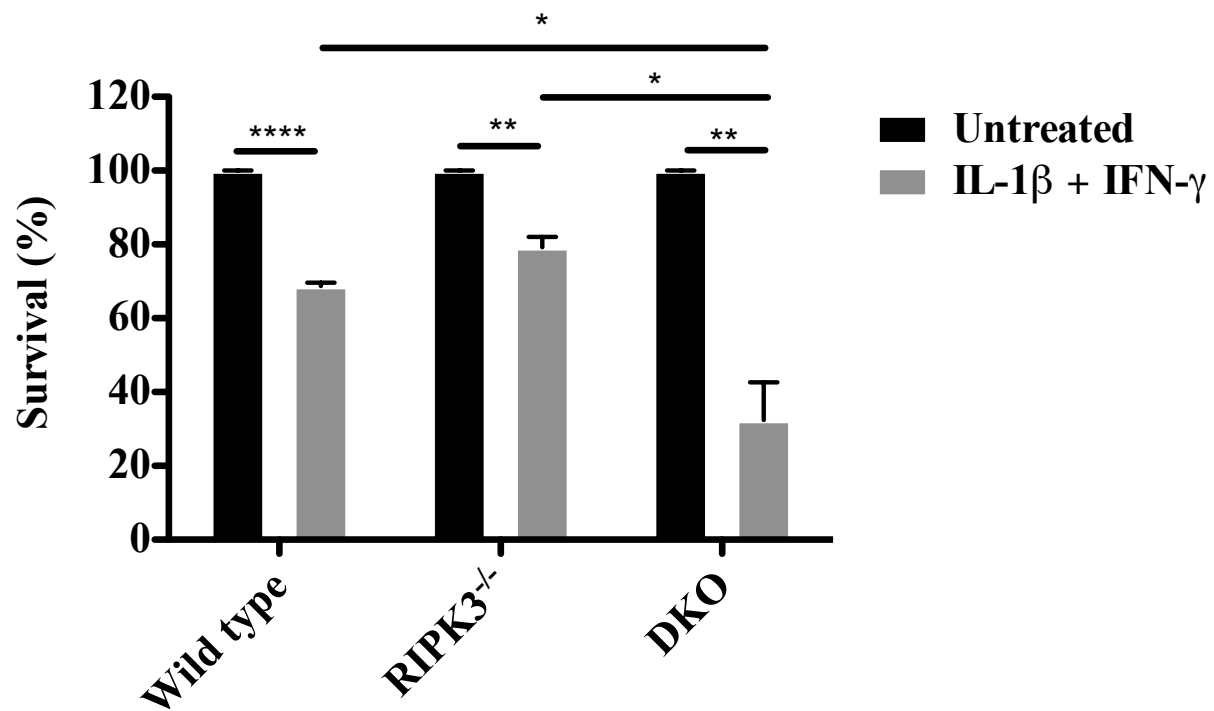


Figure 10. Absence of RIPK3 and caspase-8 sensitizes TEC to death induced by the combination of IL-1 β and IFN- γ

Wild type, RIPK3^{-/-}, and DKO TEC were cultured as described and treated with combination of 4 ng/mL of IL-1 β and 120 ng/mL of IFN- γ for 24 hours. Cell viability was measured and quantified using MTT assay analyses. This figure represents one of four independent experiments that showed the same responses (n=4, *: p < 0.05, **: p<0.01, ****: p<0.0001, Two-Way ANOVA).

3.6 Absence of RIPK3 and caspase-8 result in basal level mRNA up-regulation of pro-apoptotic BAX and BAK

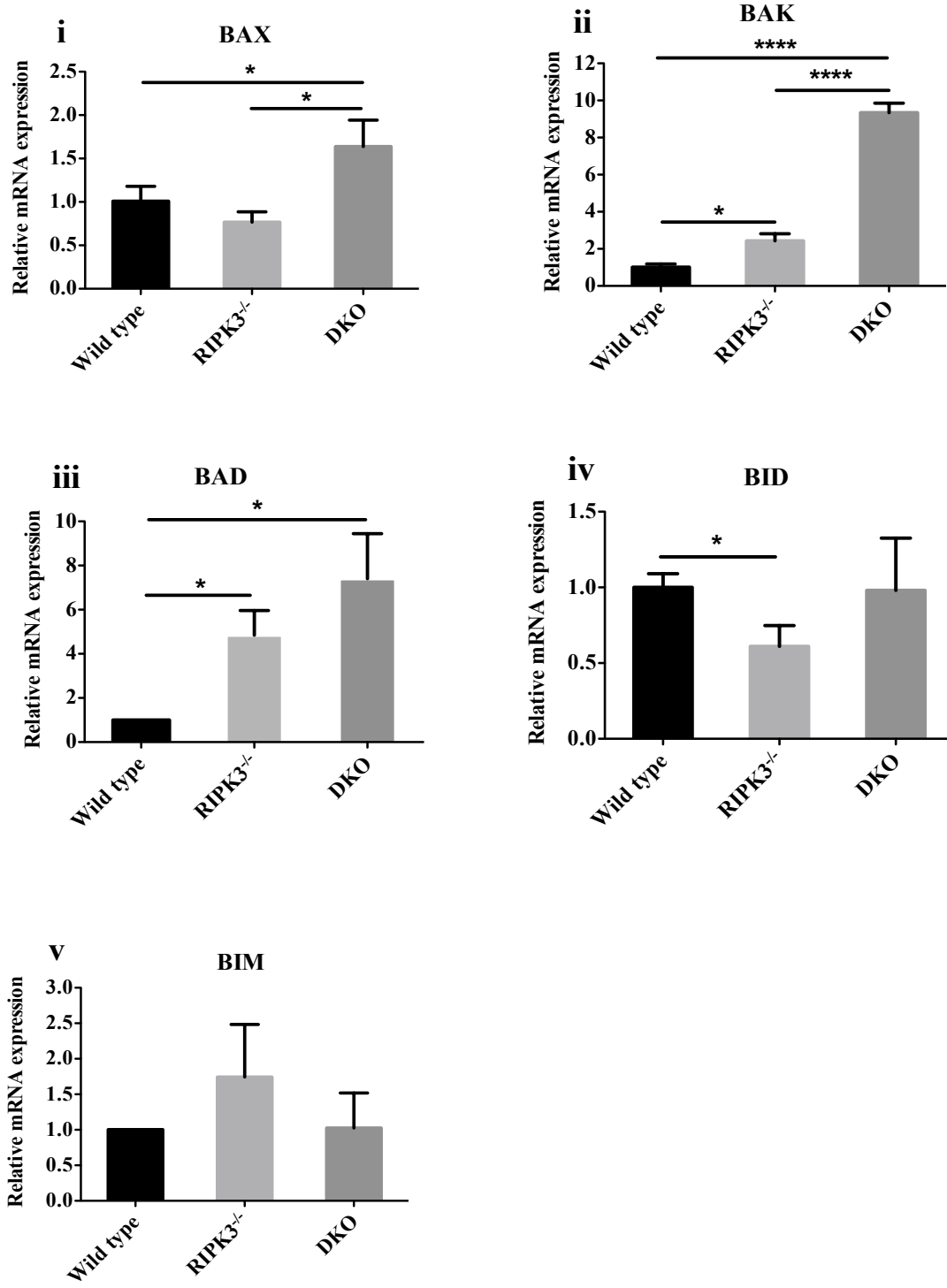
BCL-2 family proteins are key mitochondrial mediators and regulators of apoptosis²⁵⁰, and their roles in regulating TEC death in IRI have been well documented^{212,213}. Studies have shown that apoptotic stimuli such as IL-1 β + IFN- γ mediate the activation of pro-apoptotic BAX, BAK, and BAD^{243,273,274}. Upon our observation that DKO TEC are significantly more sensitized to IL-1 β + IFN- γ induced death (Figure 10), we hypothesized that this may involve transcriptional level differences of mitochondrial factors. We therefore assessed basal mRNA levels of BCL-2 family members in each of the cell lines using RT-PCR. We noted that basal levels of BAX, BAK, and BAD in DKO TEC were higher than wild type or RIPK3^{-/-} TEC. As shown in Figure 11Ai-ii, compared to RIPK3^{-/-} TEC, DKO TEC showed significant basal level up-regulation of pro-apoptotic BAX (1.7 ± 0.2 vs. 0.8 ± 0.1 , fold of change, $n=3/\text{group}$, $p=0.036$, Figure 11Ai) and BAK (9.4 ± 0.3 vs. 2.4 ± 0.3 , fold of change, $n=3/\text{group}$, $p<0.0001$, Figure 11Aii). Level of BAD was higher in DKO TEC compared to RIPK3^{-/-} TEC (7.4 ± 2.0 vs. 4.9 ± 1.1 , fold of change, $n=3/\text{group}$, $p=0.33$) but was significantly higher than wild type TEC (9.4 ± 0.3 vs. 1 ± 0 , fold of change, $n=3/\text{group}$, $p=0.035$, Figure 11Aiii). Level of BID was decreased in RIPK3^{-/-} TEC compared to wild type TEC (0.61 ± 0.1 vs. 1 ± 0 , fold of change, $n=3/\text{group}$, $p=0.015$, Figure 11iv), and BIM showed no difference across the cell lines (Figure 11v).

There was no significant difference of BCL-2 expression in DKO TEC compared to wild type (155.1 ± 76.75 vs. 1 ± 0 , fold of change, $n=3/\text{group}$, $p=0.115$) and RIPK3^{-/-} TEC (155.1 ± 76.75 vs. 1.3 ± 0.2 , fold of change, $n=3/\text{group}$, $p=0.116$, Figure 11Bi).

RIPK3^{-/-} TEC showed reduction in BCL-xL compared to wild type TEC (0.5 ± 0.1 vs. 1 ± 0 , fold of change, $n=3/\text{group}$, $p=0.007$, Figure 11Bii) but showed no difference compared to DKO TEC. Level of MCL-1 was higher in DKO TEC compared to RIPK3^{-/-} TEC (2.9 ± 0.2 vs. 2.2 ± 0.4 , fold of change, $n=3/\text{group}$, $p=0.03$) but significantly higher than wild type TEC (2.9 ± 0.2 vs. 1 ± 0 , fold of change, $n=3/\text{group}$, $p=0.0003$, Figure 11Biii).

These results suggest that in absence of RIPK3 and caspase-8, transcription of certain pro- and anti-apoptotic members of BCL-2 family is altered, for reasons that are not clear. Higher levels of pro-apoptotic BAX, BAK, and BAD, as well as anti-apoptotic MCL-1 and BCL-2 were observed in DKO TEC. Our results show that the sensitization to intrinsic apoptosis displayed by DKO TEC is due to predisposition from transcriptional level up-regulation of pro-apoptotic BAX, BAK, and BAD. Even in normal growth media, it is possible that signaling from surface receptors may alter mitochondrial proteins in the absence of cell death by caspase-8 mediated apoptosis or by RIPK3-mediated necroptosis.

