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RIPK3 Regulates Microvascular Endothelial Cell Necroptosis and Cardiac Allograft Rejection

Alexander William Pavlosky, *The University of Western Ontario*

Supervisor: Dr. Zhuxu Zhang, *The University of Western Ontario*
Joint Supervisor: Dr. Anthony Jevnikar, *The University of Western Ontario*
A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pathology

RIPK3 REGULATES MICROVASCULAR ENDOTHELIAL CELL NECROPTOSIS AND CARDIAC ALLOGRAFT REJECTION

(Thesis format: Monograph)

by

Alexander Pavlosky

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

Abstract

Cell death results in tissue damage and ultimately donor graft rejection and can occur as an active molecular process through apoptotic, necrotic and newly identified Receptor Interacting Protein 1 and 3 kinase (RIPK1/3) mediated necroptotic pathways. Necroptosis leads to the release of inflammatory molecules and activation of immune cells which can potentially threaten the graft and has yet to be studied in cardiac transplantation. We have found that necroptosis was induced in murine cardiac microvascular endothelial cells (MVEC) under anti-apoptotic conditions following TNFα treatment and results in the release of the danger molecule high mobility group box 1 (HMGB1). Necroptosis was inhibited by the RIPK1 inhibiting molecule necrostatin-1 and by genetic deletion of RIPK3. In addition, tissue necrosis, release of HMGB1 and graft cell infiltrate were attenuated in RIPK3 null heart allografts following transplantation. Finally, a brief sirolimus treatment markedly prolonged RIPK3 null cardiac allograft survival in allogeneic BALB/c recipients as compared to wildtype C57BL/6 donor grafts (95 ± 5.8 vs. 24 ± 2.6 days, $P<0.05$). This study has demonstrated that RIPK1/3 contributes to MVEC death and cardiac allograft survival through necroptotic death and the release of danger molecules. Our results suggest that targeting RIPK-mediated necroptosis may be an important therapeutic strategy in transplantation.

Keywords

Necroptosis, endothelial, heart transplantation, RIPK

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List of Abbreviations

ACR	Accute cellular rejection
AMR	Antibody mediated rejection
APC	Antigen presenting cell
CDAMPS	Cellular danger associated molecular patterns
cIAP	Cellular inhibitors of apoptosis
CMV	Cytomegalovirus
CTLA4	Cytotoxic T-lymphocyte-associated-antigen 4
CYLD	Cylindromatosis
DC	Dendritic cell
EC	Endothelial cell
FADD	Fas associated death domain
FKBP12	FK binding protein 12
GLUD1	Glutamate dehydrogenase
GLUL	Glutamine synthase
GVD	Graft vascular disease
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1
HSP	Heat shock protein
hTNFα	Human tumour necrosis factor α

ICAM-1	Intracellular adhesion molecule-1
IFNγ	Interferon γ
IL-1	Interleukin-1
IRI	Ischemia reperfusion injury
ISHLT	International society of heart and lung transplantation
JNK	c-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin
MVEC	Microvascular endothelial cell
NEMO	NF-kappa B essential modulator
PYGL	Glycogen phosphoylase
RIPK	Receptor interacting protein kinase
ROS	Reactive oxygen species
TCR	T cell receptor
TNFα	Tumour necrosis factor α
TRAF	TNF receptor associated factor
TLR	Toll-like receptor
TRADD	TNF receptor associated death domain
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand

VCAM-1	Vascular cell adhesion molecule-1

Chapter 1

1 Introduction

1.1 Current status of cardiac allograft transplantation

Following irreversible cardiac damage, transplantation is the most beneficial and highly pursued treatment option available. Cardiac transplantation is most commonly used to treat diseases related to cardiomyocyte dysfunction, coronary artery disease, congenital defects and valvular dysfunction[1]. In past decades, advances in therapeutic immunosuppression and biopsy techniques have allowed adequate control of host immune cell-mediated rejection, improving heart allograft half-life times from 9.6 years in 1982-1992, to 12.0 years in 1993-2003[1]. However, following 2003, improvements in heart transplant survivability have been the result of enhanced one-year survival due to superior therapeutic management of acute rejection, rather than novel chronic rejection therapy, as indicated by conditional post-one-year survival rates[1]. The understanding of the mechanisms involved in chronic rejection of heart allografts continues to fall behind that of acute rejection, necessitating further research in the chronic rejection field.

