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Cell-free DNA Release During Programmed Cell Death in Kidney Ischemia Reperfusion Injury

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Abstract

Transplantation is invariably associated with ischemia reperfusion injury (IRI) which causes organ dysfunction. IRI is also directly linked to several forms of programmed cell death including apoptosis and necroptosis, which increase kidney dysfunction, promote inflammation and may contribute to premature graft failure. The contribution of necroptosis and apoptosis following kidney IRI to cell-free DNA (cfDNA) generation and the potential of cfDNA to activate effectors such as NK cells involved in kidney IRI have not been defined. Our data indicate that necroptotic microvascular endothelial cells (MVECs) release considerably more cfDNA than apoptotic MVECs or untreated controls ($p<0.0001$). cfDNA was readily detected in serum following kidney IRI *in vivo*. RIPK3 deficiency modestly inhibited cfDNA release post-IRI. Levels in genetically altered mice incapable of both apoptosis and necroptosis (RIPK3$^{-/-}$/caspase-8$^{-/-}$, DKO) were comparable to sham controls ($n=7$, $p<0.0001$). cfDNA also appears to be a sensitive biomarker of injury as serum levels were reduced even with small reduction in IRI model temperatures ($n=4$, $p<0.01$). Furthermore, cfDNA was detectable in subclinical IRI at 24-28°C, in which serum creatinines remained in the normal range ($n=4$, $p<0.0001$). Lastly, NK cells, well described effectors of IRI, upregulated key activation markers Granzyme B ($p<0.0001$), IFNγ ($p<0.0001$), and TNFα ($p<0.01$) when treated with cfDNA ($n=4$). As such, cfDNA is a sensitive biomarker of cellular injury that is also able to activate NK cells and can promote IRI-associated inflammation. Strategies that reduce inflammatory injury and necroptosis that release cfDNA would be expected to attenuate IRI and improve kidney function following transplantation.

Keywords

cfDNA, Necroptosis, Apoptosis, Natural Killer Cell, Ischemia Reperfusion Injury

Summary for Lay Audience

Transplantation is invariable associated with injury to the donor organ. During this injury various forms of cell death occur, and contribute to organ injury. During this cell death, molecules are released from dying cells in a controlled fashion. One such molecule that has generated interest is termed cell-free DNA (cfDNA), as it is released from dying cells, and is also capable of stimulating the immune system. We have shown that cfDNA is released by dying cells by a variety of different cell death programs. We have also shown that cfDNA is released into the blood in a mouse model of transplant-associated organ injury, and that this release of cfDNA can be limited when certain forms of cell death are genetically impaired. We have also shown that cfDNA is a more sensitive marker of organ injury than currently used clinical standards, such as the measurement of serum creatinine levels. Lastly, we have shown that immune cells, NK cells in particular, are activated by cfDNA, which may therefore be promoting inflammation in the transplant recipient. As such, we have shown that cfDNA is a sensitive biomarker of cellular injury that is also capable of activating the immune system, thereby promoting inflammation. Strategies to reduce the release of cfDNA and the inflammatory response would be expected to improve kidney function following transplantation.

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List of Abbreviations

ANT	Adenine nucleotide translocator
APAF-1	Apoptotic protease activating factor-1
ARF	Acute renal failure
ATP	Adenosine triphosphate
B6	C57BL/6
BAX	BCL-2-associated X protein
BCL-2	B cell lymphoma 2
cfDNA	Cell-free DNA
CKD	Chronic kidney disease
cNP	Cationic nanoparticle
CypD	Cyclophilin D
DAMP	Damage associated molecular pattern
DCD	Donation after cardiocirculatory death
ddcfDNA	Donor-derived cell-free DNA
DISC	Death-inducing signaling complex
DKO	Double knockout
ESRD	End stage renal disease
FADD	Fas-associated death domain protein
FASL	Fas ligand
haMSC	Human adipose-derived mesenchymal stem cell
HLA	Human leukocyte antigen
HMGB1	High-mobility group box-1
HSP	Heat shock protein
IAP	Inhibitor of apoptosis protein

ICAM-1	Intracellular adhesion molecule-1
IETD	Z-Ile-Glu-Thr-Asp-Fluoromethylketone
IFN-γ	Interferon-gamma
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-10	Interleukin 10
IRI	Ischemia reperfusion injury
MHC	Major histocompatibility complex
MLKL	Mixed lineage kinase domain-like
MOM	Mitochondrial outer membrane
MPTP	Mitochondrial permeability transition pore
MVEC	Microvascular endothelial cell
Nec-1	Necrostatin-1
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
PCR	Polymerase chain reaction
PI	Propidium iodide
qPCR	Quantitative PCR
RA	Rheumatoid arthritis
RIPK	Receptor interacting protein kinase
ROS	Reactive oxygen species
TEC	Tubular epithelial cell
TLR	Toll-like receptor
TNFα	Tumour necrosis factor-alpha
TNFR	Tumour necrosis factor receptor
TRADD	TNF receptor-associated death domain

TRAF	TNF receptor-associated factor
VCAM-1	Vascular cell adhesion molecule-1
VDAC-1	Voltage-dependent anion channel-1

Chapter 1

1 INTRODUCTION

1.1 Challenges in Kidney Transplantation

Chronic Kidney Disease (CKD) is a global health problem that imposes a large burden on the healthcare system and on society (1). During the early stages of CKD, some forms of kidney pathology can be reversed and kidney function can improve with a change in diet, lifestyle, as well as with the administration of medications (2). However, once kidney function drops below 15%, complications of CKD begin to occur, and these include hypertension, malnutrition due to poor reabsorption of nutrients from the blood, bone disease, heart failure, and neuropathy (3). If not treated, progression will lead to End Stage Renal Disease (ESRD) which requires treatment to avoid death (3). Once the patient has progressed to ESRD, only two treatment options are available: dialysis and kidney transplantation (4). Numerous clinical studies indicate that kidney transplantation is by far the preferred treatment for most patients with ESRD, with transplant recipients experiencing decreased mortality as well as increased quality of life when compared to patients who receive dialysis (5,6). Indeed, the data show that receiving a kidney transplant can prolong life expectancy by 10-15 years compared to dialysis alone (7). Despite the overwhelming superiority of transplantation to dialysis, transplantation-related complications can arise that can lead to kidney allograft loss or injury (7).

The success of a renal transplant is multifactorial, with both immunological and non-immunological factors coming into play. Non-immunological factors that affect renal transplant success include drug toxicity, donor organ viability, and host/recipient factors (8). The viability of the donor organ can be affected by age of the donor, renal mass, the amount of time the donor organ is deprived of blood supply, presence or absence of donor brain injury, and whether the donor was living or deceased at the time of procurement (8). Similarly, the overall health of the recipient directs survival. If the recipient of the transplant has a lipid disorder (i.e. hypercholesterolemia), diabetes, high blood pressure, or

any circulatory obstructions (i.e. an atherosclerotic plaque), then the likelihood of success of the graft can be reduced (8). Immunological factors that affect transplant success include cellular immunity, antibody-mediated (formerly referred to as humoral) immunity, and infection (8). Cellular immunity is triggered by direct or indirect allorecognition and from a failure of immunosuppression to adequately reduce the alloimmune response of the recipient (8). Antibody immunity is related to antibody-mediated rejection of the donor tissue, or if the recipient has been sensitized to donor antigens prior to the transplant (8). Lastly, although immunosuppression is critical and unavoidable in the setting of a transplant, it also has the unintended side-effect of a 'weakened' host immune system, increasing the risk of opportunistic viral, bacterial, and fungal infections that would typically not be of concern in a non-immunocompromised individual.

Preventing allograft responses and rejection by the recipient immune system remains a key target in kidney transplantation (9). The recipient immune system recognizes the donor organ as foreign by targeting the highly variable major histocompatibility complex (MHC) molecules [aka human leukocyte antigens (HLA), as they are referred to in humans] (10). For this reason, HLA matching between organ donor and recipient is important, as matching will significantly reduce the chance of graft rejection, but it is of less priority than medical urgency or waiting time (10).

1.2 Ischemia Reperfusion Injury

One particularly critical early type of allograft injury that occurs invariably with transplant is ischemia reperfusion injury (IRI) (11). IRI is a major cause of delayed graft function in transplant recipients, and can even lead to early graft loss via severe acute renal failure (ARF) (12). Ischemia occurs when the blood vessels in the donor tissue are clamped, thereby removing blood supply to the organ, and creating an environment that lacks both oxygen and nutrients (13). Reperfusion occurs when blood flow is restored to the organ. This influx of oxygen during the reperfusion stage results in the formation of reactive oxygen species (ROS) which promote inflammation (13). The oxidative stress resulting in increased levels of ROS causes the upregulation of pro-inflammatory cytokines and chemokines. The upregulation of these cytokines and chemokines causes increased vascular permeability as well as an increase in the expression of endothelial cell adhesion

molecules, which will act to recruit and activate infiltrating leukocytes to the graft (14, 15). Furthermore, oxidative damage caused by ROS will trigger the activation of toll-like receptor (TLR) and complement arms of the innate immune system, furthering the damage to the graft (16). For example, the release of endogenous ligands, known as damage associated molecular patterns (DAMPs) from damaged cells during IRI will bind and activate numerous TLRs of resident dendritic cells (17). Complement induction is mediated through the reduced expression of complement regulatory proteins on the surface of tubular epithelial cells (TECs) and microvascular endothelial cells (MVECs) of the allograft (18, 19). Both the induction of the complement system and the activation of TLRs will induce resident dendritic cells of the allograft to produce even more pro-inflammatory chemokines and cytokines, triggering earlier and more robust immune responses against the allograft (16). Part of this immune response to IRI is the infiltration of macrophages, neutrophils, and natural killer (NK) cells into the graft (20-22). These infiltrating innate immune cells can release more ROS, as well as proteases, cytokines and chemokines which will recruit additional immune cells and will induce undesirable and unbalanced programmed cell death of the resident tissue cells (22), all contributing to increased inflammation, increased IRI, and increased acute allograft injury in a cyclical fashion. It is a cycle of inflammation that requires control, yet there are no direct therapeutics to prevent or attenuate IRI.

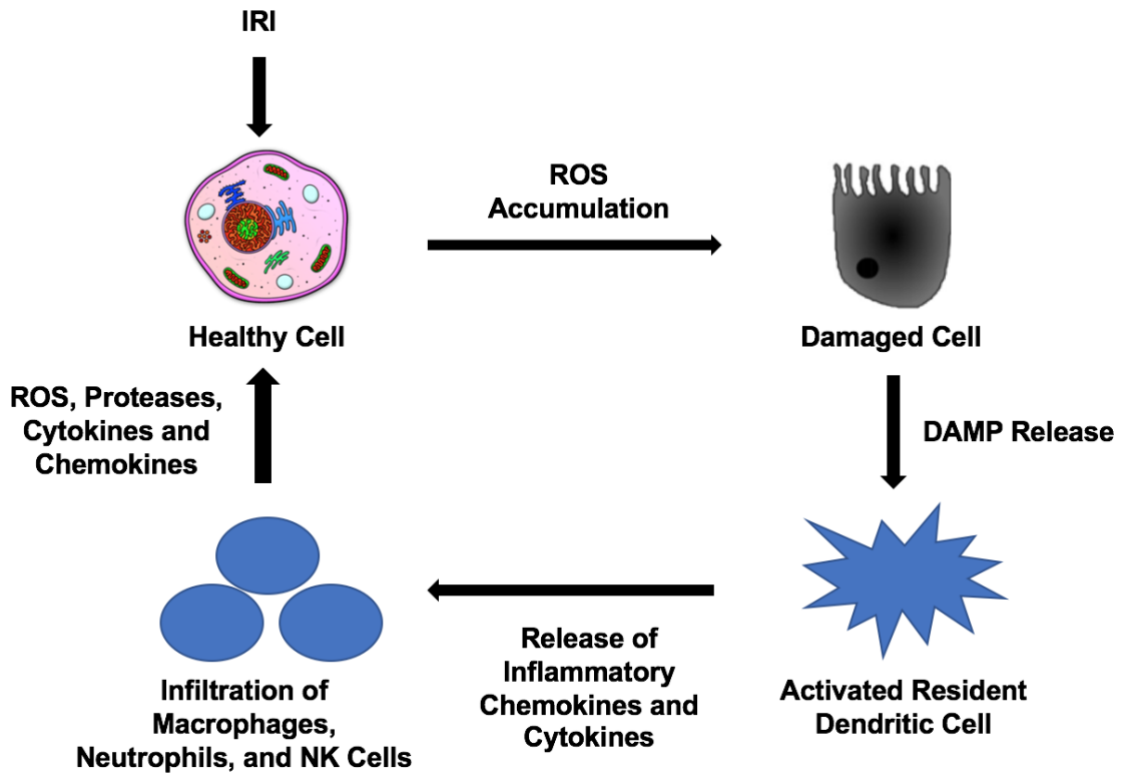


Figure 1. Innate inflammatory cycle during IRI.

During IRI, cells of the kidney allograft are injured as a result of increased ROS as well as due to host immune responses, leading to DAMP release from cells of the allograft. Released DAMPs then bind to TLRs on resident dendritic cells which will release pro-inflammatory chemokines and cytokines, resulting in the infiltration of macrophages, neutrophils and NK cells. Infiltrating innate cells release even more ROS, as well as proteases, cytokines and chemokines which induce further cell death of resident tissue cells.

1.3 Leukocyte Function and Biology in IRI

A diverse population of leukocytes are implicated in the damage caused by IRI. Firstly, $CD4^+$ T cells infiltrate the kidney allograft as early as 4 hours post-ischemia (20). In addition to this early recruitment of T cells, $CD19^+$ B cells, resident dendritic cells, macrophages, neutrophils, and NK cells also infiltrate the kidney allograft (21). Macrophages are recruited by activated resident dendritic cells and will infiltrate the graft and secrete cytokines such as interferon-gamma (IFN-γ) (22). Cytokine release by macrophages will induce programmed cell death of cells of the allograft, as well as recruit neutrophils. Neutrophils will also cause damage to the graft as they are potent effector cells that cause damage by obstructing the renal microvasculature, as well as by the release of ROS and proteases (20). Of all these infiltrating leukocytes implicated in the damage caused by IRI, NK cells are particularly understudied.