A



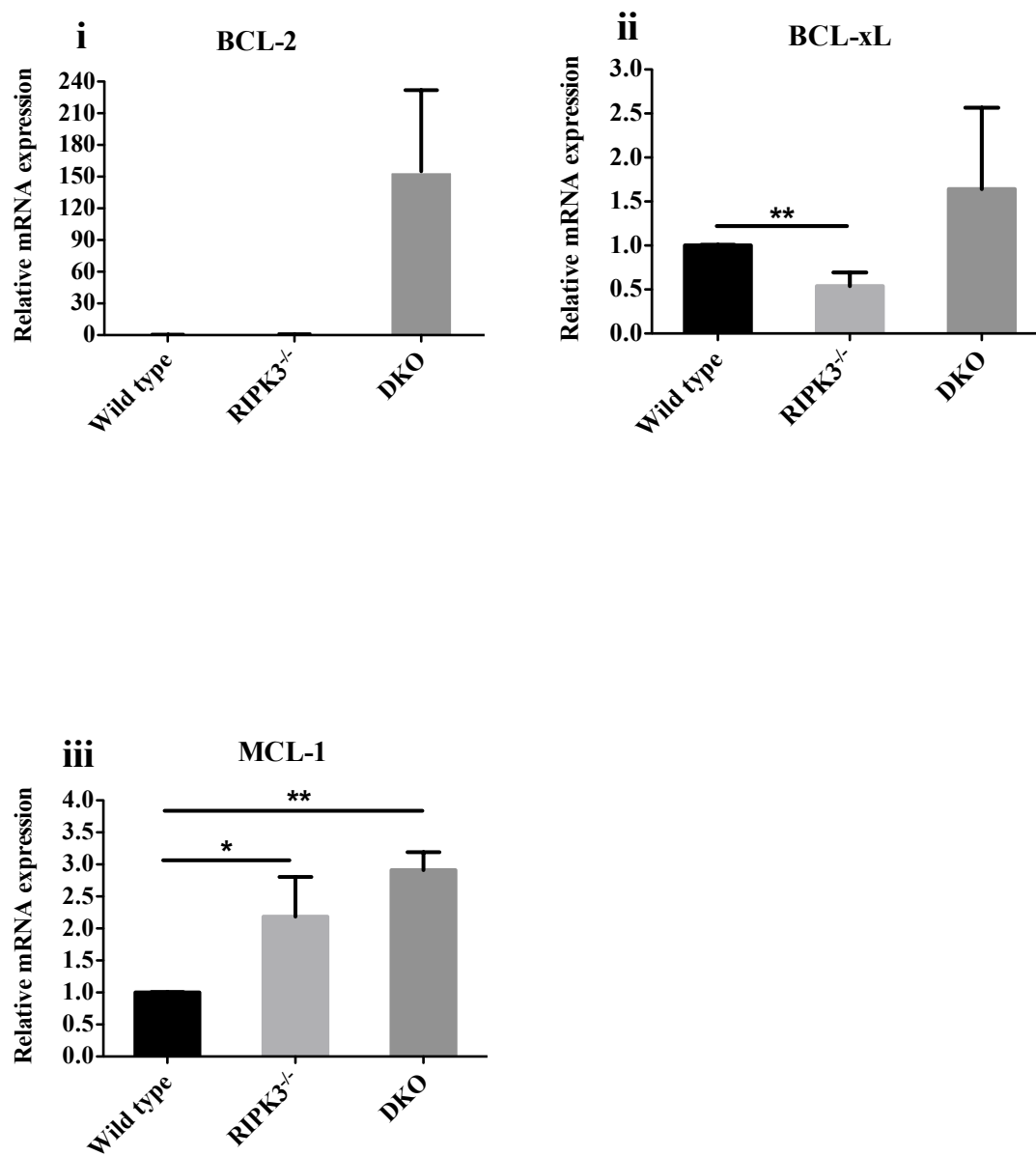
B

Figure 11. Absence of RIPK3 and caspase-8 affects basal level mRNA regulation of BCL-2 family members in TEC

TEC were cultured as described in the methods. mRNA levels of **Ai-v.** pro-apoptotic BAX, BAK, BAD, BID, BIM and **Bi-iii.** anti-apoptotic BCL-2, BCL-xL, and MCL-1 were measured by RT-PCR as described in the methods. β -actin was used as loading control.

Figure represents one of three independent experiments that show the same result (n=3, *: p < 0.05, **: p<0.01, ****: p<0.0001, One-Way ANOVA). Similar results were observed in primary TEC.

3.7 Loss of RIPK3 and caspase-8 augments BAX-mediated intrinsic apoptosis in TEC

After observing the up-regulation of basal levels of BAX, BAK, and BAD in DKO TEC and increased sensitivity to IL-1 β + IFN- γ induced death, we next hypothesized that death by intrinsic apoptosis in DKO TEC was dependent on BAX, as been shown in other cell types. To confirm this, we exposed TEC to IL-1 β + IFN- γ with or without BIP to inhibit BAX. Consistent with our previous data (Figure 10), TEC treated with IL-1 β + IFN- γ showed decreased viability (survival 51.7 ± 2.1 % in treated wild type vs. 100 in untreated wild type, $n=3$, $p<0.001$; 39.7 ± 5.5 % in treated RIPK3 $^{-/-}$ vs. 100 in untreated RIPK3 $^{-/-}$, $n=3$, $p=0.0004$; 17.9 ± 1.8 % in treated DKO vs. 100 in untreated DKO, $n=3$, $p<0.001$, Figure 12Ai). The IL-1 β + IFN- γ treatment was thus most effective in killing DKO TEC compared to wild type or RIPK3 $^{-/-}$ TEC. Survival of TEC using the BAX inhibitor BIP (50uM BIP) was not different between the cell lines (survival 32.4 ± 5.1 in wild type vs. 25.2 ± 3.2 in RIPK3 $^{-/-}$, $n=3$, $p=0.29$; 32.4 ± 5.1 in wild type vs. 28.3 ± 4.3 in DKO, $n=3$, $p=0.57$; 25.2 ± 3.2 in RIPK3 $^{-/-}$ vs. 28.3 ± 4.3 in DKO, $n=3$, $p=0.58$, Figure 12Aii). However, DKO TEC showed the greatest improvement in relative survival with BIP treatment compared to wild type (relative improved survival %: 156 ± 7.9 vs. 62.1 ± 7.9 , $n=3$, $p=0.0011$) and RIPK3 $^{-/-}$ TEC (156 ± 7.9 vs. 69.0 ± 7.4 , $n=3$, $p=0.0013$, Figure 12Aiii). Therefore, although there was more death in the DKO TEC, the addition of BAX inhibition restored the survival of DKO TEC much more than in wild type or RIPK3 $^{-/-}$ TEC, supporting the previous data of enhanced intrinsic apoptosis in DKO TEC.

We also used Sytox Green reagent in conjunction with an Incucyte ZOOM live cell imaging system as described to label and observe late apoptotic and early secondary

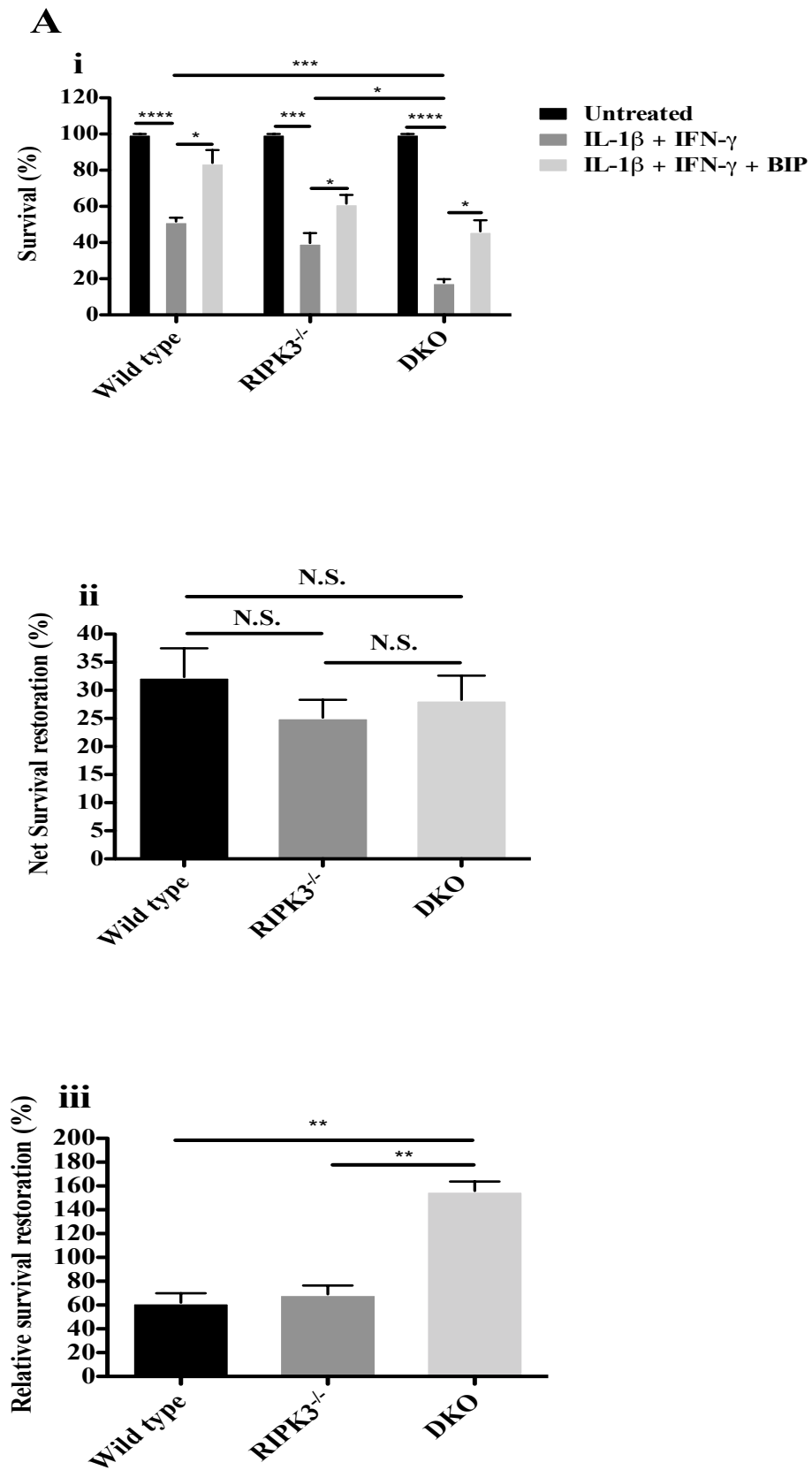
necrotic TEC in real time over a span of 24 hours. Sytox uptake is increased in cells that are undergoing cell death including necrosis and late apoptosis/early necrosis. IL-1 β + IFN- γ treatment induced cell death in all three cell lines (Sytox uptake 22940 ± 1998 in treated wild type vs. 6881 ± 457.5 in untreated wild type, $n=3$, $p<0.001$; 23740 ± 3269 in treated RIPK3^{-/-} vs. 4323 ± 419.9 in untreated RIPK3^{-/-}, $n=3$, $p=0.0042$; 33790 ± 2334 in treated DKO vs. 901 ± 341.1 in untreated DKO, $n=3$, $p<0.002$, Figure 12Bi-iv). The highest Sytox uptake after IL-1 β + IFN- γ treatment was consistently observed in DKO TEC. Furthermore, the addition of BIP decreased Sytox uptake in all cell lines, although this achieved statistical significance in DKO TEC (6398 ± 2022 vs. 33790 ± 2334 , $n=3$, $p=0.009$) but not in wild type (17780 ± 523 vs. 22940 ± 1998 , $n=3$, $p=0.067$) or RIPK3^{-/-} TEC (13400 ± 4007 vs. 23740 ± 3269 , $n=3$, $p=0.12$, Figure 12Bi).