1.2 Cardiac allograft rejection

Transplantation invariably results in organ injury mediated by host immune cell activation and graft infiltration. Nearly every branch of the immune system has been implicated in allograft rejection, including innate immunity[2, 3], antibody mediated rejection (AMR) resulting in hyperacute accelerated graft rejection and chronic rejection[4], and alloimmune recognition of donor MHC/HLA resulting in adaptive

immune responses. Each of these branches poses a threat to the graft independently and collectively.

Acute cellular rejection (ACR) in cardiac grafts is predominantly a T-cell mediated response characterized by the infiltration of lymphocytes and macrophages into the graft followed by myocyte necrosis and eventual loss of graft function. Host immune cell infiltration can be focal or diffuse depending on the rejection grade defined by the International Society of Heart and Lung Transplantation (ISHLT). ISHLT grading spans from Grade 1, characterized by perivascular or interstitial infiltration with up to one focus of myocyte damage, to Grade 4, characterized by diffuse polymorphous infiltrate with extensive myocyte damage, edema, hemorrhage and vasculitis[5, 6]. According to the current models of ACR pathogenesis, ACR is initiated by the maturation of recipient CD4+ T helper cells, CD8+ cytotoxic T cells and the transformation of host B lymphocytes into antibody producing plasma cells. These cell types can attack the graft directly or indirectly through inflammatory responses that involve monocytes and macrophages. This process is orchestrated by soluble cytokines, such as Tumor Necrosis Factor α (TNFα)[7], that can activate host immune cells or initiate cell death in target cells. Clinically, over 60% of patients will experience one or more acute rejection episodes within 6 months of transplantation. However, due to the advancement of biopsy techniques and immunosuppressive management, acute rejection survival rates have drastically improved[1].

In contrast to ACR, chronic cardiac allograft rejection presents as severe, diffuse vascular intimal hyperplasia involving the entire vessel wall, often resulting in compromised vascular flow and eventual ischemia, similar to non-transplant atherosclerosis. This phenomenon is known as Graft Vascular Disease (GVD). Although the pathogenesis of GVD is not fully understood, the process is known to be mediated by the diffuse accumulation of smooth muscle cells within the vessel intima in response to vascular damage. In the early stages of developing GVD lesions, subendothelial accumulation of mononuclear inflammation occurs in conjunction with increased markers of endothelial cell (EC) activation[8, 9]. As a result of low-level injury caused by recipient infiltrate, host immune cells as well as dysfunctional graft endothelial and smooth muscle cells secrete growth factors to recruit and activate smooth muscle cells[8, 10, 11]. Interestingly, the mechanism of vascular damage seems to be irrelevant in the progression of GVD. Indeed, non-immune graft injury such as IRI can exacerbate GVD through endothelial necrosis, resulting in danger molecule release[12] and EC activation[9, 13, 14]. Interestingly, intimal thickening does not cross the suture line between graft and host vessels, implying an alloimmune role also exists in the pathogenesis of GVD. CD4+ T cells have been indirectly implicated in the progression of GVD through the disruption of nitrous oxide production in ECs by interferon γ (IFNγ) release[15] and cytokine/chemokine regulation by TNFα[16] after activation by antigen presenting cells (APC). Although it is unlikely that any single mechanism causes GVD, the incidence of GVD correlates with CD4+ T cell presence with indirect antidonor allospecificity[17], suggesting that indirect allorecognition is the major pathway of GVD development[18]. In contrast with ACR, GVD is unresponsive to immunosuppressive treatment due to the plethora of biological

mechanisms resulting in its pathogenesis. Consequently, GVD is currently the limiting factor of cardiac allograft transplantation and future therapies, such as the one described here, may instead rely on preventative treatment and endothelial preservation to extend graft survival times.

Currently, several immunosuppressive methods exist to prevent cellular and antibody mediated rejection. As mentioned, T cells often play a critical role during immune responses against the allograft[19]. In order to achieve T cell activation, several molecular pathways must be activated. These include antigen recognition through the T cell receptor (TCR) (signal 1), verification by co-stimulators (signal 2), and cytokine (particularly IL-2) induced proliferation and maturation. Many immunosuppressive therapies target these pathways by preventing either APC activation or lymphocyte activation and proliferation.