NK cells are a subset of leukocytes that are derived from bone marrow-resident lymphoid progenitor cells, and are a component of the innate immune system (23). As their name suggests, NK cells are 'killer' cells that are typically capable of killing abnormal host cells, such as tumour cells and viral-infected cells, as well as foreign cells (24). NK cells are able to induce cytotoxicity via the release of perforin and granzyme as well as through the expression of Fas ligand (25). Perforin is a pore-forming protein that essentially creates holes in target cells which allows for the delivery of various granzyme family members (25). Granzyme is a protein family consisting of structurally-related serine proteases that are capable of triggering apoptotic and ultimately necrotic cell death upon perforin-assisted delivery to target cells (25). In addition to participating directly in the killing of target cells by the release of perforin and granzyme, NK cells also contribute to the global inflammatory response by releasing chemokines and cytokines such as IFNγ and tumour necrosis factor-alpha (TNFα), which will recruit and activate additional innate immune cells (26, 27).

Subsequent to kidney transplantation, many adaptive and innate immune cells infiltrate the graft and contribute to allograft injury. NK cells contribute to the innate immune response in the allograft and participate in IRI by lysing cells of the allograft using

perforin and granzyme in an antigen-independent manner, as well as by upregulating pro-inflammatory cytokines (28, 29). NK cells are particularly interesting in a transplant setting because NK cells not only possess constitutive and potent cytotoxicity allowing them to kill target cells without pre-sensitization, but are also able to distinguish between allogeneic and self MHC antigens (30). Therefore, NK cells are capable of directly contributing to allograft loss via direct cytotoxicity, as well as indirectly exacerbating IRI by influencing the alloreactivity of T cells.

While the specific role of NK cells in renal IRI is poorly defined to date, Zhang *et al.* have demonstrated that NK cells play an important role in both acute and chronic kidney allograft injury (28, 31). NK cells are shown to be capable of directly killing TECs following renal IRI, and adoptive transfer of NK cells into mice lacking all lymphoid immune cells increased the severity of injury following IRI (28). These and other data demonstrate clearly that NK cells worsen the injury during IRI, independent of the adaptive immune system that is targeted by conventional immunosuppressants. This finding was also supported by more recent studies in mouse models (32-35) as well as in human studies (36, 37)

Furthermore, a recent study that makes use of a B and T cell deficient Rag$^{-/-}$ transplantation model shows that NK cells induce chronic kidney injury and that depletion of NK cells ameliorated long term kidney injury (6), demonstrating that NK cells can directly react to donor allografts independent of B cell and T cell tolerance. Lastly, current immunosuppression therapies used in renal transplantation do not target NK cells directly (38). It may therefore be prudent to gain a better understanding into the role of NK cells in renal IRI in order to design immunosuppressive therapies that directly address NK cells to limit the damage caused by them in the allograft.

1.4 Endothelium and IRI

MVECs are the cells that comprise the inner-most layer of blood vessels and as such they are a particularly important cell type due to their critical role in maintaining vascular homeostasis (39). MVECs are critical regulators of smooth muscle contractions, are directly responsible for the exchange of macromolecules and fluids between the blood

and surrounding tissues, and also participate in immune surveillance (39). MVECs are particularly interesting in a transplant setting as they are the primary barrier between graft and host following any solid organ transplant. In fact, MVEC activation during transplantation has been shown to be an important factor and can result from IRI (40, 41). Activated MVECs mediate graft injury via increased vascular permeability, platelet adhesion, the expression of adhesion molecules, and through the release of pro-inflammatory molecules such as interleukin 1 (IL-1) and TNFα (42). Cumulatively, the activation of MVECs leads to increased host immune infiltration and accelerated graft rejection (42).

MVECs are also capable of inducing the activation and proliferation of T cells through the expression of active costimulatory molecules, including CD40 (43). T cell activation by CD40 results in the production of IFN-γ, which induces the expression of HLA class-II molecules on MVECs, leading to a sustained alloimmune reaction (44). Furthermore, the expression of P- and E-selectins, vascular cell adhesion molecule-1 (VCAM-1), and intracellular adhesion molecule-1 (ICAM-1) by MVECs has been shown to correlate with graft damage and rejection (45), as these molecules allow for host leukocyte infiltration of the graft (46). As the primary biological barrier between graft and host, and as an activator of leukocytes, MVECs play a critical role in the alloimmune responses that follow any solid organ transplant.

1.5 Programmed Cell Death in IRI

IRI ultimately causes cells of the kidney allograft to undergo various different forms of programmed cell death, all of which contribute to the graft being damaged. These cell death pathways include caspase-dependent extrinsic apoptosis (47), intrinsic apoptosis (48, 49) and programmed necrosis, including necroptosis (50, 51) as well as pyroptosis (52, 53) and ferroptosis (54). As these programmed cell death pathways are a major cause of cell death during IRI, a thorough understanding of the mechanisms and consequences of these cell death pathways is crucial.

1.5.1 Caspase-8-dependent Extrinsic Apoptosis in Renal IRI

Apoptosis is a well-studied cellular process that is required for the non-pathological remodelling and repair of tissues and organs. In fact, apoptosis is the predominant form of cell death in a healthy kidney, with metabolically active TECs and MVECs undergoing particularly rapid turnover (55). However, under the setting of severe renal IRI it is possible for apoptosis of cells of the graft to exceed the regenerative capacity of the kidney, this will intuitively lead to loss of graft function and ultimately will lead to graft failure (56).

Within the inflammatory environment of IRI, cytokines such as TNFα and Fas ligand (FASL) (56, 57), as well as signalling molecules such as nitric oxide (58), will lead to the activation of caspase-8, a cysteine-aspartic acid protease that is responsible for extrinsic (aka death receptor-mediated) apoptosis. The initiation of extrinsic apoptosis is similar to the initiation of necroptosis, in which Complex I assembly leads to the poly-ubiquination of receptor interacting protein kinase (RIPK)1, which leads to the formation of Complex II. The recruitment of caspase-8 to Complex II is where necroptosis and extrinsic apoptosis diverge. In extrinsic apoptosis, activated caspase-8 will cleave both RIPK1 and RIPK3, thereby eliminating the possibility of the necroptosis pathway and committing the cell towards extrinsic apoptosis (59). In addition to inactivating RIPK1 and RIPK3, caspase-8 will also cleave downstream effector caspases into their activated forms (60). These activated ‘executioner’ caspases then proceed to dismantle the cell from the inside: DNA is fragmented, chromatin is condensed, and the characteristic membrane blebbing of apoptosis follows (61). Since caspase-8 promotes apoptosis in cells of the kidney allograft, and therefore graft damage during IRI, it would be of benefit to define strategies to inhibit caspase-8 activity in donor kidneys (62). However, apoptosis is an essential cell death pathway in a healthy organ, as is evidenced by the fact that caspase-8 knockout mice are embryonically lethal (63). Therefore, attempts to minimize apoptosis by interfering with caspase-8 activity must be confined to short periods, as to avoid organ infection or cancer.

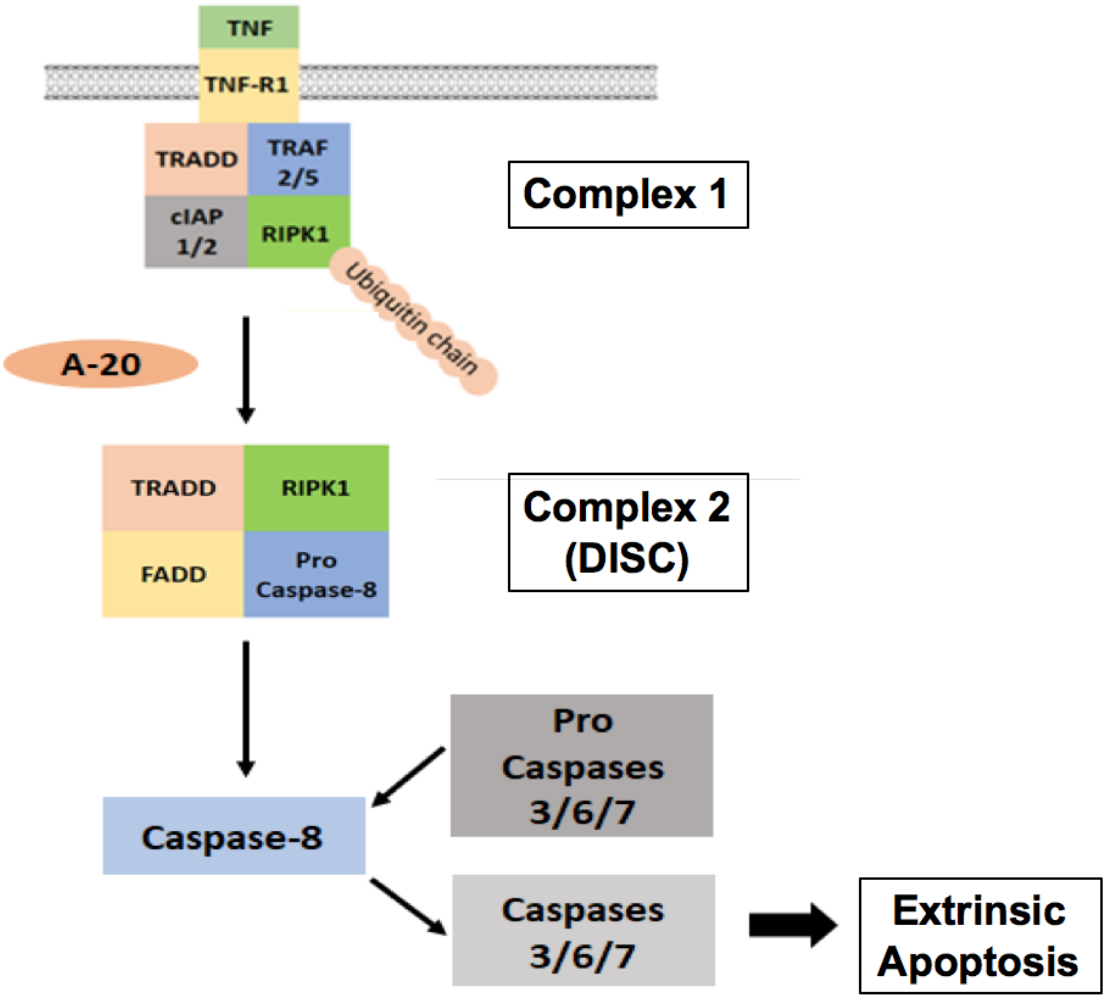


Figure 2. Mechanism of extrinsic apoptosis.

Upon TNFα binding to its receptor, TNFR-1, assembly of Complex I at the cytosolic tail domain of TNFR-1 occurs. Complex I consists of TRADD, cIAPs, TRAF2/5, and poly-ubiquitinated RIPK1. Removal of the ubiquitin tail from RIPK1 by A-20 allows for the formation of DISC/Complex II from Complex I. The DISC complex then directly cleaves and activates pro-caspase-8, which cleaves and activates pro-caspases-3, -6, and -7, known as executioner caspases. Activation of executioner caspases leads to DNA fragmentation and apoptosis.

1.5.2 Intrinsic Apoptosis in Renal IRI

Another form of regulated cell death that is prominent during renal IRI is intrinsic apoptosis. During renal IRI, cells of the allograft experience a variety of stressful stimuli, including acidic pH (64) and intracellular ROS production (65). Under such stress, cells of the kidney allograft will start to upregulate the expression of B cell lymphoma-2 (BCL-2) family members, including the pro-apoptotic effector BCL-2 associated X protein (BAX) (66), which increases most dramatically of all family members, and remains increased as long as 7 days after transplant (67).

Stressful stimuli cause BAX to become activated and translocate to organelle membranes to promote apoptosis (68), the most important of which is the mitochondrial outer membrane (MOM) (69). At the MOM, BAX causes the opening of voltage-dependant anion channel-1 (VDAC-1) (70), which becomes associated with cyclophilin D (CypD) and adenine nucleotide translocator (ANT) to form the mitochondrial permeability transition pore (MPTP) (71, 72). The formation of the MPTP complex leads to the loss of mitochondrial membrane potential, which leads to swelling, mitochondrial inner membrane permeability, and subsequent rupture of the MOM (73). The rupture of the MOM is known as the ‘point of no return’ of intrinsic apoptosis, as it results in the release of massive amounts of cytochrome C (74) which binds to apoptotic protease activating factor 1 (APAF-1) to form the apoptosome complex (75). The assembly of the apoptosome complex will cause the activation of caspase-9 (76), which is responsible for cleavage and activation of downstream executioner caspases-3, -6, and -7 (77). The activated executioner caspases lead to the fragmentation of DNA and the degradation of many key cellular proteins (61), ultimately leading to cell death.

Intrinsic apoptosis of cells in the kidney allograft is very well studied, with a considerable amount of evidence pointing to the involvement of intrinsic apoptosis of graft cells in exacerbating renal IRI and kidney graft damage (78). Therefore, much like extrinsic apoptosis, strategies to transiently limit intrinsic apoptosis would confer survival benefits to cells of a renal allograft, thus improving overall survival of the graft.