Since BAX-mediated intrinsic apoptosis relies on apoptosome-mediated caspase-9 activation²²⁸, which leads to downstream executioner caspase-3 activation¹⁹³, we tested caspase-9 and caspase-3 activity in TEC to more definitively determine whether cell death was indeed intrinsic apoptosis. Caspase-9 activity was measured by luciferase assays as described. TEC were treated with IL-1 β + IFN- γ in the absence or presence of 50uM BIP for 24 hours to induce intrinsic apoptosis. Caspase-9 activity was measured 24 hours after treatment and was quantified by luminescence intensity. TEC treated with IL-1 β + IFN- γ showed up-regulation of caspase-9 activity (luminescence intensity 13730 ± 601.2 in treated wild type vs. 6261 ± 467.3 in untreated wild type, $n=3$, $p=0.0006$; 10670 ± 1881 in treated RIPK3^{-/-} vs. 3734 ± 292.4 in untreated RIPK3^{-/-}, $n=3$, $p=0.022$; 19370 ± 2397 in treated DKO vs. 4119 ± 1236 in untreated DKO, $n=3$, $p=0.0048$, Figure 12C). Caspase-9 activity of IL-1 β + IFN- γ treated TEC was thus higher in DKO than

RIPK3^{-/-} TEC (19370 ± 2397 vs. 10670 ± 1881 , $n=3$, $p=0.046$), although statistical significance was not achieved when compared to wild type TEC (19370 ± 2397 vs. 13730 ± 601.2 , $n=3$, $p=0.085$). Interestingly, addition of BIP led to decreased caspase-9 activity only in DKO TEC (11070 ± 1342 vs. 19370 ± 2397 , $n=3$, $p=0.039$) but not in wild type (13710 ± 2308 vs. 13730 ± 601.2 , $n=3$, $p=0.99$) or RIPK3^{-/-} TEC (11650 ± 2285 vs. 10670 ± 1881 , $n=3$, $p=0.76$).

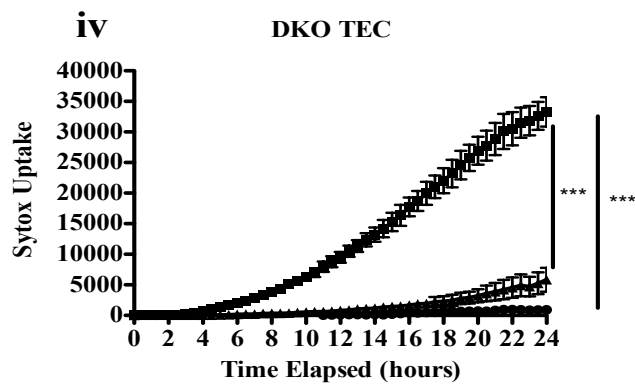
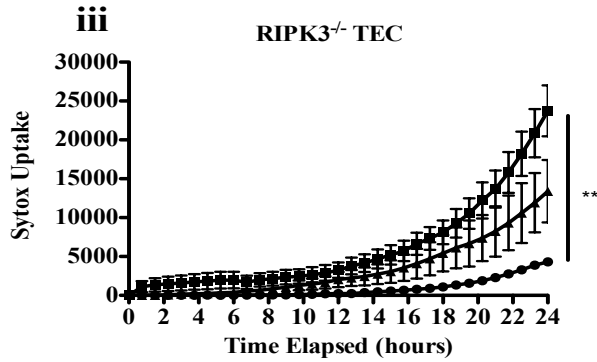
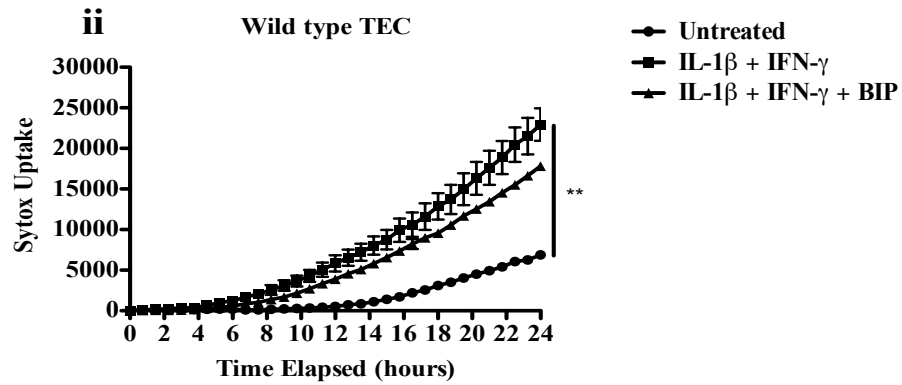
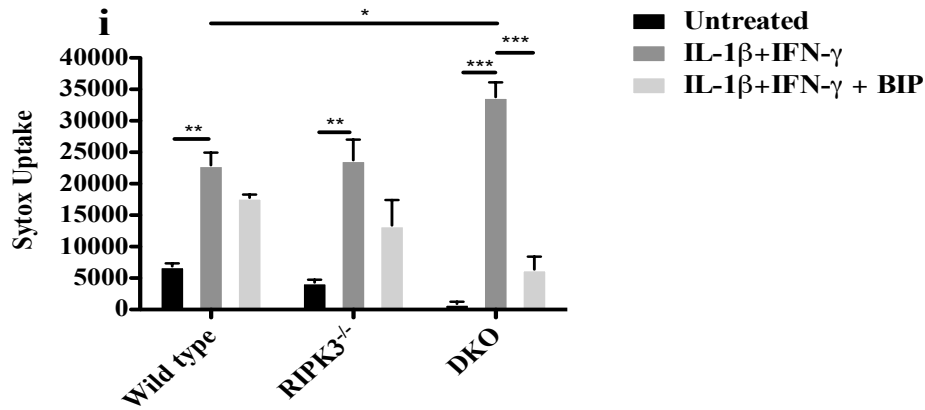
To confirm whether up-regulation of caspase-9 activity resulted in the expected up-regulation of downstream caspase-3 activity, we measured caspase-3 activity as described. 5 μ M of the CellPlayer™ Kinetic Caspase-3/7 Apoptosis Assay Reagent was added to treated and untreated TEC. Incucyte ZOOM live cell imaging analyses were used to scan at 30 minute intervals over 24 hours. The pan-caspase inhibitor Z-vad-fmk was added to confirm caspase mediated apoptotic cell death. IL-1 β + IFN- γ treatment induced caspase-3 activity in all three cell lines (caspase-3 positivity 7636 ± 806.1 in treated wild type vs. 4503 ± 120 in untreated wild type, $n=3$, $p=0.018$; 6605 ± 631.4 in treated RIPK3^{-/-} vs. 3207 ± 933.7 in untreated RIPK3^{-/-}, $n=3$, $p=0.039$; 62760 ± 3808 in treated DKO vs. 4310 ± 400.7 in untreated DKO, $n=3$, $p<0.001$, Figure 12Di-iv). Consistent with our caspase-9 results, DKO TEC showed the greatest increase in caspase-3 activity post-treatment compared to wild type (62760 ± 3808 vs. 7636 ± 806.1 , $n=3$, $p=0.001$) or RIPK3^{-/-} TEC (62760 ± 3808 vs. 6605 ± 631.4 , $n=3$, $p=0.001$ Figure 12Di). The addition of BIP led to results similar to those observed in caspase-9 assays. Caspase-3 activity decreased in DKO TEC (8199 ± 1078 vs. 62760 ± 3808 , $n=3$, $p=0.0002$) but not in wild type (8075 ± 1465 vs. 7636 ± 806.1 , $n=3$, $p=0.8$) or RIPK3^{-/-} TEC (8573 ± 1887 vs. 6605 ± 631.4 , $n=3$, $p=0.37$). Z-vad-fmk was effective in all of wild type (285.7

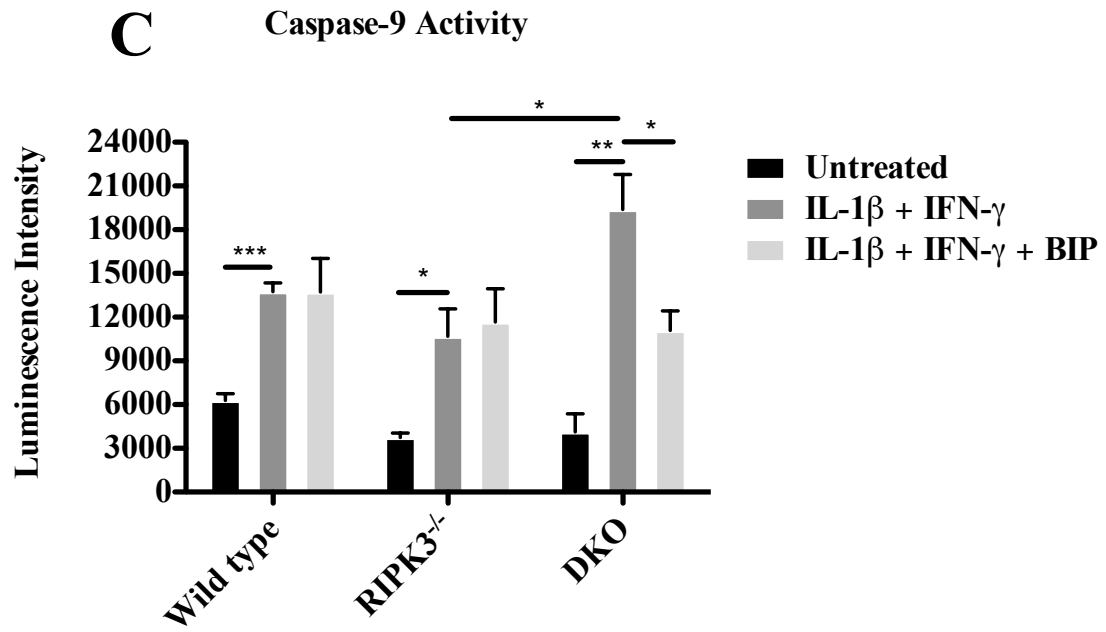
± 140 vs. 7636 ± 806.1 , $n=3$, $p=0.0008$), $\text{RIPK3}^{-/-}$ (-521 ± 389 vs. 6605 ± 631.4 , $n=3$, $p=0.0007$), and DKO TEC (511.3 ± 49.74 vs. 62760 ± 3808 , $n=3$, $p<0.0001$), and addition achieved near-complete reduction of caspase-3 activity. Our data indicates the loss of both RIPK3-mediated necroptosis and caspase-8-mediated extrinsic apoptosis results in augmentation of caspase-9 and caspase-3 activities in TEC, which can be recovered by addition of BIP and Z-vad-fmk. These results support that enhanced intrinsic apoptosis occurs in DKO TEC.