During immune reactions, T cells recognize foreign antigens presented on APCs using a TCR/CD3 protein complex. To achieve T cell activation, survival and clonal expansion, an additional signal, known as a costimulatory signal, must also be provided. Failure to provide this signal will result in T cell anergy[20]. Several transplant-relevant costimulatory receptors and ligands have been identified[21]. These include the CD28/B7 pathway and the CD40/CD154 pathway. The CD28/B7 costimulatory pathway is one of the most important T cell activation pathways in both mice and humans and activation failure of this pathway results in T cell anergy, rendering T cells functionally inactive[22, 23]. Upon

engagement with its ligands B7.1 (CD80) and B7.2 (CD86), CD28 initiates a T cell activation signal, allowing for T cell survival, proliferation and cytokine production. This activation pathway is negatively regulated by the CD28 analog cytotoxic T-lymphocyte-associated-antigen 4 (CTLA4), which is also expressed on the surface of T cells. CTLA4 binds B7.1 and B7.2 with greater affinity than CD28 and down-regulates T cell responses[24, 25] and knockout of either CTLA4 or CD28 results in severe lymphoproliferative disorders. Blockade of CD28 directly results in unwanted clinical side effects and therefore an antibody fusion protein CTLA4Ig is instead used to inhibit CD28 activation[26]. Another important costimulatory pathway involves CD40 and CD154 interaction. CD40 is a TNFR superfamily member and is expressed on the surface of APCs. Binding of CD154 on T cells to CD40 is critical for dendritic cell (DC) activation and upregulation of MHC, B7 molecules and cytokine production[27]. CD40/CD154 activation is important during plasma cell isotype switching[28], and indirect activation of T cells through the promotion of CD80 or CD86 expression[29]. This pathway can be inhibited using an anti-CD40 antibody, preventing CD40/CD154 binding, and has shown efficacy in extending allograft survival but not in preventing GVD[30].

Lastly, T cell specific immunosuppression can be achieved by inhibiting IL-2 cytokine production. The advent of the calcineurin inhibitors cyclosporine and tacrolimus, which bind cytosolic proteins cyclophilin and FK binding protein 12 (FKBP12) respectively, greatly advanced the field of transplantation in the 1980's. Binding of cyclophilin and FKBP12 inhibits calcineurin phosphatase, preventing transcription of cytokines (including IL-2) and T cell expansion. Sirolimus, which has a similar molecular structure

to tacrolimus, also binds to FKBP12 but does not inhibit calcineurin phosphatase. Instead, sirolimus binds to the mammalian target of rapamycin (mTOR), a downstream effector of both the CD28/B7 costimulatory pathway and IL-2, effectively making sirolimus an inhibitor of both signal 2 and signal 3[31]. In this study, both sirolimus and CD40 costimulatory blockade are used to promote allograft tolerance.

1.3 Immunophysiology of endothelial cells in transplantation

Microvascular endothelial cells (MVEC) are the first barrier between the graft and host after any solid organ transplantation. Consequently, MVECs are a key player during graft recognition by host immune cells and organ rejection. MVEC activation has been shown to be an important factor in transplantation and can result from donor brain death[32], ischemia reperfusion injury (IRI)[33, 34], cyclosporine toxicity[35] and, most importantly, host immune responses to donor antigens.

Following their activation, MVECs mediate graft injury directly or indirectly through platelet adhesion, increased vascular permeability, expression of adhesion molecules and the release of proinflammatory molecules, such as TNFα or interleukin 1 (IL-1), leading to a loss of vascular integrity and enhanced tissue damage[36]. These mechanisms may provide the first trigger of graft injury, resulting in increased host immune infiltration and accelerated graft rejection.

Interestingly, cultured human MVECs have been shown to cause direct activation and proliferation of T cells in the absence of professional APCs through the expression of active costimulatory molecules such as CD40[37] and promote the differentiation of monocytes into competent APCs[38]. Activation of CD4+ T cells induces expression of HLA class-II molecules on MVECs through IFNγ production, eliciting a sustained alloimmune reaction[39] similar to delayed-type hypersensitivity reactions. MVECs also contribute to GVD and smooth muscle cell recruitment both directly and indirectly. Expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and the E and P-selectins on MVECs has been directly correlated to rejection and GVD[40, 41] as expression of these molecules allows for adhesion and rolling of host leukocytes on the EC barrier of the graft. Lastly, as previously mentioned, EC dysfunction can exacerbate GVD through growth factor and danger molecule release. As the first biological barrier between graft and host and a key mediator of lymphocyte activation, ECs play an important role in alloimmune responses following cardiac transplantation.