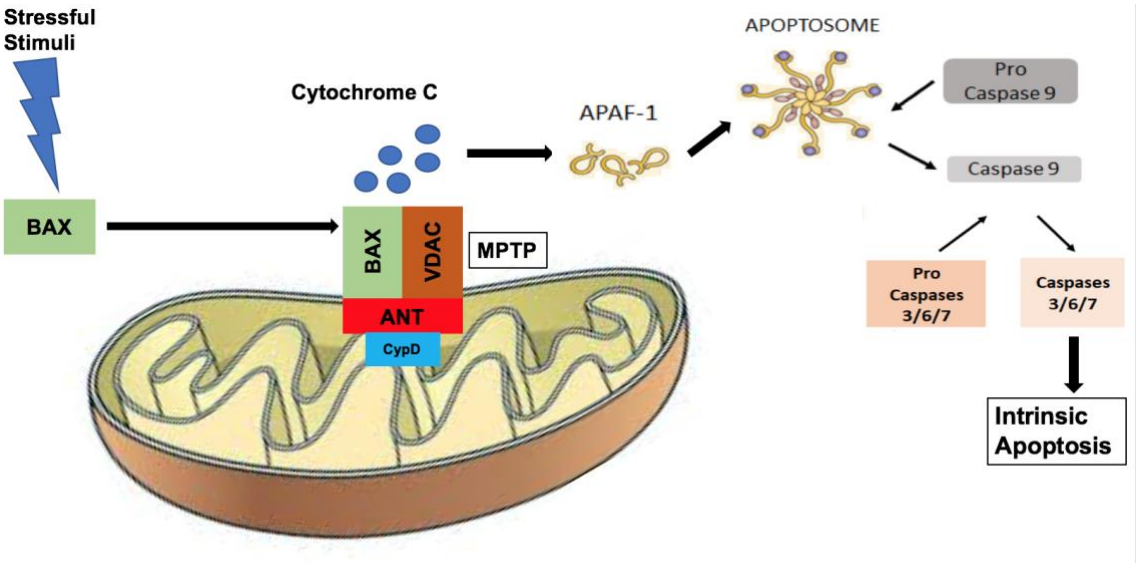


Figure 3. Mechanism of intrinsic apoptosis.

Stressful stimuli, such as an accumulation of ROS that occurs during IRI result in the activation of BAX. Activated BAX will then translocate to the MOM and become associated with VDAC-1, which then recruits ANT and CypD to form the MPTP. The formation of the MPTP leads to cytochrome C release which binds to APAF-1 to form the apoptosome complex. The apoptosome complex activates caspase-9 which cleaves and activates executioner caspases-3, -6, and -7. The activated executioner caspases lead to DNA fragmentation and apoptosis.

1.5.3 Receptor Interacting Protein Kinase 3 (RIPK3)-mediated Necroptosis in Renal IRI

Necroptosis is a regulated form of necrosis that occurs in kidney allografts. Recent data implicates necroptosis as a major factor contributing to cell death in kidney allografts and in the worsening of IRI (79). Necroptosis is not distinguishable from necrosis biochemically or morphologically (80, 81), as both of these cell death pathways involve the swelling of the mitochondria and the cytoplasm, both due to ATP depletion, as well as loss of plasma membrane integrity, and eventual cell lysis (82, 83). Necroptosis is similar to apoptosis in that it is programmed, but necroptosis also resembles necrosis generally in that cell lysis and the leakage of intracellular immunogenic DAMPs occurs (84).

Necroptosis in kidney allografts can be triggered by many different ways, and typically involves specific death receptors binding to their ligands. Death receptors that trigger necroptosis upon ligand binding include: TNF receptor 1 (85), FAS receptor (86), TNF-related apoptosis inducing ligand receptor (87, 88) and TLR-4 (89). All of these receptors can trigger necroptosis upon ligand binding via different downstream pathways (90, 91). A commonality between necroptotic pathways is the activation of receptor interacting protein kinase 1 (RIPK1) and RIPK3, which are serine/threonine kinases that form into a necrosome complex (92-94). Upon ligand binding to its corresponding death receptor, several different adaptor proteins (95) are recruited to the cytoplasmic domain of the activated death receptor, including poly-ubiquinated RIPK1 (96), which together with the death receptor form necrosome Complex 1 (97). After initiation of complex 1, the ubiquitin tail of RIPK1 is cleaved by enzymatic effectors of necroptosis such as A20 (98) or cyclindromatosis (99, 100). The cleavage of RIPK1's ubiquitin tail leads to the formation of Complex II from Complex I, which includes both RIPK1 and RIPK3 (80, 101). After the formation of necrosome Complex II, RIPK3 will phosphorylate and activate downstream molecules, including mixed lineage kinase domain-like (MLKL) (102). Phosphorylated-MLKL will then cause pore formation in the plasma membrane, leading to cell lysis and the release of DAMPs from the cell (103). This resultant release of intracellular immunogenic DAMPs will recruit other cytotoxic immune cells, including NK cells (20). The release of DAMPs and recruitment of immune cells that occurs during

IRI highlights the fact that necroptosis is problematic in a transplant setting. IRI causes death of allograft cells which leads to DAMP release, which leads to the recruitment of additional immune cells, which contribute to further injury in an auto-amplification fashion. For this reason, limiting or controlling the release of DAMPs during IRI, or preventing the released DAMPs from recruiting cytotoxic immune cells, may prove to be an effective strategy to attenuate damage caused during IRI. One DAMP that is particularly interesting and is receiving much attention recently in transplant, is cell-free DNA (cfDNA). As DNA is present in all cell types (excluding erythrocytes), production is multi-cellular.

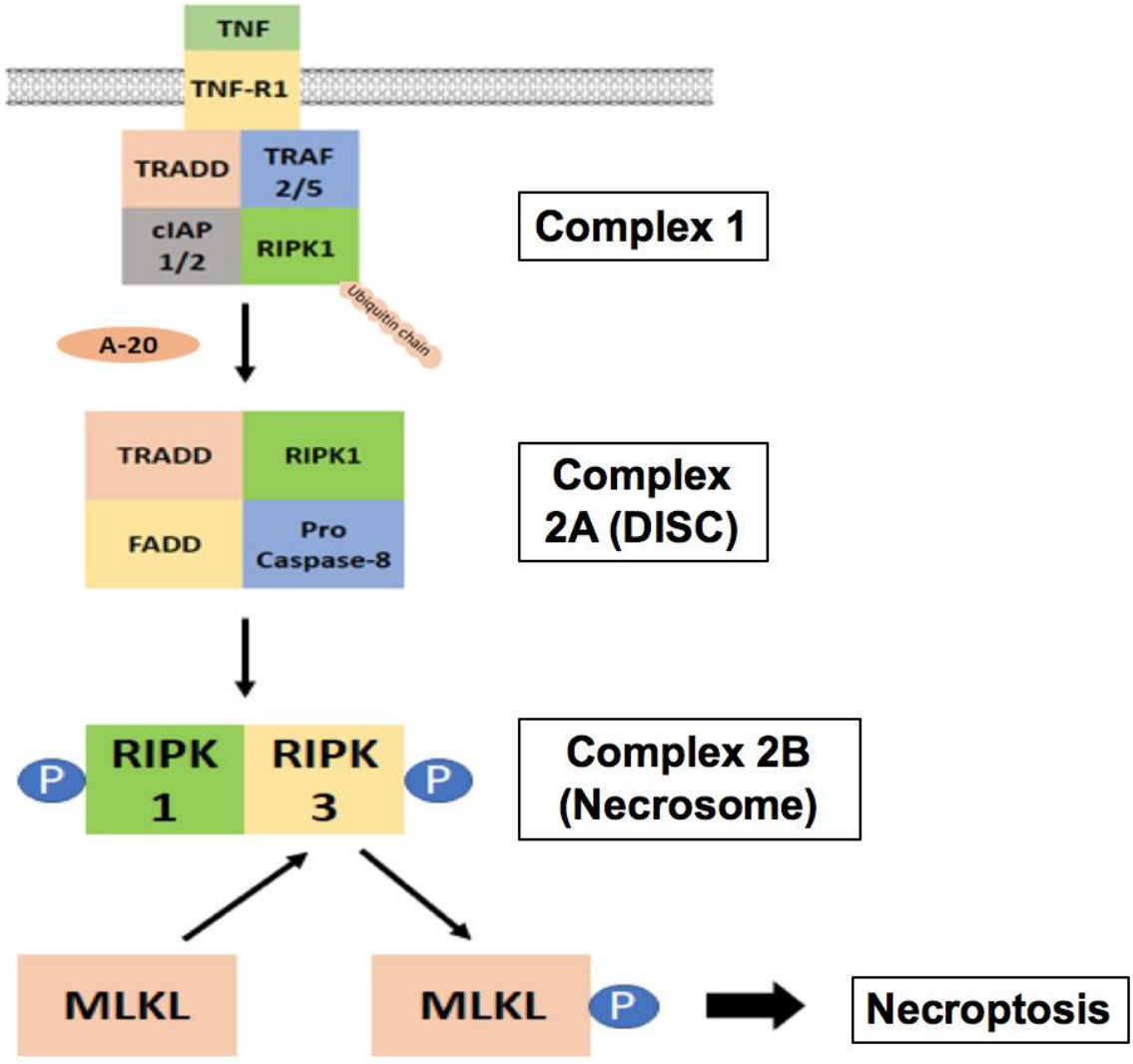


Figure 4. Mechanism of necroptosis.

Upon TNFα binding to its receptor, TNF-R1, assembly of Complex I at the cytosolic tail domain of TNF-R1 occurs. Complex I consists of TRADD, cIAPs, TRAF2/5, and poly-ubiquitinated RIPK1. Removal of the ubiquitin tail from RIPK1 by A-20 allows for the formation of the DISC/Complex II from Complex I. In the absence of caspase-8 activation, RIPK1 and RIPK3 become associated and form the Necrosome Complex. Once the Necrosome Complex is formed, RIPK3 phosphorylates MLKL. Phosphorylated MLKL induces mitochondrial permeability, cell rupture, and necroptosis.

1.6 Cell-free DNA in Transplantation

As the name suggests, cfDNA are degraded fragments of DNA that have been released into the bloodstream and circulate extracellularly. Circulating nucleic acids were first discovered in 1948 (104), and in 1966 it was shown for the first time that cfDNA levels are higher in diseased patients compared to healthy individuals, a discovery that was made first in Lupus patients (105). Since its discovery, cfDNA has become a useful diagnostic tool, particularly in diagnosing pre-eclampsia (106), fetal chromosome abnormalities (106), and various forms of cancer (107). In fact, the ability to detect circulating tumor DNA led to the development of the 'liquid biopsy', allowing for non-invasive diagnosis and screening of various cancers (108). As research progresses, it has become evident that cfDNA is not just a biomarker for a few specific diseases, but rather it is a marker of general cellular injury. This position is supported by the recent finding that cfDNA levels become measurably higher with age (109).

The current gold standard for monitoring graft function in kidney transplant recipients is the serial measurement of serum creatinine levels (110). Unfortunately, a deterioration in graft function can only be detected after significant damage to the graft has occurred (111). As such, a more sensitive biomarker of kidney graft function is needed. Furthermore, subclinical rejection (graft damage in the *absence* of a measurable increase in serum creatinine) can be detected only at present by surveillance biopsies which are costly and invasive (112). As such, a less invasive and more efficient detection of subclinical rejection is needed. A potential solution to this problem is donor-derived cell-free DNA (ddcfDNA). ddcfDNA is detectable in both the blood and urine of transplant recipients (113) and is a direct result of damage to the graft (114). For these reasons, ddcfDNA is a potential biomarker of graft injury. The potential to isolate and quantify ddcfDNA from urine or serum may lead to new, less-invasive diagnoses of acute rejection and graft damage. Indeed, high levels of ddcfDNA post-transplantation have been reported and may be caused by unbalanced programmed cell death during IRI (115-117).

Despite the aforementioned intrigue, little is known about the mechanism of cfDNA release from the graft. Apoptosis (118-121), necrosis (121) and active secretion (122-124) have all been postulated as mechanisms of cfDNA release into the blood. Furthermore,

even less is known of the biological function of cfDNA. Most of the research on cfDNA is as a potential biomarker for injury. However, it has also been shown that cfDNA is more GC-rich than genomic DNA (125) and that cfDNA is capable of binding TLR9, leading to the activation of the NF-kB pathway and the secretion of TNFα and Interleukin-10 (IL-10) in human adipose-derived mesenchymal stem cells (haMSCs) (126). Clearly cfDNA is not just a biomarker for cellular injury, but is also a biologically active molecule that is capable or participating in and influencing immune responses. Greater insights on the release and mechanism of action of cfDNA in transplant may alter diagnosis of early injury as well as potentially direct new therapies.

1.7 Hypothesis

We hypothesize that cfDNA is released by MVECs during programmed cell death with ischemia reperfusion injury, and that cfDNA acts as both a biomarker for cellular injury as well as a biologically active molecule capable of amplifying inflammation and organ injury.

1.8 Objectives

Our first objective is to determine if MVECs release cfDNA during programmed forms of cell death. Our second objective is to determine if cfDNA is released during ischemia reperfusion injury *in vivo*, and whether it can be prevented with cell death inhibition. Our third and final objective is to determine if cfDNA is capable of activating NK cells *in vitro*.

Chapter 2

2 MATERIALS AND METHODS

2.1 Animals

Wild-type C57BL/6 (B6) mice were obtained commercially from Charles River Canada Inc (St. Constant, QC). B6 RIPK3$^{-/-}$ mice were generously provided by Dr. Kim Newton (Genentech) (127). Combined B6 RIPK3$^{-/-}$ Caspase-8$^{-/-}$ double knockout (DKO) mice were generously provided by Dr. Douglas Green (St. Jude Children's Research Hospital, TN) (128). All mice were maintained in the animal care facility at Western University using approved protocols. All experimental procedures were approved by the Animal Care Committee at Western University.

2.2 Microvascular Endothelial Cell (MVEC) Culture

Microvascular endothelial cells (MVECs) were isolated from mice hearts as previously described (129). MVECs were grown in DMEM (Invitrogen), supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100IU/mL penicillin, and 100IU/mL streptomycin (Invitrogen).