B

Sytox Positivity after 24 hr





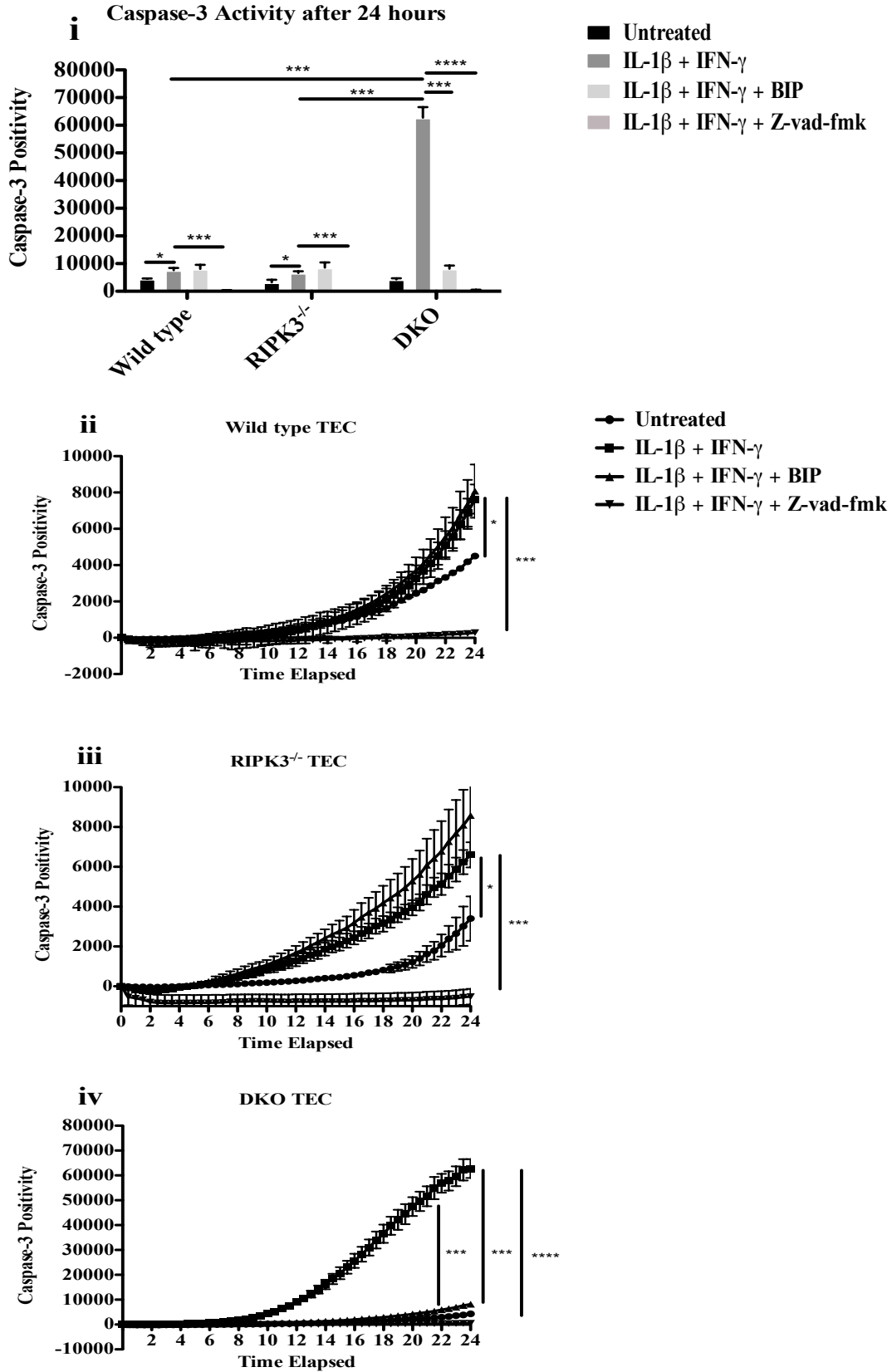
D

Figure 12. Loss of RIPK3 and caspase-8 augments BAX-mediated intrinsic apoptosis in TEC

Immortalized wild type, RIPK3^{-/-}, and DKO TEC were cultured as described and treated with combination of 4 ng/mL of IL-1 β and 120 ng/mL of IFN- γ with or without 1 hour pre-incubation of 50uM BIP, or 50uM Z-vad-fmk for 24 hours. **Ai-iii.** Cell viability was measured and quantified using MTT assay analysis, and **Bi-iv.** Sytox Green was used to stain and quantify late apoptotic and early secondary necrotic cells. **C.** Luminescence assay was used to measure caspase-9 activity and **Di-iv.** CellPlayer™ Kinetic Caspase-3/7 Apoptosis Assay Reagent was used to measure caspase-3 activity. Figures represent one of three independent experiments, which showed the same results (n=3, *: p < 0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001, Two-Way ANOVA).

3.8 MCMV completely abrogates intrinsic apoptosis in TEC

In order to inhibit host cell death, certain viruses encode proteins that inhibit programmed cell death to ensure their survival. One such virus, murine cytomegalovirus (MCMV), possesses a potent and somewhat unique capacity to inhibit multiple cell death pathways simultaneously with inhibitor proteins. Most notably, MCMV uses vCIA to inhibit caspase-8 mediated extrinsic apoptosis²⁷⁵, vMIA and vIBO to inhibit BAX and BAK-mediated intrinsic apoptosis^{198,200}, and vIRA to inhibit RIPK3-mediated necroptosis^{252,276}, and these inhibitions have been observed repeatedly in different cell lines. Elegant studies have demonstrated that loss of function mutations to these viral inhibitor proteins results in the loss of specific anti-death functions, to then allow MCMV-infected cells to die exclusively through that specific pathway. Thus, simultaneous inhibition of death pathways is required for effective survival of the virus. We utilized MCMV's capacity to alter cell death to further study intrinsic cell death in DKO TEC. To test whether the MCMV's cell death inhibition capabilities could alter TEC survival following death-inducing treatments, we measured the viability of MCMV-infected TEC post- IL-1 β + IFN- γ treatment using MTT assays. Propagation of wild-type K181 BAC MCMV strain, crude stock harvesting, and TEC infection were performed as described in methods²⁵⁵. IL-1 β and IFN- γ were used to induce intrinsic apoptosis in uninfected and MCMV-infected TEC. Uninfected TEC showed expected decreased viability with IL-1 β + IFN- γ treatment (viability 57.0 ± 9.6 in uninfected treated wild type TEC vs. 100 in uninfected untreated wild type TEC, $n=3$, $p=0.046$; 39.3 ± 3.77 in uninfected treated RIPK3^{-/-} TEC vs. 100 in uninfected untreated RIPK3^{-/-} TEC, $n=3$, $p=0.0038$; 29.3 ± 0.3 in uninfected treated DKO TEC vs. 100 in uninfected untreated DKO TEC, $n=3$, $p<0.0001$, Figure 12).

Importantly, with MCMV infection, we observed near complete salvage of viability in response to IL-1 β + IFN- γ treatment in the cell lines (viability $85.0 \pm 9.3\%$ in infected treated wild type TEC vs. $57.0 \pm 9.6\%$ in uninfected treated wild type TEC, $n=3$, $p=0.025$; $87.3 \pm 3.53\%$ in infected treated RIPK3 $^{-/-}$ TEC vs. $39.3 \pm 3.8\%$ in uninfected treated RIPK3 $^{-/-}$ TEC, $n=3$, $p=0.022$; 53.7 ± 3.93 in infected treated DKO TEC vs. 29.3 ± 0.3 in uninfected IL-1 β + IFN- γ treated DKO TEC, $n=3$, $p<0.0001$). Interestingly, MCMV infection decreased the overall viability of DKO TEC (viability $64.0 \pm 4.0\%$ in infected untreated DKO TEC vs. 100 in uninfected untreated DKO TEC, $n=3$, $p=0.012$), which was not observed in wild type or RIPK3 $^{-/-}$ TEC. In summary, our data suggests that MCMV infection improves the survival of TEC from IL-1 β + IFN- γ induced intrinsic apoptosis.