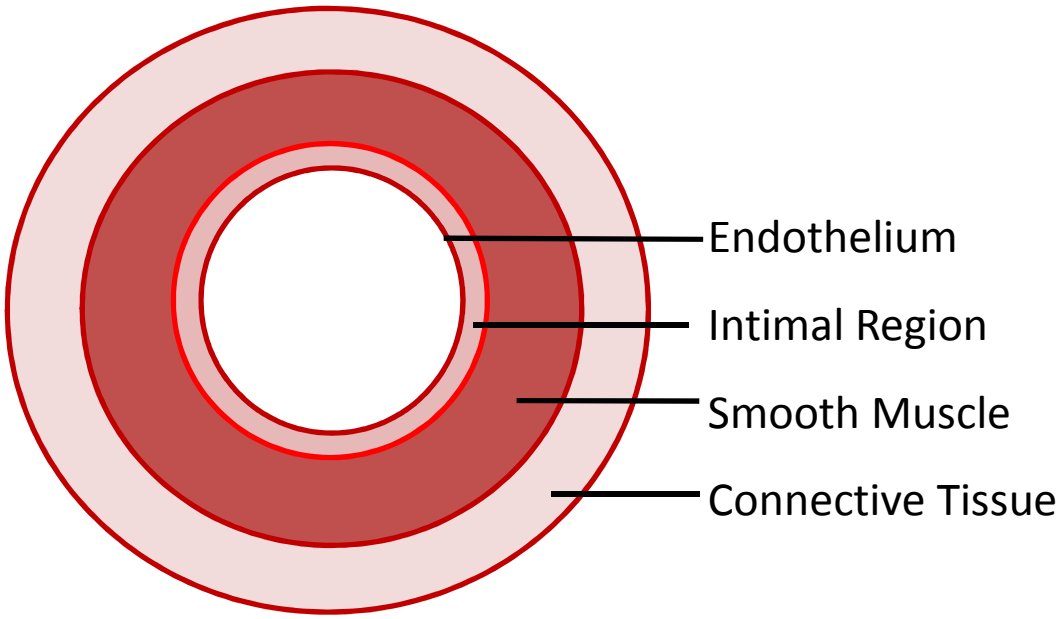


Figure 1 – Anatomy of an artery

An artery consists of several anatomical structures including the endothelium, intimal region, smooth muscle layer and is surrounded by connective tissue. The endothelial layer interacts with blood cells in the serum and plays important roles in facilitating white cell emigration from the blood into the tissue. In transplantation, the endothelial lining serves as the barrier between the graft and the host, making it an important player in immune responses against the graft.

1.4 TNFα and TNFα receptors

TNFα, also known as cachectin, was originally identified as a potent inducer of cell death in tumour cells[42] but is recognized today as a pleotropic cytokine involved in many signal transduction pathways including cell survival, proliferation, activation and cell death. TNFα has been shown to be an important contributor to heart graft rejection and GVD through the regulation of soluble TNFα[43] and TNFα receptors[44]. Furthermore, TNFα has implicated in the induction of early neutrophil and macrophage chemoattractant levels, increased immune cell infiltrate[45] and decreased graft survivability[46, 47] following cardiac transplantation.

As suggested above, TNFα can induce a variety of cellular responses. Traditionally, TNFα was thought to induce programmed apoptosis in target cells following TNFα Receptor (TNFR) 1 binding, or immune activation through transcription factor NF-κB following TNFR2 signalling[7]. Interestingly, human and murine MVECs in heart grafts constitutively express diffuse levels of death receptor TNFR1 and activating receptor TNFR2 before and after transplantation[48]. Conversely, cardiomyoctytes, which normally express high levels of TNFR1, drastically reverse TNFα receptor expression from TNFR1 to TNFR2 following rejection injury[44, 48]. This suggests that human and murine MVECs in donor grafts may be more vulnerable to TNFα mediated death than cardiomyocytes. Recently, TNFα has been shown to also initiate a second cell death pathway known as necroptosis, also initiated through TNFR1[49, 50].

1.5 Apoptosis, necrosis and necroptosis

Cell death has been classified into a variety of forms including apoptosis, necrosis, post-apoptotic secondary necrosis, necroptosis, autophagy and mitotic catastrophe[51]. The two extremes in the array of cell death are caspase-dependent apoptosis and caspase-independent necrosis. Apoptosis is characterized by DNA fragmentation and membrane blebbing induced by endogenous nucleases, whereas necrosis is characterized by swelling of the mitochondria and cytoplasm, due to ATP depletion, leading to eventual plasma membrane rupture and lysis of the cell.

Traditionally, apoptosis was viewed as the primary active and programmed form of cell death while necrosis was viewed as a passive and immunogenic form of cell death. However, this paradigm has been challenged in recent years with the discovery of necroptotic cell death, which takes on characteristics of both apoptosis and necrosis. Like apoptosis, necroptosis requires endogenous cellular machinery to initiate cell death. This process is initiated through either TNFR1[49], tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)[52, 53], Fas[54], Toll-like receptor (TLR) 3[55] or 4[56], or retinoic acid-inducible protein[57] signalling. The result is the activation of receptor interacting protein kinase (RIPK) 1 and RIPK3, together known as the necrosome, which induce necrotic-like cell death through endogenous reactive oxygen species (ROS) production.