2.3 NK Cell Culture

Murine NK cells were purified from the spleens of WT C57BL/6 mice. Adult mice were sacrificed and spleens were removed and mechanically processed through a 20μm cell strainer (BD Biosciences, Mississauga, ON). Following mechanical processing, whole splenocytes were labelled with anti-CD3ε magnetic MACS beads (Miltenyi Biotec, Auburn, CA) for 10 minutes at 4°C in 500μL of MACS buffer (PBS, 1% FBS, 0.5% EDTA). Labelled splenocytes were then passed through a magnetic MACS bead column to deplete all CD3ε^{+} cells. Subsequently, the column was washed three times with MACS Buffer. Lastly, NK cells were purified from the column flow through by CD49b^{+} selection on MACS beads columns using anti-CD49b magnetic MACS beads (Miltenyi). NK cells were cultured in RPMI media (Invitrogen), supplemented with human IL-2, 2mM

glutamine, 1mM sodium pyruvate, 10mM HEPES, 0.5mM 2-ME, 100µg/mL streptomycin, 100 IU/mL penicillin and 10% FBS. NK cell purity was confirmed to be >93% $CD49b^{+}NK1.1^{+}$, $CD3^{-}$ by flow cytometry.

2.4 B16-Bim$_S$ and B16-FADD-DD Cell Culture

B16-Bim$_S$ and B16-FADD-DD cell cultures were generously provided by Dr. Lakshman Gunaratnam (Western University, London, ON). B16-Bim$_S$ and B16-FADD-DD cell lines are derived from mouse melanoma cell line B16 as described (130), resulting in cell lines with tetracycline/doxycycline-inducible expression of either Bcl-2 family protein Bim$_S$ or Fas-associated death domain protein (FADD). The induced expression of Bim$_S$ results in rapid apoptosis (130), while the induced expression of FADD death domain (FADD-DD) results in nonapoptotic/necrotic cell death. Both cell lines were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 50µg/µL penicillin/streptomycin, 50µM 2-ME and 50µg/mL G-418 sulfate. Gene expression was induced by stimulating cells with 1µg/mL tetracycline.

2.5 Cell Death Assay

B6 MVECs were seeded on 6-well flat-bottomed plates for 24 hours in complete DMEM (Invitrogen) in order to generate a confluent mono-layer. Subsequently, MVECs were treated with human TNFα (100ng/mL, PeproTech, USA), Smac-mimetic (15nM, Selleckchem, USA), Z-Ile-Glu-Thr-Asp-Fluoromethylketone (IETD, 30µM, APExBIO, USA), and Necrostatin-1 (Nec-1, 10µM, Merck Millipore, USA) in serum free media for 24 hours. TNFα is an inflammatory cytokine that induces cell death. Smac-mimetic suppresses the function of Inhibitor of Apoptosis Proteins (IAPs) which leads to caspase activation, promoting apoptotic cell death. IETD inhibits caspase-mediated apoptosis by inhibiting caspase-8. Nec-1 is a RIPK1 inhibitor that results in inhibited necroptosis. Cell death was quantified using SYTOX® Green Nucleic Acid Stain (100nM, ThermoFisher, USA), in which cell death is correlated to fluorescence intensity of SYTOX. Fluorescent intensity of SYTOX was monitored using the IncuCyte ZOOM® System (Essen Bioscience, USA). Cell death was also quantified using flow cytometry. Briefly, cells were trypsinized (Gibco) 24 hours after treatment with the same reagents mentioned above, and

washed with PBS. Cell death was detected with propidium iodide (PI, BD Bioscience) and Annexin-V (BD Bioscience) staining and analyzed with the CytoFLEX flow cytometer (Beckman Coulter, USA).

2.6 cfDNA Isolation

cfDNA was extracted from either serum or cell culture supernatant using the NucleoSpin Gel and Polymerase Chain Reaction (PCR) Clean-up kit (Machery-Nagel), following the PCR clean-up protocol with minor adjustments. The PCR clean-up protocol is a particularly suitable method for cfDNA isolation as it avoids the use of both membrane lysis and protein degradation steps that may liberate cellular genomic DNA from live cells or DNA bound to blood proteins in the serum. Prior to cfDNA extraction, samples were centrifuged at 16000 x g for 5 minutes at room temperature in order to remove any live cells from the cell culture supernatant or serum. After centrifugation and prior to extraction, samples were kept at -80 °C. For each biological replicate, cfDNA was extracted from either 1.0mL of cell culture supernatant, or from 100µL of serum. Prior to extraction, samples were mixed with NTB binding buffer in a buffer-to-sample ratio of 2:1. Next, the samples were vortexed and added to the spin column and centrifuged at 11000 x g for 1 minute at room temperature. The columns were washed twice and dried twice, and cfDNA was eluted into 20µL of elution buffer.

2.7 cfDNA Quantification

cfDNA was quantified using the Qubit® 1.0 fluorometer (Invitrogen) in combination with the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen) according to the manufacturer's instructions. Briefly, 1µL of extracted cfDNA was diluted in 9µL of DEPC H_2O and then mixed with 90µL of PicoGreen™ working solution (200:1 mixture of TE buffer and Quant-iT™ PicoGreen™ reagent). Samples were quantified in triplicate (100µL/well) in 96-well plates. cfDNA concentration was calculated using a standard curve ranging from 0 to 100ng/µL, according to the manufacturer's protocol using Lambda DNA provided with the PicoGreen™ kit. Samples were excited at 480nm and fluorescence emission intensity was detected at 520nm.

2.8 Kidney Ischemia Reperfusion Injury (IRI) Model

The left kidney was removed and a renal clamp was applied to the right kidney pedicle of wild type B6, RIPK3$^{-/-}$, or RIPK3$^{-/-}$/caspase-8 double knockout (DKO) mice for 45 minutes (28). Body temperature during surgery was not precisely regulated, relying on a heat-cool blanket with average body temperatures during surgery being approximately 34°C, measured using a rectal thermometer, or was precisely controlled at either 24°C, 28°C, 32°C, or 36°C using the Homeothermic Monitoring System (Harvard Apparatus). Serum was collected at either 24- or 48-hours post-surgery. Serum creatinine levels were determined using the Catalyst Dx Chemistry Analyzer (IDEXX, Canada).

2.9 Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from NK cells using the RNeasy Mini kit (Qiagen, USA). The concentration and purity of isolated RNA was measured using the GENESYS™ UV-vis spectrophotometer (Thermofisher, Canada) in order to accurately generate standardized quantities of cDNA. cDNA generation from RNA was accomplished using Superscript II Reverse Transcriptase (Thermofisher, USA). Real-time quantitative PCR was performed using the Brilliant SYBR Green qPCR Master Mix™ kit (ABM, Canada) with the CFX Connect (BioRad, USA) qPCR machine. Normalized delta threshold cycle (Ct) value and relative expression levels (ΔΔCt) were calculated according to the manufacturer's protocol. Primers used are: β-actin F (CCA GCC TTC CTT CCT GGG TA), β-actin R (CTA GAA CAT TTG CGG TGCA), Granzyme B F (TGG GGG ACC CAG AGA TTA AAA), Granzyme B R (TTT CGT CCA TAG GAG ACA ATG C), IFNγ F (TGC ATC TTG GCT TTG CAG CTC), IFNγ R (GGC TTT CAA TGA GTG TGC CGT), TNFα F (GCC TCT TCT CAT TCC TGC TTG), and TNFα R (CTG ATG AGA GGG AGG CCA TT).

2.10 Statistical Analysis

Experimental values were expressed as mean + SD. Data was analyzed using 1-Way ANOVA with Tukey's Multiple Comparison test. All statistical analyses were performed using Prism 7™ software.

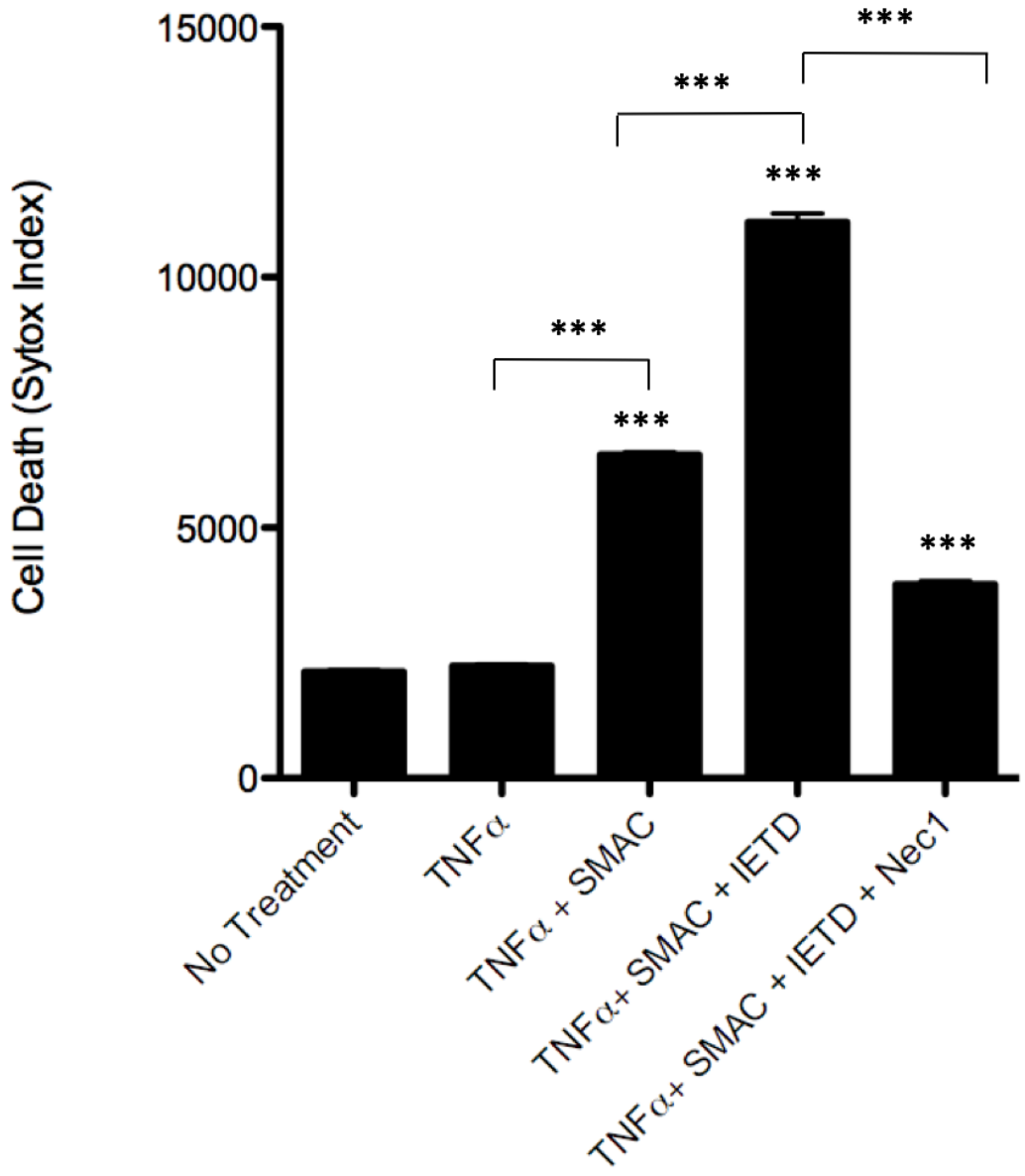
Chapter 3

3 RESULTS

3.1 MVECs Undergo Apoptosis and Necroptosis when Treated with Various Inflammatory Cytokines *in vitro*

In order to first characterize the programmed cell death of wild type B6 MVECs, we added various inflammatory cytokines to MVEC cultures. Mouse TNFα, Smac-mimetic, caspase-8 inhibitor IETD, and the RIPK1-inhibitor Nec-1 were added to wild type B6 MVEC cell cultures. Cell death was quantified by SYTOX fluorescent intensity with IncuCyte ZOOM live imaging system. Treatment of MVECs with TNFα alone did not increase cell death compared to untreated cells. The addition of Smac-mimetic, an inhibitor of inhibitor of apoptosis proteins (IAPs), enhanced TNFα-induced cell death, suggesting apoptotic cell death (Figure 5A). The addition of caspase-8 inhibitor IETD further enhanced TNFα-induced cell death. Since caspase-8 is required for apoptotic cell death, it is likely that the cell death observed with IETD treatment is not from apoptosis. Lastly, when necroptosis was inhibited in TNFα, Smac-mimetic, and IETD-treated MVECs using the RIPK1 inhibitor Nec-1, cell death was eliminated to near-baseline levels (Figure 5A). As RIPK1 is essential in necroptosis, the reduction in cell death with RIPK1 inhibition is indicative that TNFα, Smac-mimetic, and IETD-treated MVECs are undergoing necroptosis. To confirm this, treated cells were also stained with Annexin V and PI and were analyzed by flow cytometry. Annexin V positive staining supports apoptosis as the primary form of cell death, PI positive staining indicates necrosis, and double positive cells indicate late apoptosis and early necroptosis. Indeed, MVECs treated with TNFα and Smac-mimetic show a cell population that is Annexin V positive, with no cell populations that are PI positive, supporting mostly apoptotic cell death (Figure 5B). Furthermore, MVECs treated with TNFα, Smac-mimetic, and caspase-8 inhibitor IETD show a large portion of cells that are Annexin V and PI double positive, while the addition of Nec-1 eliminates this cell population, suggesting as expected, that necroptotic cell death is occurring in cells treated with TNFα, Smac-mimetic, and IETD.

A.


Cell Death (Sytox Index)
15000
10000
5000
0
***
***
***
***
***
No Treatment
TNFα
TNFα + SMAC
TNFα+ SMAC + IETD
TNFα+ SMAC + IETD + Nec1

B.