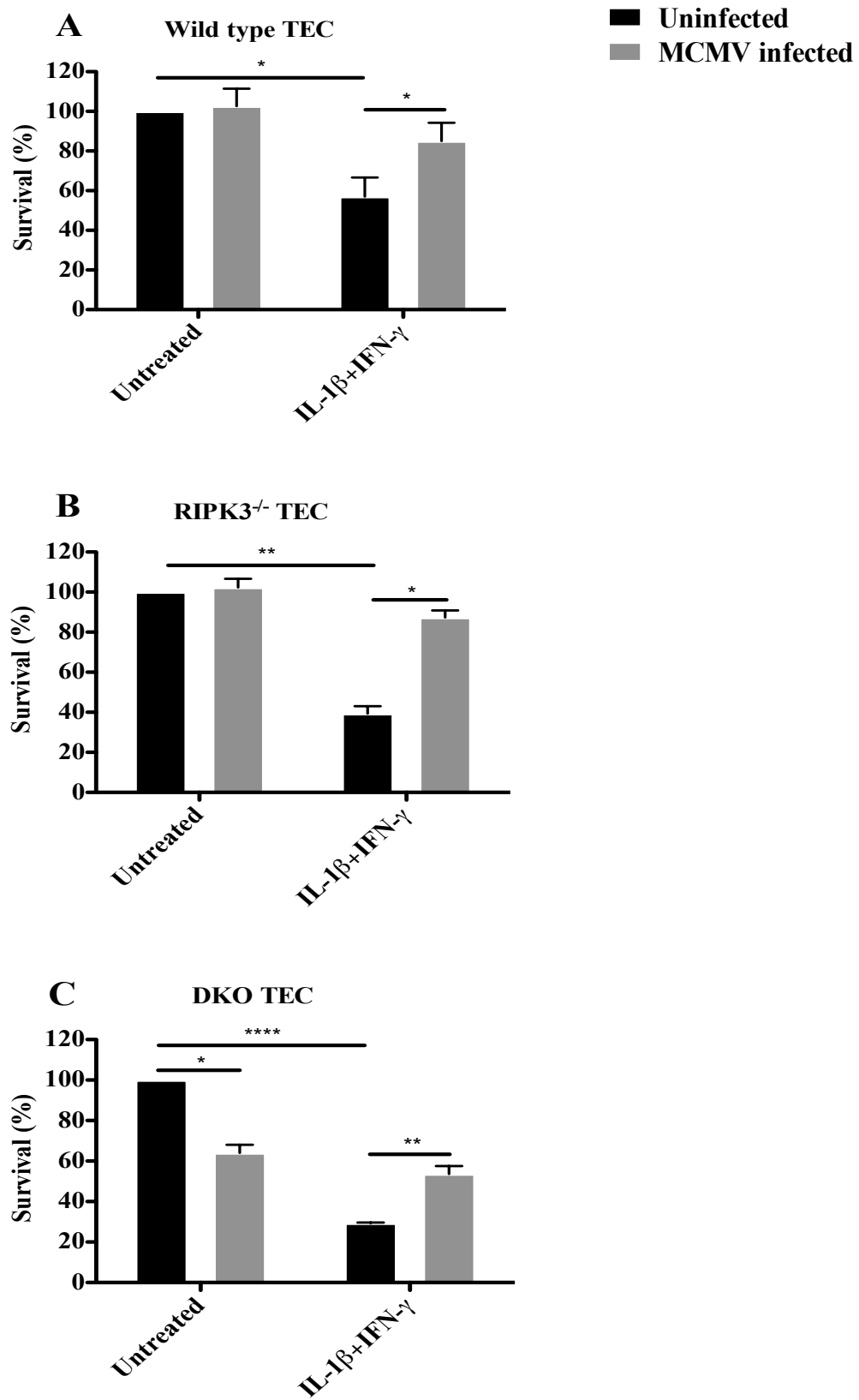


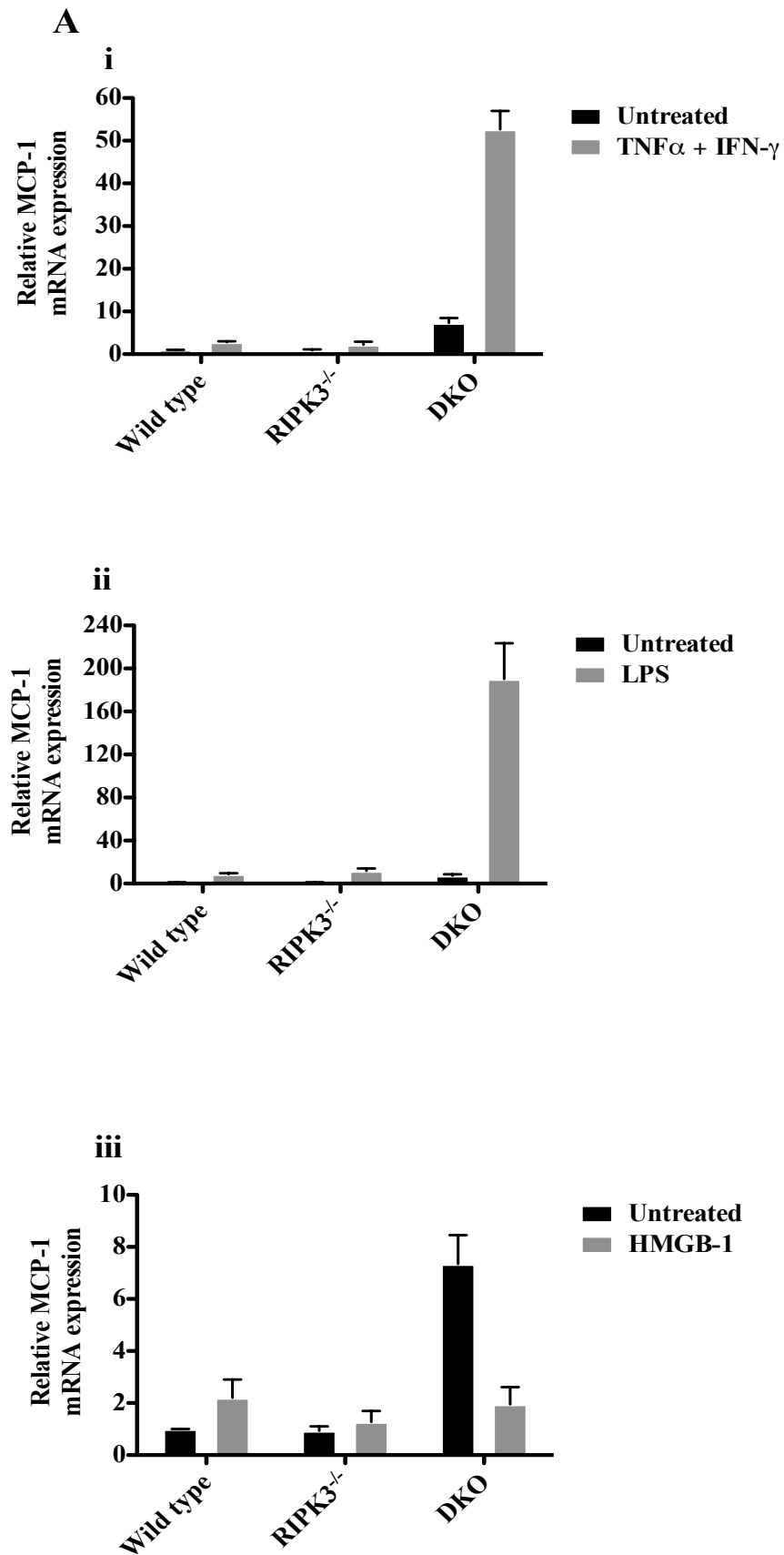
Figure 13. MCMV abrogates IL-1 β /IFN- γ induced intrinsic apoptosis in TEC

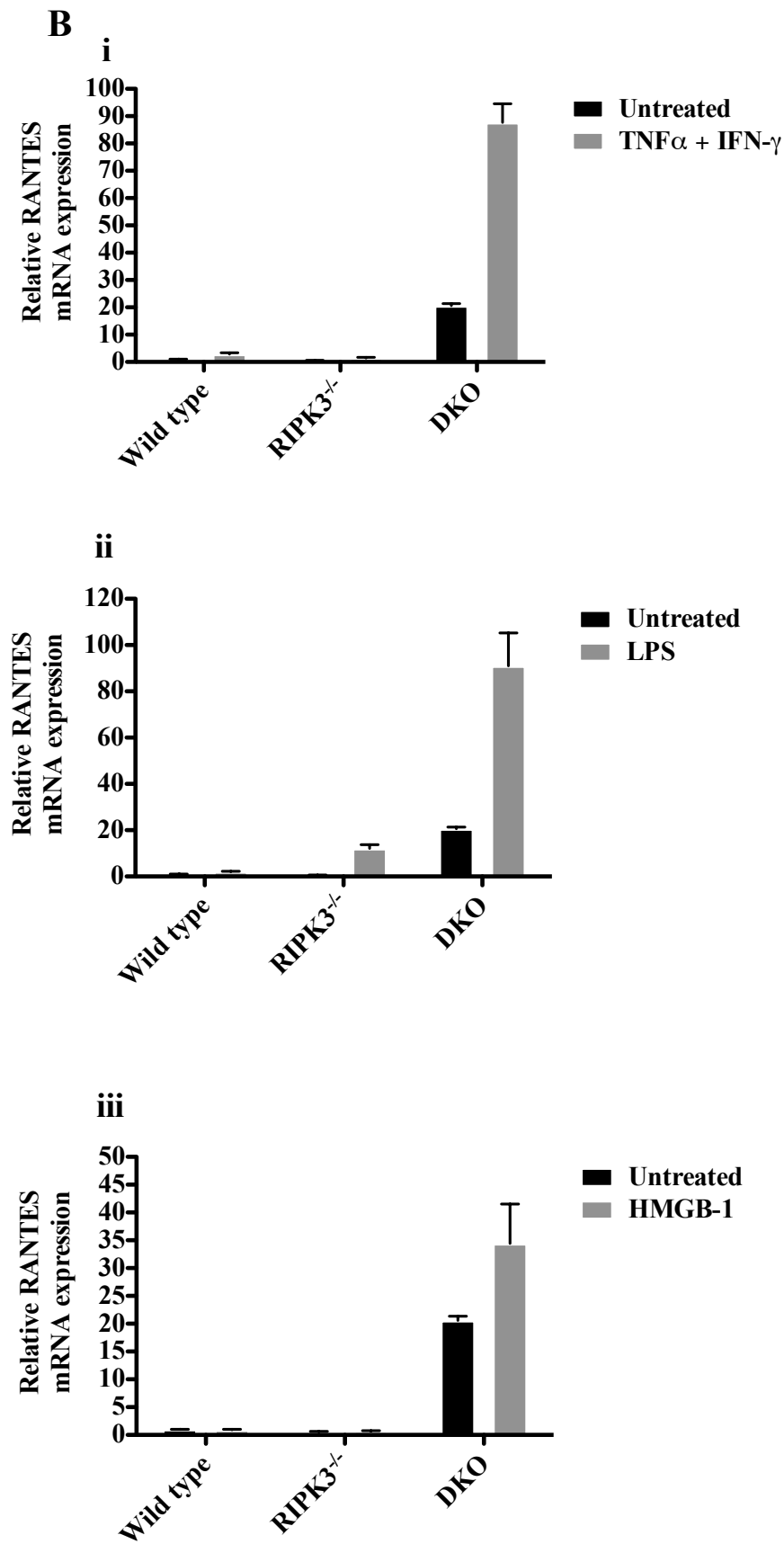
Ai-iii. TEC were cultured as described in the methods. Wild type, RIPK3^{-/-}, and DKO TEC were treated with combination of 4 ng/mL IL-1 β and 120 ng/mL IFN- γ for 24 hours in serum-free media in absence and presence of MCMV infection to induce intrinsic apoptosis. Cell viability was measured and quantified using MTT assay analyses. Figures represent one of three independent experiments, which showed the same results. (n=3, *: p < 0.05, **: p<0.01, ****: p<0.0001, Two-Way ANOVA).

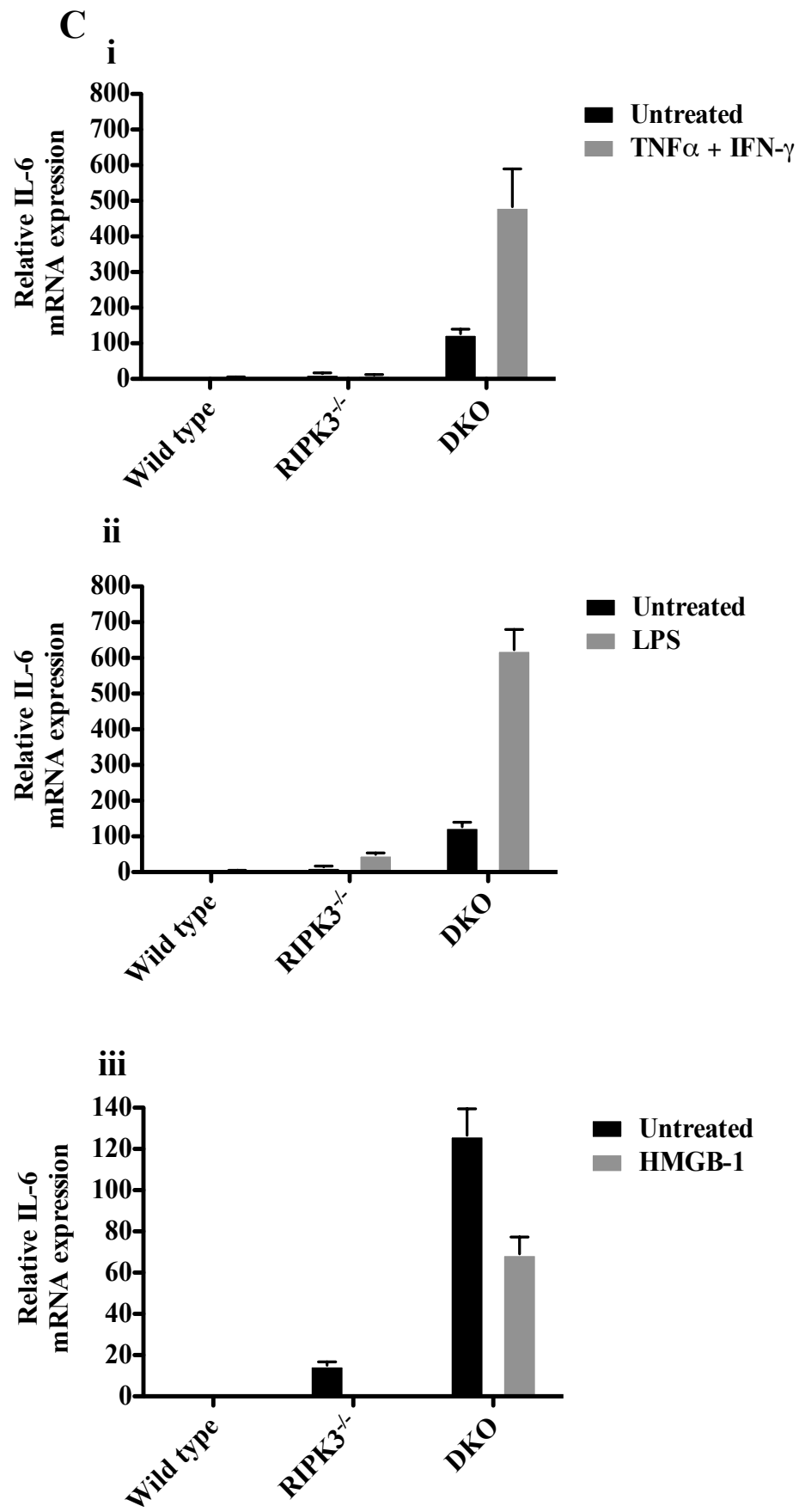
3.9 Pro-inflammatory activation of TEC is augmented in the absence of necroptosis and extrinsic apoptosis