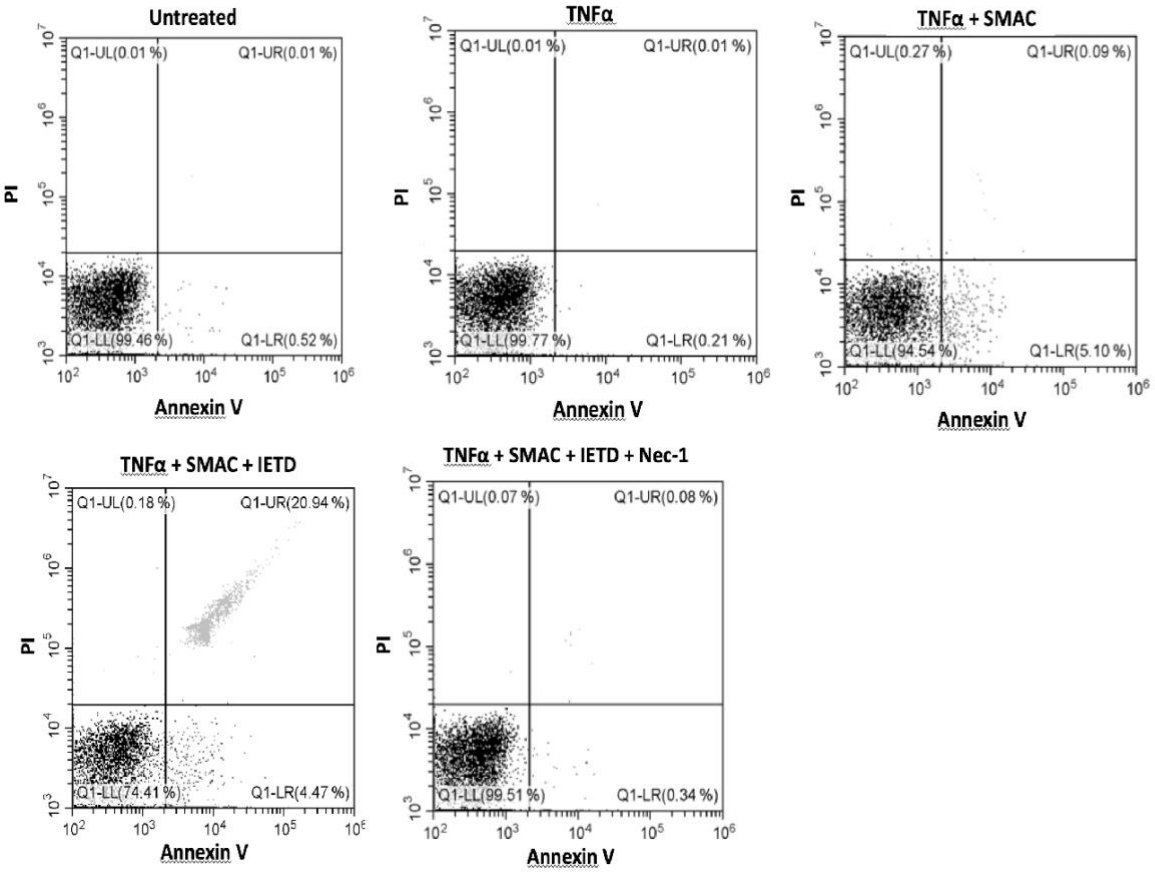


Figure 5. Treatment of MVECs with various cytokines results in apoptosis and necroptosis.

(A) B6 MVEC were plated on 6 well plates in triplicates and were treated with 100ng/mL human TNFα (T), 15nM Smac-mimetic (S), 30μM IETD (I) and 10μM Nec-1 (N) along with the addition 100nM SYTOX green. Cell death was quantified by SYTOX fluorescent intensity as measured by the IncuCyte ZOOM live imaging system. Data at 24 hours were shown as mean +SD of 6 independent experiments. ***p ≤0.0001, 1way ANOVA with Tukey's Multiple Comparison Test. (**B)** B6 MVEC were plated on 6 well plates in triplicates and were treated with 100ng/mL human TNFα, 15nM Smac-mimetic, 30μM IETD and 10μM Nec-1 for 24 hours and cell death was characterized using Annexin V and PI staining, followed by flow cytometry analysis. Data shown are representative of at least 3 independent experiments.

3.2 cfDNA is Released by MVECs During Apoptosis and Necroptosis *in vitro*

Next, we sought to investigate whether cfDNA is released from MVECs during cytokine-induced cell death. MVECs were treated with cytokines as described in Figure 5. cfDNA was isolated from the supernatant of cytokine-treated MVEC cell culture and was quantified using a PicoGreen™ assay.

It has been suggested that cfDNA is released from apoptotic cells (118-121), necrotic cells (121), as well as by active secretion (122-114). In agreement, our data indicate that cfDNA indeed is released in significantly large quantities by apoptotic (TNFα and Smac-mimetic treated) cells when compared to untreated cells (Figure 6). However, our data shows that cfDNA is released in large quantities by necroptotic (TNFα, Smac-mimetic, and IETD treated) cells when compared to untreated cells, and that necroptotic cells release more cfDNA than apoptotic cells (Figure 6). Furthermore, when both necroptosis and apoptosis are inhibited (via treatment with Nec-1 and IETD, respectively) cfDNA release is attenuated to levels comparable to untreated cells.

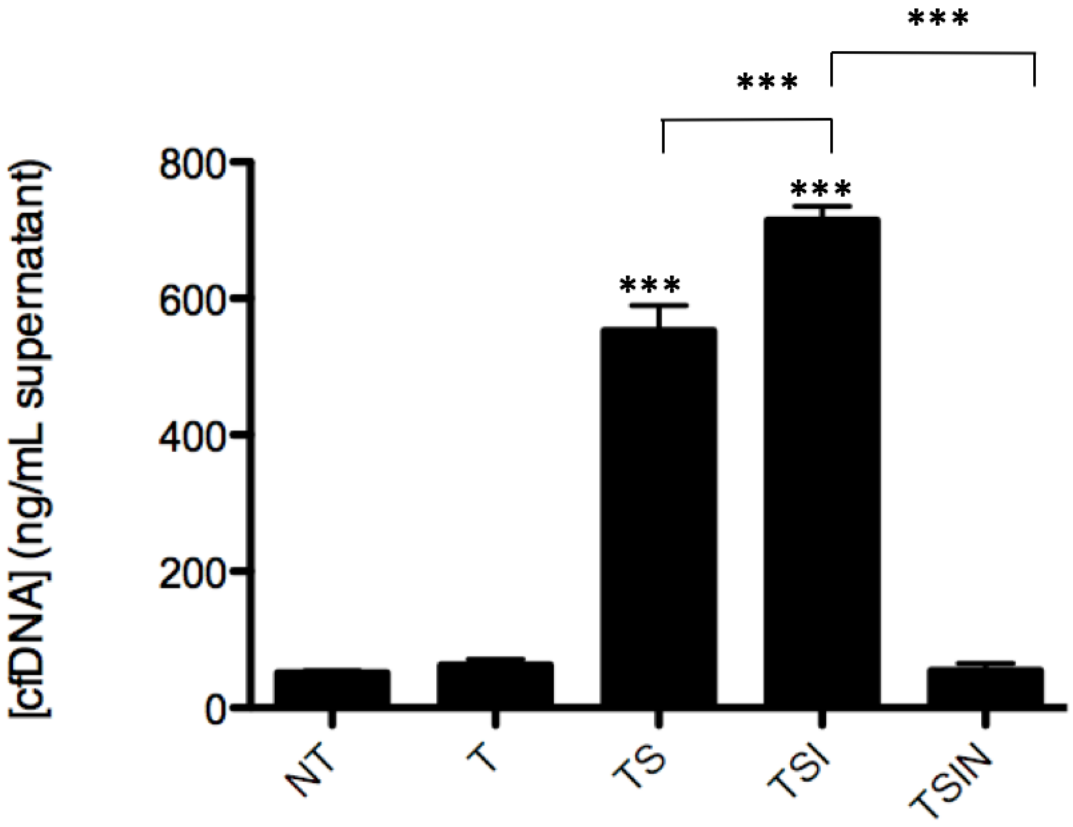


Figure 6. cfDNA is released by MVECs during apoptosis and necroptosis *in vitro.*

B6 MVECs were plated on 6 well plates in triplicates and were treated with 100ng/mL human TNFα (T), 15nM Smac-mimetic (S), 30μM IETD (I) and 10μM Nec-1 (N). cfDNA was isolated from the supernatant of treated cells and was quantified using the PicoGreen™ assay. Data is shown as Mean +SD of 8 independent experiments. ***p<0.0001, One-Way ANOVA with Tukey's Multiple Comparison Test.

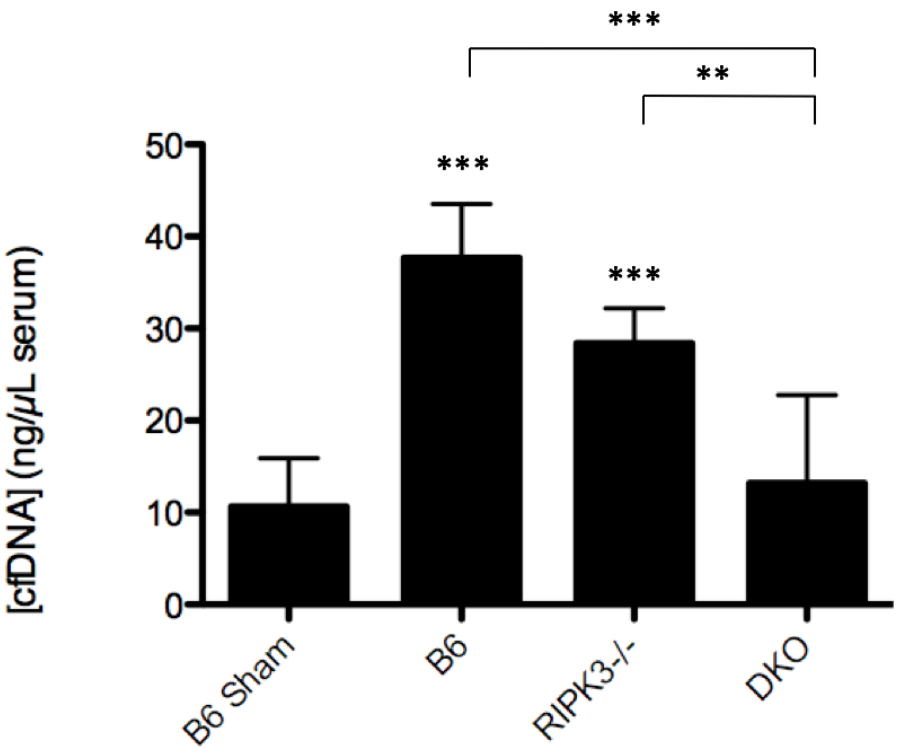
3.3 cfDNA Release is Regulated by Cell Death Programs During Ischemia Reperfusion Injury *in vivo*

To investigate whether cfDNA is released during kidney IRI, a mouse model of kidney IRI was employed. The left kidney was removed and a renal clamp was applied to the right kidney pedicle of wild type B6, RIPK3$^{-/-}$, or RIPK3/caspase-8 double knockout (DKO) mice and was removed after 45 minutes. A wild type B6 sham surgery, in which the mouse was anesthetized, surgically incised, but without renal clamping, was used as a control. Body temperature during surgery was not precisely regulated; however, a rectal probe was used at multiple intervals during the surgery in order to determine body temperature in the absence of precise regulation. Rectal probe measurements indicated an average body temperature of 34°C during the procedure when body temperature is not precisely regulated. Serum was collected at 48-hours post-surgery. Serum creatinine was measured using the Catalyst Dx Chemistry Analyzer (IDEXX). cfDNA was isolated from serum and quantified exactly as described in the isolation of cfDNA from cell culture supernatant in Figure 6.

Our results indicate that serum cfDNA is significantly elevated 48 hours post-IRI in both wild type B6 and RIPK3$^{-/-}$ mice when compared to the sham surgery, and that RIPK3 deficiency modestly reduced serum cfDNA levels post-IRI (Figure 7A). Furthermore, DKO mice show no measurable difference in serum cfDNA 48 hours post-IRI when compared to the sham surgery (Figure 7A). Serum cfDNA levels in DKO mice 48 hours post-IRI are significantly lower than serum cfDNA levels in both B6 and RIPK3$^{-/-}$ mice 48 hours post-IRI.

Furthermore, serum creatinine levels were markedly higher in wild type B6 mice 48 hours post-IRI when compared to the sham surgery (Figure 7B). Interestingly, both RIPK3$^{-/-}$ and DKO serum creatinine levels 48 hours post-IRI were not significantly different from the control sham surgery (Figure 7B).

A.


***
**
***
***
[cfDNA] (ng/μL serum)
50
40
30
20
10
0
B6 Sham
B6
RIPK3-/-
DKO

B.

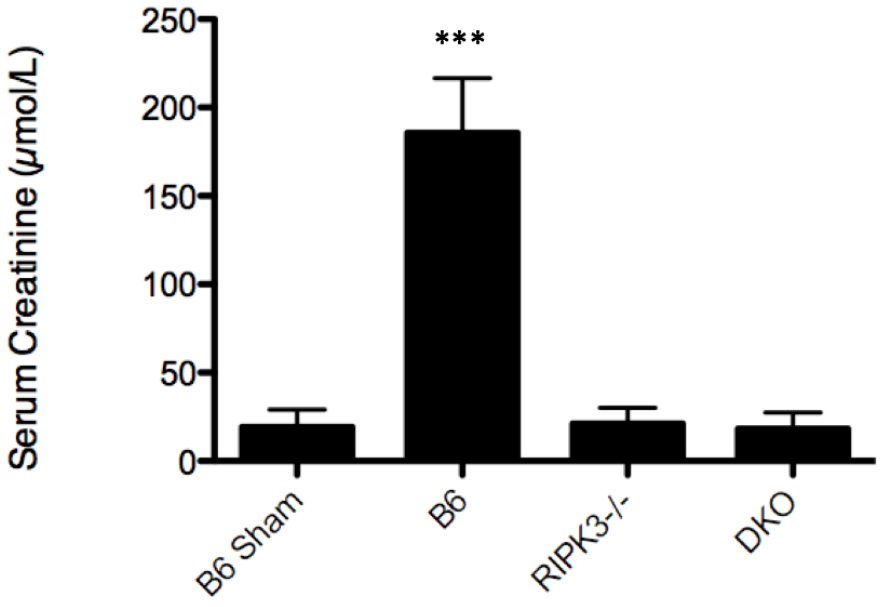

***
Serum Creatinine (μmol/L)
250
200
150
100
50
0
B6 Sham
B6
RIPK3-/-
DKO

Figure 7. cfDNA is released during IRI *in vivo* and is ameliorated in Caspase-8/RIPK3 DKO mice.

The left kidney was removed and a renal clamp was applied to the right kidney pedicle of wild type B6, RIPK3$^{-/-}$, and RIPK3$^{-/-}$ Caspase-8$^{-/-}$ (DKO) mice for 45 minutes. Average body temperature during surgery was measured at 34°C. Serum was collected 48 hours post-IRI for creatinine and cfDNA quantification. **A.** cfDNA was isolated from serum and quantified exactly as described in the isolation of cfDNA from cell culture supernatant in Figure 6. Data is shown as Mean +SD of 6 independent experiments. ***p<0.0001, **p<0.001, One-Way ANOVA with Tukey's Multiple Comparison Test. **B.** Serum creatinine levels were measured using the Catalyst Dx Chemistry Analyzer (IDEXX). Data is shown as Mean +SD of 6 independent experiments. ***p<0.0001, One-Way ANOVA with Tukey's Multiple Comparison Test.

3.4 cfDNA Release During Ischemia Reperfusion Injury *in vivo* is Correlated to the Degree of Injury

Previous data have shown that kidney IRI in rodent models (131) and in humans (134) is temperature-sensitive and that lower temperatures inhibit kidney IRI. To investigate whether cfDNA release during kidney IRI is temperature-dependant, the same mouse model of kidney IRI as described in Figure 7 was employed, except that body temperature during the surgery was precisely controlled at either 24°C, 28°C, 32°C, or 36°C using a sophisticated homeothermic monitoring system (Harvard Apparatus). Briefly, the left kidney was surgically removed and a renal clamp was applied to the right kidney pedicle of wild type B6 mice. Serum was collected at either 24- or 48-hours post-surgery. Serum creatinine was measured using the Catalyst Dx Chemistry Analyzer (IDEXX). cfDNA was isolated from serum and quantified exactly as described with isolation from cell culture supernatant in Figure 6.

Our data indicate that the body temperature at which surgery is performed is a crucial determinant of the degree of renal injury caused by IRI (Figure 8). Firstly, at an early time point of 24 hours post-IRI, cfDNA levels isolated from serum show an increase in a temperature/injury-dependant manner, with statistically significant increases in serum cfDNA occurring between 24°C and 28°C, 28°C and 32°C, as well as between 32°C and 36°C (Figure 8A). Furthermore, at the same 24-hour time point, serum creatinine levels also increase in a temperature-dependant manner. Statistical differences between 24°C and 28°C are observed, as well as between 28°C and 32°C (Figure 8B). Interestingly, no significant difference in serum creatinine levels was measured between 32°C and 36°C, nor was a significant increase detected when comparing IRI at 24°C with sham control (Figure 8B). It is worth noting that serum creatinine levels for both sham surgery and 24°C IRI were at or below the lower limit detectable using the IDEXX.

Our data show a similar, but less pronounced temperature-dependant increase in both serum cfDNA and creatinine levels when the serum is analyzed 48-hours post-IRI instead of 24-hours post-IRI (Figure 8C). Much like at 24-hours post-IRI, cfDNA levels isolated from serum following IRI is higher than sham control across all temperature groups (Figure 8C). An increase in serum cfDNA was detectable between 24°C and 32°C,

as well as between 24°C and 36°C, but not between 24°C and 28°C (Figure 8C), although a clear trend towards higher cfDNA at higher temperatures is observed.

Lastly, at 48-hours post-IRI, serum creatinine levels also increase in a temperature-dependant manner (Figure 8D). However, at 48-hours these differences are less pronounced than at 24-hours. 32°C and 36°C IRI are both statistically higher than sham control, with an appreciable difference measured between 32°C and 36°C (Figure 8D). However, no significant increase in serum creatinine levels were detected when comparing 24°C and 28°C with sham control. Again, it is worth noting that serum creatinine levels collected 48-hours post-surgery for sham surgery, 24°C IRI, and 28°C IRI were at or below the lower limit detectable using the IDEXX. Taken together, our data suggest that cfDNA is a more sensitive injury marker than serum creatinine measures.

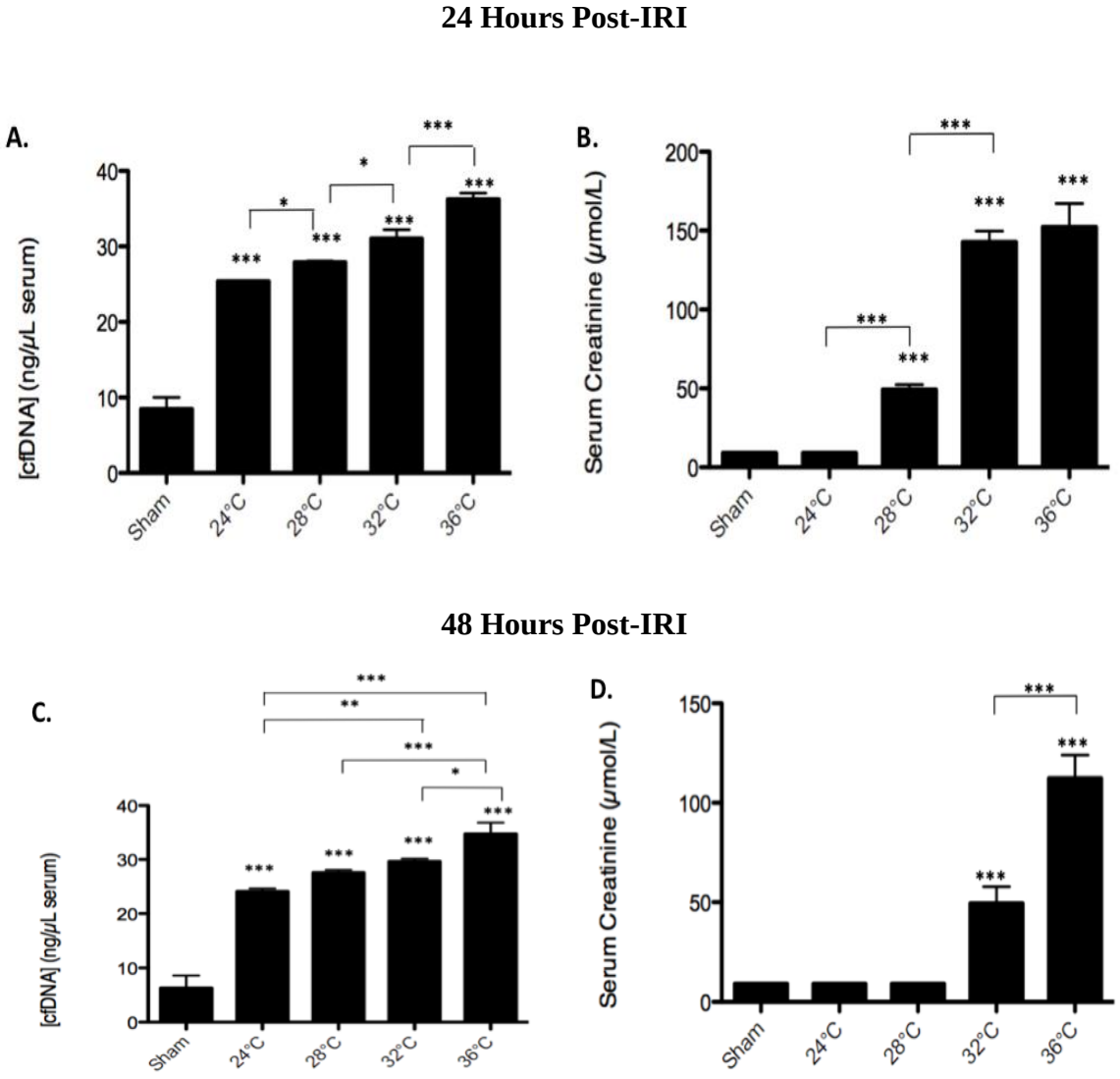


Figure 8. cfDNA is released during IRI *in vivo* in a temperature-dependent manner.

The left kidney was surgically removed and a renal clamp was applied to the right kidney pedicle of wild type B6 mice for 45 minutes. Body temperature during surgery was precisely controlled at either 24°C, 28°C, 32°C, or 36°C. Serum was collected either 24- or 48-hours post-IRI for creatinine and cfDNA quantification. **A./C.** cfDNA was isolated from serum and quantified exactly as described in Figure 6. **B./D.** Serum creatinine levels were measured using the Catalyst Dx Chemistry Analyzer (IDEXX). Data is shown as Mean +SD of 4 independent experiments. ***p<0.0001, **p<0.001, *p<0.01, One-Way ANOVA with Tukey's Multiple Comparison Test.

3.5 cfDNA is Capable of Activating NK Cells *in vitro*

Previous work from our group has established that NK cells are key regulators in cell death during IRI and it has been demonstrated that NK cells play a key role in both acute and chronic kidney allograft injury (28, 31-37). NK cells have been shown to participate directly in the killing of kidney TECs following renal IRI (28) and it is well-established that NK cells induce cytotoxicity by releasing perforin and granzyme (25). Furthermore, NK cells also contribute to the global inflammatory response during IRI by releasing chemokines and cytokines such as IFNγ and TNFα which recruit and activate additional innate immune cells that contribute to and worsen the inflammatory landscape during IRI (26, 27).

Furthermore, previous work has established that cfDNA is capable of binding to TLR9, activating the NF-kB pathway, leading to the secretion of TNFα and IL-10 in haMSCs (126). These results clearly indicate that cfDNA may not just be a biomarker of cell death, but that it may also play a role in exacerbating inflammation, thereby worsening acute allograft injury. For these reasons, we sought to characterize the response of NK cells, known mediators of IRI, to cfDNA *in vitro*.

Firstly, primary NK cells were isolated and treated with cfDNA isolated from the serum of wild type B6 mice 48-hours post-IRI. We also sought to determine whether cfDNA from necrotic cells differed from cfDNA from apoptotic cells in its ability to activate NK cells. In order to accomplish this, we also subjected NK cell culture to 100 ng/mL cfDNA isolated from programmed cell death control cell lines B16-BIMs cell culture and B16-FADD-DD cell culture, which are genetically capable of undergoing only apoptosis and necroptosis, respectively, in lieu of all other forms of programmed cell death. Positive controls, 50µg/mL PolyIC and 2000 IU/mL human IL-2 were also added to NK cell culture as these are well described activators of NK cells.

Granzyme B expression was significantly increased following treatment with 100ng/mL cfDNA isolated from B6 IRI serum, B16-BIMs cells, and B16-FADD cells (Figure 9A). Importantly, no increase in Granzyme B expression was observed in NK cells treated with elution buffer, or with 100ng/mL cfDNA + DNase (Figure 9A).

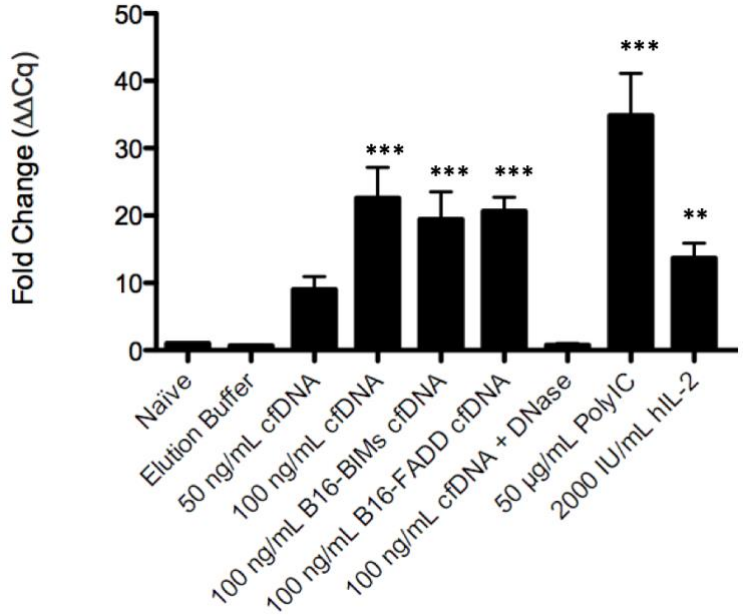
Unsurprisingly, Granzyme B expression was significantly increased following treatment with positive controls PolyIC and human IL-2 (Figure 9A).

Similarly, IFNγ expression was also significantly increased following treatment with 100ng/mL cfDNA isolated from B6 IRI serum, B16-BIMs cells, and B16-FADD cells (Figure 9B). Again, no increase in IFNγ expression was observed in NK cells treated with elution buffer, or with 100ng/mL cfDNA + DNase (Figure 9B). Lastly, IFNγ expression was significantly increased following treatment with PolyIC, but no increase in expression was observed with human IL-2 treated cells (Figure 9B).

Similar to Granzyme B and IFNγ, TNFα expression was significantly increased following treatment with 100ng/mL cfDNA isolated from B6 IRI serum and B16-BIMs cells (Figure 9C). However, although a clear trend is observable, statistically significant increases in TNFα expression were not observed in NK cells treated with 100ng/mL cfDNA isolated from B16-FADD cells (Figure 9C). Visually, it is clear that there is a trend towards upregulation, however, since TNFα expression is only modestly increased even in the highest groups (approximately 2-fold in PolyIC positive control), it is likely that a very high n value would be required for statistical significance. Importantly, no increase in TNFα expression was observed in NK cells treated with elution buffer, or with 100ng/mL cfDNA + DNase (Figure 9C). Lastly, much like IFNγ, TNFα expression was significantly increased following treatment with PolyIC but not with human IL-2 treated cells (Figure 9C).