We hypothesized that in absence of RIPK3-mediated necroptosis and caspase-8-mediated apoptosis, DKO TEC may demonstrate enhanced responses related to NF- κ B signaling. As mentioned previously, renal IRI results in the infiltration of immune effector cells into the renal parenchyma, thereby causing the activation of and up-regulation by TEC of diverse pro-inflammatory chemokines and cytokines²⁸. TNF α ²⁷⁷, IFN- γ ²⁷⁸, and HMGB-1⁵³ are prominent molecules that participate in IRI by activating TEC. TEC up-regulate pro-inflammatory NF- κ B signaling upon activation, leading to repeated cycles of inflammation that are detrimental to the graft survival (Figure 3). These pathways of activation would be expected to be intact in TEC despite loss of caspase-8 or RIPK3, and indeed may be enhanced in cells that are exposed to stimulation, yet unable to die. We therefore tested the expression of selected pro-inflammatory chemokines and cytokines produced by TEC. MCP-1, RANTES, and IL-6 have been well reported to exacerbate IRI. TEC were treated with 30 ng/mL TNF α + 30 ng/mL IFN- γ , 500 ng/mL of LPS, and 1 μ g/mL of HMGB-1 for 24 hours, and RT-PCR was performed as described. This treatment can induce cell death but is much less than what we have demonstrated with IL-1 β and IFN- γ combination that triggers intrinsic apoptosis. We used Two-way ANOVA analyses to assess differences in the cell lines. Treatments induced up-regulation of MCP-1 (Figure 13Ai-iii), RANTES (Figure 13Bi-iii), and IL-6 (Figure 13Ci-iii) mRNA in TEC, but notably the greatest increase was observed in DKO TEC (Figure 13D). These results confirm that DKO TEC retain the capacity to express pro-inflammatory chemokines and cytokines. Furthermore, in the absence of necroptosis and

extrinsic apoptosis, TEC appear to have enhanced pro-inflammatory responses following activation.







D

| Interaction Factor | Untreated vs. TNF α + IFN- γ | Untreated vs. LPS | Untreated vs. HMGB-1 |
|--------------------|--|-------------------|----------------------|
| MCP-1 | **** (p<0.0001) | **** (p<0.0001) | *** (p=0.0004) |
| RANTES | **** (p<0.0001) | *** (p=0.0001) | **** (p<0.0001) |
| IL-6 | ** (p=0.0019) | **** (p<0.0001) | ** (p=0.0017) |

Figure 14. Pro-inflammatory response is enhanced in TEC in the absence of necroptosis and extrinsic apoptosis

TEC were cultured as described in the methods. Wild type, RIPK3^{-/-}, and DKO TEC were treated with 30 ng/mL TNF α + 30 ng/mL IFN- γ , 500 ng/mL of LPS, and 1 μ g/mL of HMGB-1 for 24 hours in serum-free media. mRNA levels of **Ai-iii.** MCP-1, **Bi-iii.** RANTES, and **Ci-iii.** IL-6 were measured by RT-PCR as described in the methods. β -actin was used as loading control. D) Table of interaction factor significance from Two-way ANOVA analysis. Figures represent one of three independent experiments which show the same results (n=3, **: p<0.01, ***: p<0.001 ****: p<0.0001, Two-Way ANOVA).

Chapter 4

4 Discussion

4.1 Overview of results

The foundation of the majority of work in this thesis follows the observation that the loss of two regulated cell death pathways, which are known to independently reduce renal IRI, failed to have an additive benefit in IRI. Indeed, there was a trend to worsened renal function with IRI in DKO mice. While the kidney is composed of many cell types, TEC represent 75% of the parenchymal cells and are the cells that are most active in kidney function as well as being the primary target in most renal transplant rejection. In addition to primary cultured cells, we utilized the immortalized wild type NG, RIPK3^{-/-}, and DKO TEC cell lines for *in vitro* experiments in these studies that were designed to test the impact of removal of two cell death pathways on residual function and survival (Figure 7). In this sense, the TEC represents most but not all the responsible cells involved in IRI. Further studies would need to also include endothelial cells. We have shown basal level cell surface expressions of TNFR-1, TNFR-2, FAS, DR 5, TLR 2, and TLR 4 do not vary between cell types, nor do receptor levels change in response to activation with TNF α and IFN- γ in TEC (Figure 8). We have found that TEC also do not exhibit an increased sensitivity to CHX + TNF α and LPS (Figures 9) to cause death, and indeed TEC are generally resistant to death. However, we showed that DKO TEC exhibit enhanced sensitivity to IL-1 β + IFN- γ induced intrinsic apoptosis, which could be recovered by the addition of BIP (Figure 10). We also noted up-regulation of caspase-9 and caspase-3 activity in response to IL-1 β + IFN- γ treatment in all cells but was maximal in DKO TEC (Figure 10). DKO TEC showed up-regulation of mRNA levels of BAX and BAK (Figure

11). Following infection with MCMV, which inhibits cell death programs within cells, TEC showed near-complete abrogation of death (Figure 12). Lastly, mRNA expression levels of pro-inflammatory MCP-1, RANTES, and IL-6 were up-regulated in all TEC but was maximal in DKO TEC treated with $\text{TNF}\alpha$ + $\text{IFN-}\gamma$, LPS, and HMGB-1 as compared to wild type or $\text{RIPK3}^{-/-}$ TEC (Figure 13). The absence of RIPK3-mediated necroptosis and caspase-8-mediated extrinsic apoptosis thus favours BAX-mediated intrinsic apoptosis in TEC and promotes the expression of pro-inflammatory NF- κ B signaling. IRI continues to be a major early cause of organ injury in many clinical scenarios including sepsis-induced hypotension and in transplantation where the donor organ is invariably subjected to temporary ischemia. While there may be clinical benefit in targeting apoptosis and necroptosis, the data in this thesis suggests additional targeting of renal allograft-specific BAX, caspase-9, caspase-3, and NF- κ B might be necessary to maximize protection of the donor organ in renal transplantation.

4.2 Cell death and allograft rejection

From organ extraction to post-transplantation IRI, TEC are susceptible to cell death and injury that leads to attenuated graft function and potentially organ failure. Several factors contribute to this process, and there must be still undiscovered mechanisms that inflict critical damage to grafts. IRI is of course a prime example, but being complex and multifactorial, it has been difficult to adequately prevent IRI. One important aspect of IRI is the induction of many forms of cell death, which include but are not limited to apoptosis and regulated forms of necrosis such as necroptosis and ferroptosis.

Collectively, each of these pathways contributes to overall dysfunction of the organ, as

well as compromising the regenerative capacity of the graft. If exceeded, permanent organ dysfunction may occur and indeed the most important predictor of renal transplant survival is donor age. This might be because cell death in an aged organ that has limited regenerative capacity likely has a different implication than cell death in a young organ.

During IRI, TEC and other parenchymal cells of renal allografts are subjected to death by inflammatory cytokines and infiltrating immune cells, resulting in activation of host immune responses²⁷⁹. The specific forms of cell death are critically important to graft survival as the magnitude of immune responses during IRI can greatly influence early rejection and thus, graft survival. Apoptosis and necroptosis are distinctive forms of regulated death in TEC that orchestrate different immune responses to accelerate graft rejection.

Apoptosis has long been considered a prototypical form of programmed cell death, and it has been comprehensively studied using diverse research models in oncology, developmental biology, and transplantation models^{280,281}. Apoptosis results in formation of ‘shrinking’ Annexin-V positive cells that are ‘cleaned up’ by phagocytes, leading to minimal inflammatory consequences. Indeed within the kidney, KIM-1 bearing TEC act as the primary intrarenal phagocytes to remove phosphatidylserine (PS) bearing debris from all forms of cell death²⁸². Because of its non-immunogenic characteristics, apoptosis was once considered as not participating in worsening clinical transplantation, but research over the past decade has proven otherwise. A recent study has shown proximal TEC undergoing apoptosis during IRI express an “eat me” molecule, KIM-1, which permits efferocytosis amongst TEC²⁸², as well as infiltration of phagocytes into renal tubules^{283,284}. Furthermore, caspase-8 silencing or transgenic overexpression of the

caspase-8 inhibitor c-FLIP protected TEC against TNF- α induced apoptosis *in vitro* and renal IRI *in vivo*^{19,285}. Apoptotic cells not immediately cleared by the immune system may undergo secondary necrosis, resulting in plasma membrane rupture and release of intracellular contents that act as DAMPs on neighbouring cells to promote further inflammation and death²⁸⁰. Interestingly, while silencing caspase-8 has proven to be beneficial in renal IRI, it led to reduced allograft survival¹³⁵. While these findings highlight the important role apoptosis plays in renal IRI, they also emphasize the complex biology and the possibility of harm from strategies that do not take into account the inter-relation of these cell death pathways. In this case, our observation that the combined loss of apoptosis and necroptosis did not improve IRI *in vivo*, immediately suggests that there may be a relationship to other forms of cell death.

Contrary to apoptosis, until recently necrosis was thought to be an unregulated, “messy” form of cell death caused by sudden trauma. Studies have found that TNF superfamily receptors TNFR1 and Fas can induce a downstream transduction cascade of necrosis as well as apoptosis when stimulated^{148,286,287}, revealing a novel form of controlled, programmed necrosis termed necroptosis which is controlled by interactions of caspase-8, RIPK1, and RIPK3¹⁹². Necroptosis cannot be distinguished from unregulated necrosis or secondary necrosis based on morphology, as the pathways converge on common ‘end-point’ features such as rupture of plasma membrane integrity and release of cytoplasmic contents¹⁴⁷, which can start a devastating inflammatory cascade¹⁵². As expected, a recent study proved that knocking out RIPK3 mediated necroptosis to prevent inflammatory outcomes granted significant benefits in kidney function and reduced ATN in renal IRI model¹³⁵. In this model, inflammation and rejection are attenuated but not completely

eliminated as evidenced by the infiltration of RIPK3^{-/-} transplanted kidneys, and the detection of HMGB-1 in late samples. This would suggest that other forms of cell death exist in even long-term transplants, and the likely candidates remain- extrinsic and intrinsic apoptosis. In the current study, clearly the removal of extrinsic apoptosis and necroptosis sensitized cells to intrinsic apoptosis. While we were examining IRI as a model in this study, it would be intriguing to suggest that intrinsic apoptosis would also play a role in transplant outcomes. In preliminary studies of transplantation using DKO donor kidneys, this seems to be the case. Similarly, it would be of interest if intrinsic apoptosis inhibition could attenuate the late injury and HMGB1 release observed in the transplants with RIPK3^{-/-} kidneys.

In our study, we investigated potential benefits granted by deletion of both caspase-8 and RIPK3 in renal IRI using RIPK3^{-/-} caspase-8^{-/-} DKO mice. Here, we report that RIPK and caspase-8 deficiency does not grant additional benefits compared to RIPK3 deficiency alone, as DKO mice showed slight increase in serum creatinine levels and ATN scores were nearly identical (Figure 6). These *in vivo* data might indicate an over-compensatory mechanism by TEC where the absence of necroptosis and extrinsic apoptosis is negatively affecting kidney function without inducing ATN, suggesting presence of programmed, non-inflammatory cell death. Recently it has been reported in abstract form that IL-10 production by double negative CD3⁺ CD4⁻ CD8⁻ DNT cells have an attenuating effect on renal IRI²⁸⁸. As the DKO mice used in this study show an expansion of DNT cells that results in splenomegaly and lymphadenopathy, it may be that the magnitude of intrinsic apoptosis injury we observed was muted by the presence of these

cells. It would be of interest to test the IL-10 production in these DNT cells and perhaps use a model in which the host cells were normal, and only the kidney was a 'DKO.'

4.3 Absence of RIPK3 and caspase-8 sensitizes TEC to BAX-mediated intrinsic apoptosis

Since there were no additional benefits granted on DKO mice compared to RIPK3^{-/-} mice, we deduced that alternate forms of cell death might be enhanced with perturbations of necroptosis and extrinsic apoptosis. We confirmed that both primary and immortalized DKO TEC do not undergo apoptosis nor necroptosis induced by CHX + TNF α or LPS treatment (Figure 9), as expected in the absence of RIPK3 and caspase-8. It is also interesting to note that the CHX + TNF α and LPS treatments did not induce substantial amount of death, demonstrating the TEC have endogenous mechanisms of protection including osteopontin production, cellular FLICE-like inhibitory protein (cFLIP), and various inhibitor of apoptosis proteins (iAPs).

Many subtypes of intrinsic apoptosis have been described, with different functions and significance ranging from a role in retinoblastoma as well as p53-dependent apoptosis that controls growth development triggered in response to stress (hypoxia, radiation)²⁸⁹⁻²⁹¹. There is surprisingly little known about the role of intrinsic apoptosis in renal IRI, but limited studies have suggested intrinsic apoptosis contributes to IRI-induced graft damage²⁹². Nephrotoxicity from drugs such as cisplatin is partly due to BAX and caspase-9 mediated intrinsic apoptosis of TEC^{293,294}. In DKO TEC, the caspase-9 dependent intrinsic apoptosis pathway is intact, so we decided to investigate whether augmentation of intrinsic apoptosis was present in DKO TEC.

Intrinsic apoptosis was triggered *in vitro* in immortalized wild type, RIPK3^{-/-}, and DKO TEC using combination of IL-1 β and IFN- γ ²⁷⁴, and BIP was added to test whether the death induced was BAX-mediated apoptosis^{295,296}. While we showed DKO TEC are sensitized to IL-1 β + IFN- γ induced intrinsic apoptosis, BIP was not able to achieve complete rescue in the TEC. MTT and Sytox Green assays are limited in the ability to identify specific forms of cell death, although the uptake of Sytox would suggest membrane permeability as might be the case with late apoptosis and secondary necrosis. Caspase-9 and caspase-3 assays helped to more conclusively determine the presence of intrinsic apoptosis in TEC and that DKO TEC appear to die almost exclusively through intrinsic apoptosis, whereas wild type and RIPK3^{-/-} TEC as expected, die by other pathways as well. Caspase-8 is capable of activating pro-caspase-9 through SEPD domain cleavage²⁹⁷, and a previous study has shown IFN- γ can mediate activate FAS and caspase-8 dependent apoptosis in TEC²⁸⁵. With BAX inhibition, caspase-8 could be directly activating caspase-9, hence explaining the ineffectiveness of BIP treatment we observed in immortalized wild type and RIPK3^{-/-} TEC.

IL-1 β is a pro-inflammatory cytokine secreted by TEC and infiltrating immune cells. IL-1 β expression rapidly increases during renal IRI²⁹⁸ contributes to inflammation in renal allografts in conjunction with other cytokines²⁹⁹, triggers cytochrome c release from the mitochondria and activates caspase-9 activity. Also, IFN- γ induces BAD dephosphorylation and caspase activation^{300,301}. NLRP3^{-/-} mice that are unable to produce IL-1 β and inflammasomes showed better survival and function post IRI³⁰², highlighting the prominent negative impact resulting from IL-1 β and other inflammasome related stress in renal IRI. Interestingly, a recent study induced several different forms of

intrinsic apoptosis on wild type, RIPK3^{-/-}, and DKO thymocytes using drugs and stress stimuli and reported no sensitivity differences¹⁹², suggesting enhanced BAX-mediated intrinsic apoptosis may be a phenotype unique to TEC of DKO mice.

We used RT-PCR analyses to measure basal level transcriptional differences of BCL-2 family genes in immortalized wild type, RIPK3^{-/-}, and DKO TEC. Indeed, we found greatest up-regulation of pro-apoptotic BAX, BAK, and BAD in DKO TEC (Figure 11). However, it remains to be seen whether the transcriptional up-regulation of pro-apoptotic genes correlate to translational level, making protein analysis such as Western blotting necessary. Previous studies have observed an increase in BAX and BAK in both mRNA and proteins levels in rat proximal TEC post-IRI^{212,213}, supporting our results and validating the role of intrinsic apoptosis in renal IRI. Interestingly, one study showed acidic pH could inhibit BAX-dependent, caspase-9 mediated apoptosis in ATP-depleted TEC through disruption of apoptosome-mediated caspase-9 activation³⁰³. Acidosis is an inherent outcome of organ ischemia as cells resort to anaerobic metabolism in response to oxygen and glucose deprivation, leading to increased lactic acid production and thus a subsequent decrease in pH^{304,305}. Therefore, intrinsic apoptosis of TEC could be a prominent reperfusion-specific mechanism.

While loss of caspase-8 and RIPK3 enhances BAX-mediated intrinsic apoptosis and inhibition of BAX through BIP rescued TEC death (Figure 10), we do not have conclusive *in vivo* renal IRI data confirming the presence of heightened intrinsic apoptosis in DKO mice. Immunohistochemistry staining of caspase-9 on DKO kidney sections or *in situ* injection of BIP in DKO kidneys and observing outcomes in renal IRI

model would solidify our findings, but unfortunately we were not able to perform these reliably.

4.4 Activation of death receptors on TEC can promote inflammation

The intense inflammatory cascade upon reperfusion remains a recurring issue during kidney transplantation for its contribution to delayed graft function and promoting adaptive immune responses^{28,306,307}. The early stage inflammatory responses can severely compromise the survival of allografts. Among the diverse factors that contribute to IRI, such as IL-6, contribute to the general inflammatory state. Others, such as TNF α , IFN- γ and IL-1 β contribute to death³⁰⁷ and chemokines MCP-1 and RANTES contribute by increasing the infiltration by effector cells. These can be produced by the effector cells and by TEC^{75,76,96,99}. Innate immune responses, including but not limited to PMN, macrophage infiltration, and NK cell priming, following the secretion of pro-inflammatory cytokines and chemokines are hallmarks of renal IRI³⁰⁸. While we were not able to reproduce the complex *in vivo* situation, in this study, we treated TEC with TNF α + IFN- γ , LPS, and HMGB-1 to simulate the receptor activation-induced inflammation from IRI *in vitro* and used RT-PCR analysis to measure expression of IL-6, MCP-1, and RANTES at the mRNA transcriptional level.

TNF α and IFN- γ are classic inducers of pro-inflammatory NF- κ B signaling, and it has been shown TNF α and IFN- γ contribute to inflammatory damage by up-regulation of many chemokines and cytokines during renal IRI^{31,278}. In this study, TEC were exposed to murine TNF α and IFN- γ to ‘mimic’ the environment of IRI, using LPS to activate TLR and as a positive control. TEC showed up-regulation of IL-6, MCP-1, and RANTES, in

response to the treatments, with the DKO TEC showing a remarkable difference (Figure 13) compared to wild type or RIPK3^{-/-} TEC. This data suggest that in the absence of caspase-8 and RIPK3, DISC complex formation can proceed only to pro-inflammatory signaling (Figure 4) thus promoting significant inflammation. We, however, were not able to support the *in vivo* upregulation with *in vivo* increases in infiltration, as the total counts could only identify cells such as PMN, which can be counted in H&E stained samples. More specific phenotyping of cells would require considerable additional work, which was not done in this thesis.

HMGB-1 is an intracellular molecule that acts as a DAMP once released from necrotic cells⁶¹. Released HMGB-1 can bind to TLR 2, 4, 9, and RAGE of neighbouring TEC and immune cells to promote the production of chemokines and cytokines shown to participate in IRI and during graft rejection^{58,60,309}. Several forms of HMGB-1 have been identified^{310,311}. The reduced form of HMGB-1 is released by necrotic cells and possesses highly immunogenic capabilities, whereas apoptotic cells release oxidized HMGB-1 that has minimal immunogenicity³¹². In this study, we observed an increase in IL-6, MCP-1, and RANTES expressions of wild type and RIPK3^{-/-} TEC in response to recombinant murine HMGB-1. Interestingly, DKO TEC showed down-regulation of MCP-1 and IL-6 against HMGB-1 (Figure 13). This is a somewhat disparate finding, as previous studies have shown that HMGB-1 exerts its pro-inflammatory functions by inducing up-regulation of MCP-1, RANTES, and IL-6 and maximal protection was observed in renal IRI when HMGB-1 was unable to interact with kidney TLRs^{53,59,313}. There are no known anti-inflammatory functions of HMGB-1 in solid organ transplants, although immunosuppressive and anti-proliferative roles of HMGB-1 have been identified in

certain T cell subgroups^{314,315}. This could be a new finding that DKO TEC in fact show anti-inflammatory responses against HMGB-1. It would be interesting for future studies to investigate whether HMGB-1 could have anti-inflammatory and immunosuppressive functions in other conditions.

Another intriguing finding was that basal levels of MCP-1, RANTES, and IL-6 were significantly higher in DKO TEC compared to wild type or RIPK3^{-/-} TEC. While it may have been expected that DKO TEC could exhibit over-activation in response to pro-inflammatory treatment, the response in resting cells merits consideration of the normal ‘outside-in’ signals that cells obtain to maintain homeostasis. Nonetheless, the increased expression of chemokines and cytokines may have contributed to a reduced benefit in the DKO mice with IRI (Figure 6). Kang *et al* (2013) used Itgax-Cre system to achieve specific deficiency of caspase-8 in RIPK3^{-/-} mice that mimics our DKO mouse model. Interestingly and in contrast to our results, they reported that LPS treatment down-regulated pro-inflammatory TNF α and IL-1 β at protein level in dendritic cells³¹⁶. These findings suggest that different cell types with similar genetic deletions respond differently to similar treatments, leading to different result. It is now known that Nec-1 has many off-target effects³¹⁷.

Clearly, one of the limitations of our studies was that *in vitro* treatments would not fully simulate the *in vivo* situation of IRI. We also used mRNA analyses and it remains to be seen whether the observed up-regulation of chemokines and cytokines at transcriptional level correlates at translational level. Thus, future studies focusing on protein and *in vivo* expression of chemokine and cytokines in renal IRI should strengthen our data.