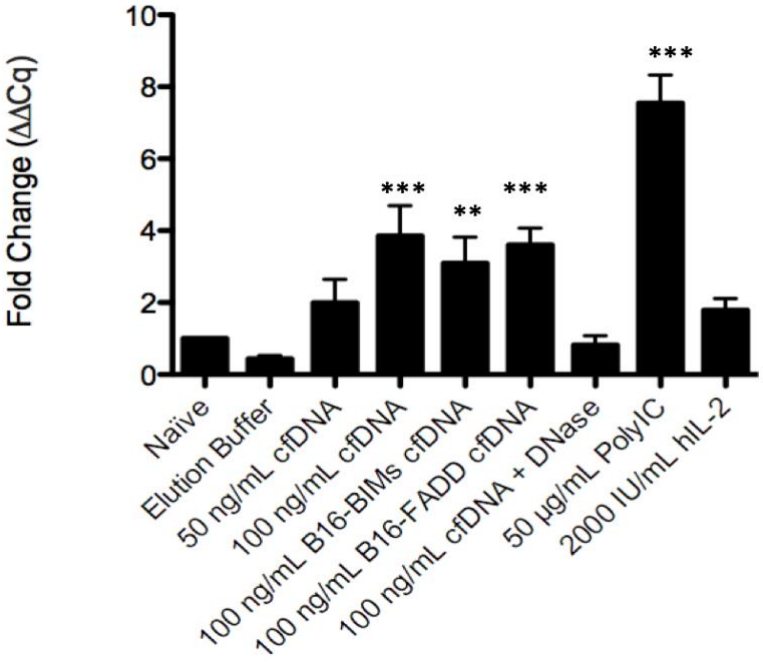
A.

Granzyme B



B.

IFNγ



C.

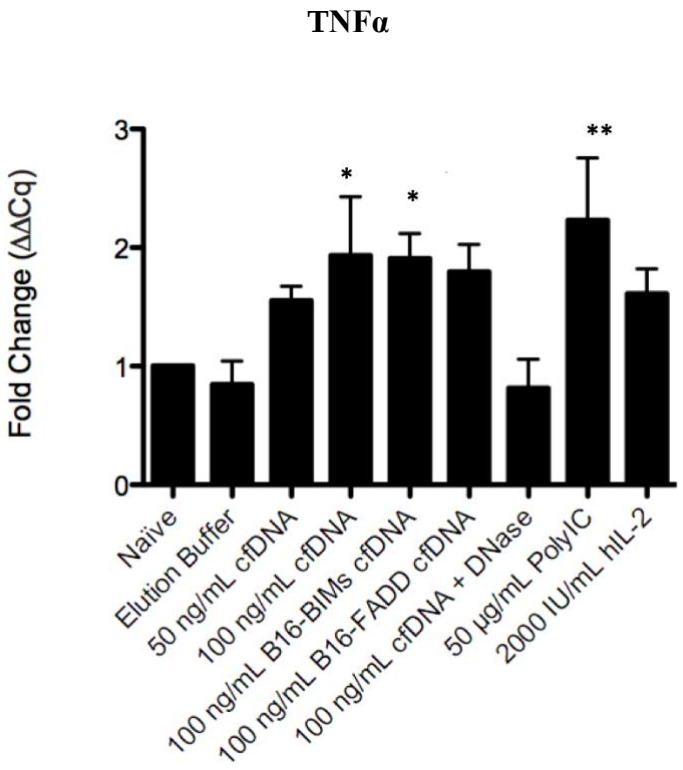


Figure 9. cfDNA is capable of activating NK cells *in vitro*.

NK cells were purified from the spleen of wild type B6 mice and were treated *in vitro* for 12 hours with elution buffer from which cfDNA is isolated as a negative control, as well as with 50 and 100ng/mL cfDNA isolated from the serum of B6 mice following IRI. NK cells were also treated with 100ng/mL cfDNA isolated from B16-BIMs and B16-FADD cells, which are genetically capable of only undergoing only apoptosis and necrosis, respectively. Lastly, NK cells were treated with 50µg/mL PolyIC and 2000 IU/mL human IL-2, two known activators of NK cells, as positive controls. RNA was isolated from cultured cells 12 hours after treatment and cDNA was generated. Relative fold change of Granzyme B (**A)**, IFNγ **(B)**, and TNFα **(C)** mRNA expression before and after treatment was determined by qPCR. Data is shown as Mean +SD of 4 independent experiments. ***p<0.0001, **p<0.001, *p<0.01, One-Way ANOVA with Tukey's Multiple Comparison Test.

Chapter 4

4 DISCUSSION

4.1 cfDNA is Released During Apoptosis and Necroptosis *in vitro*

For our interest in transplants, the foremost requirement for a biomarker that is to be used for cellular injury is that it correlates positively with cell injury or death. That is to say that when cell death increases, the marker should increase, and when cell death decreases, the marker should also decrease. Our data strongly support that cfDNA release *in vitro* is correlated with established measures of cell death, such as SYTOX fluorescence intensity and staining with PI and Annexin V. MVECs treated with TNFα and Smac-mimetic to induce apoptosis showed a substantial increase in cell death as measured by SYTOX intensity, and similarly showed a substantial increase in cfDNA release. Furthermore, MVECs treated with TNFα, Smac-mimetic, and IETD to induce necroptosis also showed a substantial increase in cell death compared to untreated controls, and similarly showed an increase in cfDNA release as well. Importantly, MVECs treated with TNFα, Smac-mimetic, IETD, and Nec-1, a treatment regimen that inhibits both apoptosis and necroptosis, showed a substantial reduction in both cell death and cfDNA release. From our data, it is evident that cfDNA release mirrors traditional cell death measures and thus may be a potential biomarker of cellular injury that is useful in diverse forms of organ injury in transplantation.

Another objective was to establish whether apoptosis or necroptosis differ in their release of cfDNA, as cell death during organ injury may be polarized to one form or another. Additionally, defining the pathways involved would be critical to selection of pharmacologic inhibitors of specific forms of cell death. It has been previously postulated that both apoptosis and necroptosis may contribute to cfDNA release (118-121) but this has not been determined conclusively to date. Our data clearly indicate that necroptotic MVECs (TSI treated) released considerably more cfDNA than apoptotic MVECs (TS treated). Briefly, we are confident that TS treated cells undergo apoptosis for the following

reasons. Firstly, TNFα is a known inducer of apoptosis. MVECs, as endothelial cells, are particularly hardy cells that are resistant to programmed cell death. As such, in our model, in order to induce significant levels of apoptosis, cells are also treated with Smac-mimetic. Smac-mimetic suppresses the function of IAPs, and this leads to caspase activation and the inhibition of RIPK1 polyubiquination, thereby promoting cell death. This is confirmed by the fact that TS treated cells show a population of Annexin V positive cells, with no population of PI positive cells. Although there is overlap, it is generally accepted that Annexin V primarily labels early apoptotic cells while PI labels necrotic or late-apoptotic cells. Therefore, we are confident that TS treated cells are primarily undergoing apoptosis. The addition of IETD to TS treated cells inhibits caspase-8, a key molecular effector of apoptosis. When caspase-8 is inhibited, cells can't undergo apoptosis and as a result the cell is programmed to shift to necroptosis (132). In fact, necroptosis has been postulated as a 'failsafe' evolutionary response to infections from caspase-8 inhibiting viruses (133). This is reflected in the fact that TSI treated cells show a significant population that are PI and Annexin V double positive, an indicator of necroptosis. Furthermore, the addition of Nec-1 inhibited necroptosis and cfDNA release. In conclusion, necroptotic MVECs released significantly more cfDNA than apoptotic MVECs.

It was not determined by our studies whether cfDNA from apoptotic cells is different than cfDNA from necroptotic cells in sequence, size, or biological effect. It is well accepted that while necrosis promotes inflammation (84), apoptosis is relatively 'silent' in its effect on immune cells and can contribute to immune tolerance (61). Further studies on cfDNA sequence and packaging within lipid bound vesicles may identify such a difference. During IRI, cells dying by apoptosis will fragment organelles and DNA, which are packaged into exosomes in a controlled manner along with other DAMPs and cellular materials (61). The resultant exosomes are non-immunogenic as their contents are encased in exosomes and kept secluded from the immune system. In contrast, cells dying by necroptosis experience cell lysis and the leakage of intracellular DAMPs (84) which are detected by resident dendritic cells (17) and results in the recruitment of additional immune cells, including T cells (20), B cells, macrophages, neutrophils, and NK cells (21). Due to the release of DAMPs and the recruitment of immune cells, necroptosis promotes inflammation (84). In the setting of IRI, perhaps cfDNA released from apoptotic cells is

encased in membrane-bound exosomes, while cfDNA released from necroptotic cells is not. This distinction would explain why apoptosis is non-inflammatory and necroptosis is pro-inflammatory. Although our data show no biological difference between apoptotic and necroptotic cfDNA, perhaps our method of cfDNA extraction is disrupting the exosomes that are produced by apoptotic cells. Further studies on the presence and contents of these exosomes in both serum and cell culture supernatant following apoptosis, necroptosis, or IRI, may clarify this.

4.2 cfDNA is a Sensitive Biomarker for Kidney Injury

Shortly following organ transplantation, donor-derived cfDNA can be detected in the blood or urine of the recipient (113). As such, cfDNA is increasingly being viewed as a potential way to detect allograft injury in clinical transplantation, particularly with rejection (114). In contrast to apoptosis, there is currently no information regarding the role of necroptosis in generating cfDNA during organ injury and transplantation. Our next step in determining the potential of cfDNA as a biomarker of cellular injury was to translate insights from our *in vitro* results to an *in vivo* model of mouse IRI. cfDNA is readily detected in serum following kidney IRI; B6 IRI serum contains considerably more cfDNA than serum from B6 sham surgery. These findings were supported by results in which serum creatinine was also significantly elevated in B6 IRI when compared to sham control. This finding supports the concept that cfDNA is a biomarker of cellular injury that occurs during IRI. In support of this, DKO mice, in which caspase-8 and RIPK3 are genetically knocked out, show significantly reduced levels of both serum cfDNA and creatinine when compared to wild type B6 IRI, and are comparable to cfDNA and creatinine levels in sham controls. It is worth noting that RIPK3$^{-/-}$ IRI mice trend towards lower serum cfDNA levels than IRI in B6 mice, but statistical significance was not achieved. Larger test numbers may have clarified this. This suggests that inhibition of necroptosis alone may attenuate but not abrogate IRI, in keeping with previous results reported. However, inhibition of both apoptosis and necroptosis together appears to be much more effective. Indeed, these results make sense when taken into context with results in which MVECs with both apoptosis and necroptosis inhibition (TSIN treated) showed a drastic reduction in cell death when compared to cells undergoing apoptosis (TS) or necroptosis (TSI) alone. Taken together,

these results indicate that any attempts to reduce cell death during IRI should practically target both apoptosis and necroptosis, as inhibiting only one form of cell death will likely not be sufficient. It is also worth noting that these findings are likely not applicable just to IRI in a transplant setting. Any disease that involves ischemia, such a myocardial infarcts or strokes, may similarly benefit from simultaneous inhibition of apoptosis and necroptosis.

Lastly, RIPK3$^{-/-}$ IRI mice showed serum creatinines that were comparable to sham, while cfDNA levels were significantly higher than those using sham surgery. This finding lead us to consider that cfDNA may be a more sensitive biomarker of kidney injury than serum creatinines, which are currently used clinically as measures of kidney dysfunction. In order to investigate this possibility further, we employed a temperature-dependant IRI model in order to characterize cfDNA release during subclinical IRI.

Indeed, as temperature during IRI was increased in 4°C intervals, from 24°C to 36°C, significant differences in serum cfDNA were detected at each interval, with cfDNA increasing as temperature increases. This finding is valuable for two reasons. Firstly, it is clear that the temperature at which ischemia is induced plays a significant role in the degree of injury to the kidney, with small reductions in temperature resulting in measurable reductions in cell death. These findings recapitulate an important finding by Niemann *et al.* (134), showing that mild hypothermia in DCD donors significantly reduced the rate of delayed graft function among kidney transplant recipients. Secondly, it is clear that cfDNA is a very sensitive biomarker of injury, as serum levels were reduced even with small reductions in IRI model temperatures. Furthermore, cfDNA was detectable in subclinical IRI at 24°C in which serum creatinines remained in the normal range 24 hours post-IRI. At 48 hours post-IRI, cfDNA was detectable in subclinical IRI at 24-28°C, in which serum creatinines also remained in the normal range. These findings indicate that cfDNA is a more sensitive biomarker of kidney cellular injury than serum creatinine levels. These findings are particularly important because serum creatinine measurements are the current gold standard for monitoring graft function in kidney transplant recipients (110), but it has been noted that deterioration in graft function can only be detected after significant damage to the graft has occurred (111). Therefore, a more sensitive biomarker of kidney injury that

can be detected before significant damage to the graft occurs would be very valuable. Furthermore, subclinical rejection (graft damage in the absence of a measureable increase in serum creatinine) is currently detected exclusively via surveillance biopsies that are both costly and invasive (112). Since cfDNA can be detected in both the blood and urine of transplant recipients (113), and because we have shown that it is more sensitive than serum creatinines, cfDNA shows promise as a less invasive and more sensitive method of detecting subclinical rejection. It is also worth noting that differences in both cfDNA and creatinine are more pronounced at 24 hours than 48 hours post-IRI. This is logical as the more time that has passed following injury, the more time there is for phagocytes to clear DAMPs such as cfDNA. It remains to be tested whether the changes we observed with cfDNA in the absence of a change in serum creatinine has an impact on long term kidney function. This will be key to any strategy that uses cfDNA as a surrogate measure of injury

4.3 cfDNA is a Biologically Active DAMP that is Capable of Exacerbating Inflammation and IRI

After characterizing the release of cfDNA during programmed cell death *in vitro* and during IRI *in vivo*, our third and final objective was to determine if cfDNA is capable of activating NK cells *in vitro*. Previous research has shown that cfDNA is capable of binding to TLR9 to induce the secretion of TNFα and IL-10 in haMSCs (126), demonstrating clearly that cfDNA has the potential of being immunogenic. Furthermore, NK cells are well-described effectors of IRI (28, 31), however, they are currently not targeted directly by any currently used immunosuppression therapies clinically (38), and the effect of cfDNA on NK cells has not been described.