Furthermore, performing immunohistochemistry staining of macrophage and NK cell infiltrates may support that the DKO TEC enhance chemotaxis *in vivo*.

TNF α + IFN- γ treatment did not induce any changes in the cell surface death receptor expression levels (Figure 8). Cells normally respond to TNF α + IFN- γ activation by up-regulating surface death receptor expressions both *in vitro*^{91,318} and *in vivo*^{319,320}. This result may suggest that different cells respond to different concentrations of cytokines, and perhaps TEC require higher amounts to change cell surface death receptors. Also, this indicates that RIPK3 and caspase-8 do not participate in receptor shuttling as DKO TEC showed similar basal level receptor expressions.

Another interesting finding of our death receptor results is that TEC show high basal level expression of DR 5. DR 5 is a family of TNF receptor superfamily, and serves as a receptor TRAIL^{321,322}. DR5 is one of the best-characterized receptors in oncology due to its roles in tumour cell apoptosis. Most cancer cells down-regulate DR 5 expression to minimize vulnerability to apoptosis. Several cancer therapeutic drugs act to up-regulate DR5 expression of the cancer cells and sensitizes them to TRAIL-mediated apoptosis^{323,324}. While the knowledge of DR 5 and TRAIL in cancer research is vast, our current understanding of their potential role in renal IRI is limited. TRAIL activation of DR 5 is known to induce caspase-8-dependent apoptosis. As mentioned before, caspase-8 also inhibits RIPK3-mediated necroptosis and promotes apoptosis. In acidic pH conditions however, TRAIL was found to induce a form of necrosis³²⁵. Surprisingly, this was dependent on caspase-8³²⁶, revealing yet another surprising role of caspase-8. During renal ischemia, TEC are under hypoxic stress due to lack of oxygen and resort to anaerobic metabolism that leads to lactic acid accumulation and subsequent acidic pH

environment inside the allograft^{327,328}. With high level of DR5 of TEC in conjunction with acidic pH during ischemia, it is possible that TEC similarly undergo DR 5 mediated necrosis. Future studies investigating the importance of TRAIL and DR 5 in renal IRI could reveal additional mechanism of TEC death.

Finally, innate immunity has been increasingly associated with acute phase damage post-transplantation^{82,329,330}. Two key components, namely TLR 2 and TLR 4, have been extensively studied for their prominent roles in triggering pro-inflammatory responses from TEC during renal IRI^{54,55,331,332}. With high basal level expression of TLR 2 and TLR 4, physiological stress during IRI could contribute to TEC activation.

4.5 MCMV influences cell death pathways

Human CMV infection has been the most common and challenging viral infection in solid organ transplantation. Previous studies prior to antibiotics, have shown that symptomatic CMV infection can occurs in up to 60% of renal transplant recipients³³³. Ganciclovir treatment remains the most widely used prophylactic strategy³³⁴, but it is expensive³³⁵, often only delays rather than prevents the onset of the CMV disease^{336,337}, and may promote viral resistance³³⁸. Symptoms of CMV infection include episodic fever, fatigue, and abdominal pain. CMV can infect broad range of cells and tissues, and eventually cause organ failure and death³³⁹. Moreover, CMV infection immunosuppresses patients, exposing them to other life threatening pathogens^{340,341}.

Murine CMV encodes various cell death inhibitor proteins that are made during the immediate early phase of infection. Due to species differences however, MCMV cannot circumvent immune defense of humans, which makes humans resistant to MCMV

infection. Even with large exposure, MCMV is quiescent in human cells and replication is at a level that does not induce pathological symptoms^{255,342}. In our study, we infected immortalized TEC with wild-type K181 BAC strain of MCMV²⁵³ and tested whether MCMV generated death inhibitor proteins conferred survival benefit on TEC under death-inducing stimuli. From MTT assay results, we saw near-complete salvage of MCMV-infected TEC treated exposed to IL-1 β + IFN- γ (Figure 12). This was consistent with previous findings that MCMV can inhibit intrinsic apoptosis via BAX and BAK inhibition through vMIA and vIBO, respectively^{198,343}. Interestingly, MCMV was more effective in restoring cell viability across all three cell lines compared to BIP, which was the most effective in DKO TEC (Figure 10). This suggests DKO TEC may undergo BAX-mediated intrinsic apoptosis exclusively. Wild type and RIPK3^{-/-} TEC that may be undergoing other forms of cell death would be expected perhaps to be more responsive to MCMV salvage. We also saw that MCMV infection was not able to salvage survival in DKO TEC to levels observed with wild type or RIPK3^{-/-} TEC. This may suggest that other forms of cell death remain in DKO TEC when RIPK3, caspase-8, BAX, and BAK are all simultaneously inhibited. Since we used MTT assays to measure cell viability, it is impossible to clearly identify what type of cell death the TEC are undergoing.

At this time, our interest in MCMV is as a tool to understand cell death pathways, as it is remarkable that this virus has exploited an ability to control all known forms of regulated cell death in TEC. However, as the MCMV genome is very well characterized, it may be possible to manipulate this virus to allow localization to renal allografts and control cell death for a limited period of time in donor organs prior to transplant, i.e. during IRI. A previous study has shown that IRI-induced TNF-mediated NF- κ B signaling and ROS

production activates the enhancer region of immediate-early phase promoter region of HCMV³⁴⁴. IRI could induce the production of CMV-derived cell death inhibitor proteins if infection could be targeted and limited. Obviously this sort of viral based strategy would require extreme caution in the clinical scenario.

4.6 Renal transplants, immunosuppressive and cell death drugs

Over the past several decades, acute rejection and survival rates of renal allografts have been improving; this has been mainly attributed to increased first-year survival among patients³⁴⁵. Despite the advances in the short-term survival, long-term survival remains as the most significant challenge, as there has not been a substantial improvement in long-term outcomes in kidney transplants^{124,346}. This compounds the current problem of having too few organ donors, as recipients with premature transplant failure return to the transplant waiting list, along with patients who have never received a previous transplant. It remains unclear what contribution early damage makes to reduced long-term graft survival, but it is intuitive that modifying early injury is not likely to harm, and more likely to improve early and perhaps late graft function. Therefore, this study was focused on IRI, which is an invariable and early consequence of organ donation and is amenable to intervention. Strategically, as this laboratory is focused on regulated cell death, we were interested in attenuating multiple forms of cell death, in order to attenuate IRI.

IRI orchestrates a cascade of innate and adaptive immune responses that exacerbate allograft damage by stimulating cellular rejection, antibody production, and immune cell-mediated killing that results in parenchymal cell death. Collectively or individually, these compromise allograft survival^{43,126,279}. We have shown that simply blocking both

caspase-8 and RIPK3 to eliminate extrinsic apoptosis and necroptosis, respectively, did not have the intended effect of reducing IRI more than what we had observed by single pathway elimination. This is therefore unlikely to be effective as a strategy in transplants and indeed in our preliminary results using DKO donor kidneys, there was no benefit. It may be that the enhanced injury from augmented intrinsic apoptosis may be even more apparent in transplant recipients having normal circulating T cells, in contrast to what we observed in the DKO mice. Recent reports have shown that CD3⁺ CD4⁻ CD8⁻ DNT cells are found within the kidney post-IRI and that kidney resident DNT cells up-regulate anti-inflammatory IL-10 and IL-27, thus suggesting a potential protective role for these cells in IRI²⁸⁸. Importantly, the DKO mice we used develop lymphadenopathy due to a high number of spontaneously generated DNT cells¹⁹². These may have actually attenuated the amount of injury we observed with IRI. Similarly, it would be interesting to see whether DNT cells from DKO mice might mitigate intrinsic apoptosis injury with adoptive transfers to other mice *in vivo*.

Current immunosuppressive drugs act as double-edged sword, as the use of tacrolimus and sirolimus disables T lymphocyte function and proliferation yet can induce nephrotoxicity through fibrosis and perhaps cell death^{347,348}. With recent advances in elucidating mechanisms of cell death, specific forms of cell death during early stages of transplantation may generate alternative strategies to alter immune responses, i.e. through reduction of cell death and innate immunity. At present, there are no clinically approved pharmacological inhibitors of cell death that could be administered along with immunosuppressive therapies, although Nec-1 has considerable potential at the preclinical stage. All of these agents could be considered for organ preservation solutions.

While Nec-1 can be used to target necroptosis along with caspase inhibitors to target apoptosis, it may be possible to tailor strategies that affect intrinsic apoptosis more than extrinsic apoptosis. While we administered BIP *in vitro* and this could be given *in vivo*, a potential side effect of intrinsic apoptosis inhibition might be up-regulation of ferroptosis, a newly described form of regulated necrosis dependent on iron³⁴⁹. Contrary to intrinsic apoptosis requiring VDAC opening, ferroptosis needs ‘closed’ VDAC³⁵⁰, which can be achieved using erastin to block VDAC opening³⁵⁰. A recent study has shown that TEC can undergo synchronized, rapid, post-ischemic ferroptosis, which can be inhibited by ferrostatins¹³⁷. Due to the nature of intrinsic apoptosis and ferroptosis that depend on opposite states of VDAC, future studies would need to investigate effects of BIP inhibition *in vivo*, and the relation of intrinsic apoptosis to ferroptosis in renal IRI. BIP, in conjunction with Nec-1, could potentially become an effective combination to improve graft survival in patients.

4.7 Conclusion

In this study, we report for the first time that the loss of two forms of regulated cell death, namely RIPK3 mediated necroptosis and caspase-8 mediated apoptosis, which are known to independently reduce renal IRI, failed to provide a benefit in IRI, when combined. This was not related to altered surface expression of death receptors on TEC, but is correlated to enhanced BAX dependent, caspase-9 mediated intrinsic apoptosis. The mechanism is unknown but may be related to the loss of control of intrinsic apoptosis by caspase-8 and RIPK3 or the downstream proteins they control. We also show that DKO TEC can become ‘over-activated’ in response to treatments with cytokines and DAMP, leading to up-regulation of pro-inflammatory cytokines and chemokines. This has

considerable implications in clinical strategies that target cell death in IRI and transplantation as multiple pathways may require inhibition for effect. Furthermore, we demonstrate for the first time that MCMV infection completely abrogates intrinsic apoptosis in TEC. Therefore, inhibition of necroptosis, extrinsic apoptosis, and intrinsic apoptosis may be necessary to confer maximal protection in IRI and in renal allografts. Inhibition of pro-inflammatory NF- κ B signaling also must be taken into consideration to minimize repeated cycles of inflammation cascade that could persist and lead to graft rejection.

4.8 Future directions

Future studies will be needed to fully investigate the significance and importance of caspase-9 mediated intrinsic apoptosis in IRI *in vivo*, which will consider more than TEC responses. Drug strategies may be designed to target intrinsic apoptosis-related proteins such as caspase-9, BAX, and BAK in an organ-specific manner along with targeting necroptosis and caspase-8 apoptosis. This will be critical to develop applicable treatments in renal transplantation. The remarkable ability of CMV to block specific forms of cell death may be exploited in a clinical strategy, but will require caution in targeting organs in isolation from the recipient and in a time limited form. Finally, these studies need to be extended to transplantation models that add alloinjury to the ischemic injury. These ultimately are the major goal of this work.

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