Our data clearly indicate that NK cells upregulate key activation markers Granzyme B, IFNγ, and TNFα when treated with cfDNA. In particular, Granzyme B and IFNγ mRNA expression increased markedly from naïve controls when treated. The effect of cfDNA was the same when isolated from the serum of B6 mice 48 hours post-IRI, as well as cfDNA isolated from B16-BIM$_S$ and B16-FADD-DD cell lines, which were genetically induced (via tetracycline) to undergo apoptosis and necrosis, respectively. Importantly, this effect was abrogated completely with the addition of DNase, and no activation was observed when cells were treated with the elution buffer used to extract cfDNA. It is noteworthy that

a significant increase in mRNA expression was not detected for any of the activation markers in NK cells treated with 50ng/mL cfDNA. However, a clear trend is evident, and statistical significance may have been achieved with a higher n value. TNFα mRNA expression increased only modestly with the addition of cfDNA from B6 IRI mice and from apoptotic B16-BIM$_S$ cell culture, but not from necrotic cfDNA extracted from B16-FADD-DD cell culture. Again, a clear trend is evident and statistical significance may have been achieved with a higher n value, if time had permitted. Lastly, it is clear that PolyIC is a more potent activator of NK cells than is human IL-2. This finding may not have obvious biological implications, but it supports the notion that PolyIC may represent the near maximal positive control for NK cell activation in future experiments.

Prior to these experiments, we had considered whether cfDNA released from necrotic vs apoptotic cells would differ in its ability to activate NK cells. Our result clearly shows that the source of cfDNA does not appear to be relevant and that cfDNA released from apoptosis and necroptosis activate NK cell function equivalently at the same dose. More cfDNA is released by necroptotic cells than apoptotic cells, and therefore necroptosis may be a more important target than apoptosis from this quantitative standpoint, however, as explained above, strategies that target both apoptosis and necroptosis simultaneously will likely be most effective.

The cumulative injuries accrued during IRI, from ischemic conditions to the release of reactive oxygen species before and upon reperfusion, cause cells of the kidney allograft to undergo various forms of programmed cell death, which cumulatively contribute to further injury and organ loss (47-51). During this injury, intracellular DAMPs, including cfDNA, as well as heat shock proteins (HSPs) and high-mobility group box-1 (HMGB1), are released and enter the circulation to have local and distant effects (103). We have demonstrated the release of cfDNA with programmed cell death *in vitro* and *in vivo* and that cfDNA can activate NK cells. Therefore, as IRI causes cell death, which in turn leads to DAMP release that can activate NK cells and potentiate their function as cytotoxic effectors with IRI, sets up an auto-amplification program that will result in more cell death, and the further release DAMPs (Figure 10). It is intuitive that strategies that can reduce cell death and the resultant DAMP release during IRI would be very beneficial. It is also

evident from these results that strategies to prevent cfDNA from activating NK cells would also be beneficial.

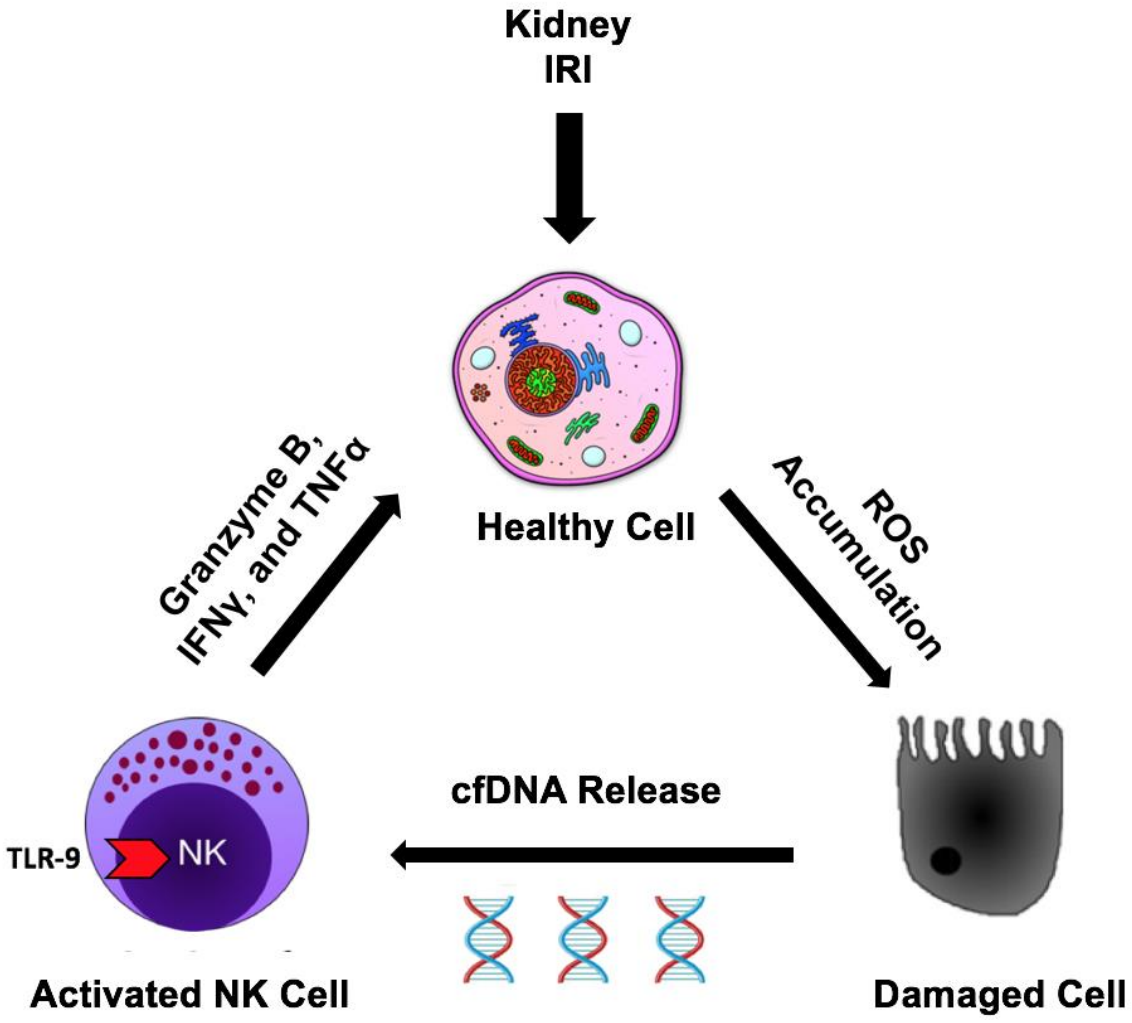


Figure 10. Proposed mechanism of inflammatory cycle during kidney IRI.

Transplantation-associated IRI causes cell death in the kidney allograft, leading to the release of cfDNA from apoptotic and necroptotic cells. Released cfDNA binds to and activates NK cells, which as cytotoxic effector cells will result in more cell death and more cfDNA release in a cyclical fashion.

4.4 Strategies to Reduce cfDNA Release

In an attempt to block the cyclical inflammatory pattern of cell death leading to DAMP release leading to immune cell activation and more cell death, it is perhaps most logical to stop this problem at its start. Thus, any strategy that prevents IRI will be beneficial, although in the case of transplant it is more complex in that surgical removal of the organ from the donor is always required. Our results also support that inhibition of both apoptosis and necroptosis simultaneously may be of impact clinically. Therefore, strategies to simultaneously inhibit apoptosis and necroptosis in kidney allografts should be explored. It is worth noting that any of these strategies would likely be limited to early time periods, as permanent elimination might result in cancerous cell growth. It is noted that in the case of necroptosis, there is no cancer phenotype for RIPK3 null mice, which suggests that the risk for cancer might be exaggerated. In contrast, long-term inhibition of caspase-8 has not been tested in a genetic deletion, as this is embryonic lethal. Transient inhibitors of apoptosis and necroptosis could certainly be considered as additives to organ perfusion solutions. Our results as well as others also show a temperature-dependant reduction in tissue injury, with less cell death occurring as the temperature at which ischemia occurs is lowered. This is consistent with the current trend in clinical transplantation towards using sub-normothermic perfusion of to-be-transplanted donor organs.

4.5 Strategies to Reduce the Biological Activity of cfDNA

As we have shown that cfDNA activates NK cells, which are known effectors of IRI (28), it would also be prudent to explore strategies that prevent cfDNA from activating NK cells. Previous work has shown that cfDNA is more GC-rich than genomic DNA (perhaps because GC-rich regions of DNA are more stable in blood) (125), and cfDNA is therefore capable of binding to and activating TLR9 (126). NK cells are known expressers of TLR9, and TLR9 activation in NK cells results in the induction of cytotoxicity and cytokine release (135). Therefore, it would be useful to explore options to inhibit TLR9 in NK cells in the setting of IRI. However, much like the inhibition of cell death pathways, permanent inhibition of TLR9 in organ transplant recipients may not be desirable as it would further suppress immunity and increase vulnerability to bacteria and viruses. Therefore, transient antagonism of TLR9 could be considered before and immediately after

transplantation in order to prevent NK cell activation during IRI. As TLR9 is an intracellular receptor, it would also be useful to study the uptake of cfDNA into NK cells. Perhaps this is another avenue of intervention that should be explored. Furthermore, as cfDNA is clearly an activator of immune effector cells, perhaps simply adding DNase to organ perfusion solutions will limit immune cell activation and subsequent cell death in the perfused organ.

Lastly, an elegant approach to limiting cfDNA-induced inflammation in a mouse model of rheumatoid arthritis (RA) was described by Liang *et al*. (136). As cfDNA is obviously anionic (due to the negatively charged phosphate backbone of DNA), Liang *et al*. utilized 40nm cationic nanoparticles (cNP) to scavenge cfDNA and prevent activation via TLR9 on various immune effector cells. Injection of cNPs inhibited the activation of monocytes in the synovial fluid, and relieved bone and cartilage damage, as well as tissue and ankle swelling. A similar model of cNP injection could be explored using a mouse transplant model to assess its effect on acute graft injury.

4.6 Conclusions

Our data indicate that cfDNA is released from MVECs during apoptosis and necroptosis *in vitro*. While necroptotic MVECs release more cfDNA than apoptotic MVECs, biological effects are similar in NK cell activation, and any differences elicited may be due to quantitative rather than qualitative issues. cfDNA was also readily detected in serum following kidney IRI in a mouse model *in vivo*, with levels in DKO mice, incapable of undergoing apoptosis or necroptosis, comparable to sham controls. We also show that cfDNA is a particularly sensitive biomarker of injury, as serum levels were reduced even with small reductions in IRI model temperatures, with cfDNA being detectable at low temperatures in which serum creatinines remained in the normal range. Lastly, we have shown that NK cells, well described effectors of IRI, upregulate key activation markers Granzyme B, IFNγ, and TNFα when treated with cfDNA. Therefore, cfDNA is not only a biomarker of kidney injury; it also has inflammatory properties.

4.7 Future Directions

As cfDNA appears to be a very sensitive marker of subclinical cellular injury and IRI, it is plausible that one day we may see the development of real time cfDNA sensors that can be employed during *ex vivo* organ perfusion. This is an obvious engineering challenge, but if executed, would provide a clinician with real time measures of cfDNA as a proxy of graft viability. This would allow a clinician to make real time judgement calls on whether a potential donor organ (particularly from deceased donors) is of sufficient quality for transplantation. Furthermore, as cfDNA is particularly sensitive, it is possible for the development of liquid biopsies to replace current surgical biopsies, where subclinical acute rejection can be diagnosed at an earlier time point than is currently possible. This would allow for earlier intervention during subclinical acute rejection, and is also less invasive for the patient.

More work is also required to understand the mechanism of cfDNA incorporation into NK cells as well as other immune effector cells such as macrophages and monocytes. As mentioned, cfDNA binds to TLR9 which is an intracellular receptor. What is not clear is how cfDNA gets from the blood into the cytoplasm of NK cells. There are many receptors on the surface of NK cells that may allow for cfDNA incorporation into the cytoplasm, and these receptors are potential therapeutic targets. Lastly, similar to the injection of cNPs into a RA model, a similar method could be explored in a mouse model of transplant or IRI.

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2015 Dean's Honor List, Western University

2014 Dean's Honor List, Western University

2013 Dean's Honor List, Western University

Platform Presentations:

Abstract Title: Cell-free DNA Release During Programmed Cell Death of IRI - *Alexander Dionne, Adnan Qamar, Patrick McLeod, Yuexia Ma, Zhu-Xu Zhang, and Anthony M. Jevnikar*

- Medical Resident Research Day 2019 – London, Ontario

Conferences:

Abstract Title: Cell-free DNA Release During Programmed Cell Death of IRI - *Alexander Dionne, Adnan Qamar, Patrick McLeod, Yuexia Ma, Zhu-Xu Zhang, and Anthony M. Jevnikar*

- Canadian Society of Transplantation 2019 – Banff, Alberta
- London Health Research Day 2019 – London, Ontario
- Pathology Research Day 2019 – London, Ontario
- Western Research Forum 2019 – London Ontario