TNFα mediated death signalling requires the formation of a complex of proteins on the inner membrane domain of TNFR1 known as Complex I (Figure 1). This complex

includes TNFα receptor associated death domain (TRADD), TNFα receptor associated factor (TRAF), cellular inhibitors of apoptosis (cIAP) 1 and 2, and poly-ubiquitinated RIPK1. Binding of NF-kappa B essential modulator (NEMO) to the ubiquitin tail of RIPK1 will result in the downstream activation of NF-κB, mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinases (JNK), resulting in survival of the cell. Alternatively, deubituitination of RIPK1 by enzymes A20 or cylindromatosis (CYLD) will result in formation of complex II, which includes RIPK1, RIPK3, TRADD and the Fas Associated Death Domain (FADD)[58-60]. Caspase-8 recruitment into Complex II will result in the cleavage of RIPK1 and RIPK3 followed by caspase-8 mediated apoptosis. In the absence or inhibition of caspase-8, RIPK3 will autophosphorylate itself and phosphorylate RIPK1, initiating a downstream cascade resulting in up-regulation and activation of three metabolic enzymes: Glutamine Synthase (GLUL), Glutamate Dehydrogenase (GLUD1) and Glycogen Phosphorylase (PYGL)[50, 60]. PYGL is responsible for the catabolism of glycogen to glucose-1-phosphate, directly fuelling glycolysis, while GLUL catabolises glutamate to glutamine, stimulating gluconeogenesis, glutathione production and lipogenesis. GLUD1 is subsequently involved in glutamine catabolism, increasing the cellular pool of α-ketoglutarate to feed the TCA cycle. RIPK mediated increases in cellular energy metabolism through activity of these enzymes results in excessive reactive oxygen species (ROS) production within the mitochondria, eventually resulting in cytotoxicity of the cell[61]. Indeed, mitochondrial derived ROS have been implicated in the ultrastructural changes seen in cellular organelles during programmed necrosis[62]. This provides strong evidence that the current model of TNFα induced necroptosis is mediated through increased ROS production. Consequently, like

traditional necrosis, necroptosis is considered to be immunogenic, potentially making it an important player during transplantation.

1.6 Inhibiting TNFα mediated cell death

The discovery of TNFα mediated necroptosis lead to the discovery of chemical compounds that allosterically inhibited the kinase activity of RIPK1, known as necrostatins[63, 64]. These compounds do not affect the activation of NF-κB, MAPK or JNK signalling following TNFR1 activation, confirming the bifurcation between cell survival and cell death following TNFR1 activation. Necrostatins have been shown in experimental models to be beneficial inhibitors of cell death following ischemic brain injury[63], myocardial infarction[65] and chemotherapy-induced cell death[66]. Conversely, because the induction of TNFα mediated necroptosis is dependent on caspase-8 inactivity, compounds that inhibit caspase-8, such as the pan-caspase inhibitor zVAD-fmk, can be used to induce necroptosis following TNFα treatment[60]. zVAD-fmk works by binding the active site of caspase-8[67], preventing caspase-8-ligand interactions.

Although synthetic caspase inhibitors such as zVAD-fmk do not exist in biological systems, caspase inhibition can occur naturally in a variety of instances. In fact, simple changes in the redox status of the active cysteine residue in the caspase active site can render caspase activation impossible[68]. A study by Cho and colleagues has also revealed that TNFα treated cells infected with vaccinia virus, which encodes the viral caspase inhibitor Spi2, also undergo necroptosis and knockout of RIPK3 in this model results in

higher vaccinia virus titers compared with wildtype mice[69]. This data implies that RIPK signalling likely has a physiological role as a backup mechanism in viral protection where the viral target is caspase-8. This protective effect is then amplified by the release of cellular danger associated molecular patterns (CDAMPS) following necroptosis, which attract and activate the innate and adaptive immune system[70]. Interestingly, it seems that some viruses, such as cytomegalovirus (CMV), have evolved to escape this mechanism. CMV does this through expression of viral proteins M36, an inhibitor of caspase-8 activation[71], and M45, an adaptor protein that interferes with RIPK1 and RIPK3 interaction[72] and NF-κB activation[73], rendering TNFR1 signaling inert. Physiologically, necroptosis appears to be another player in the biological arms race between infecting organisms and the immune system.

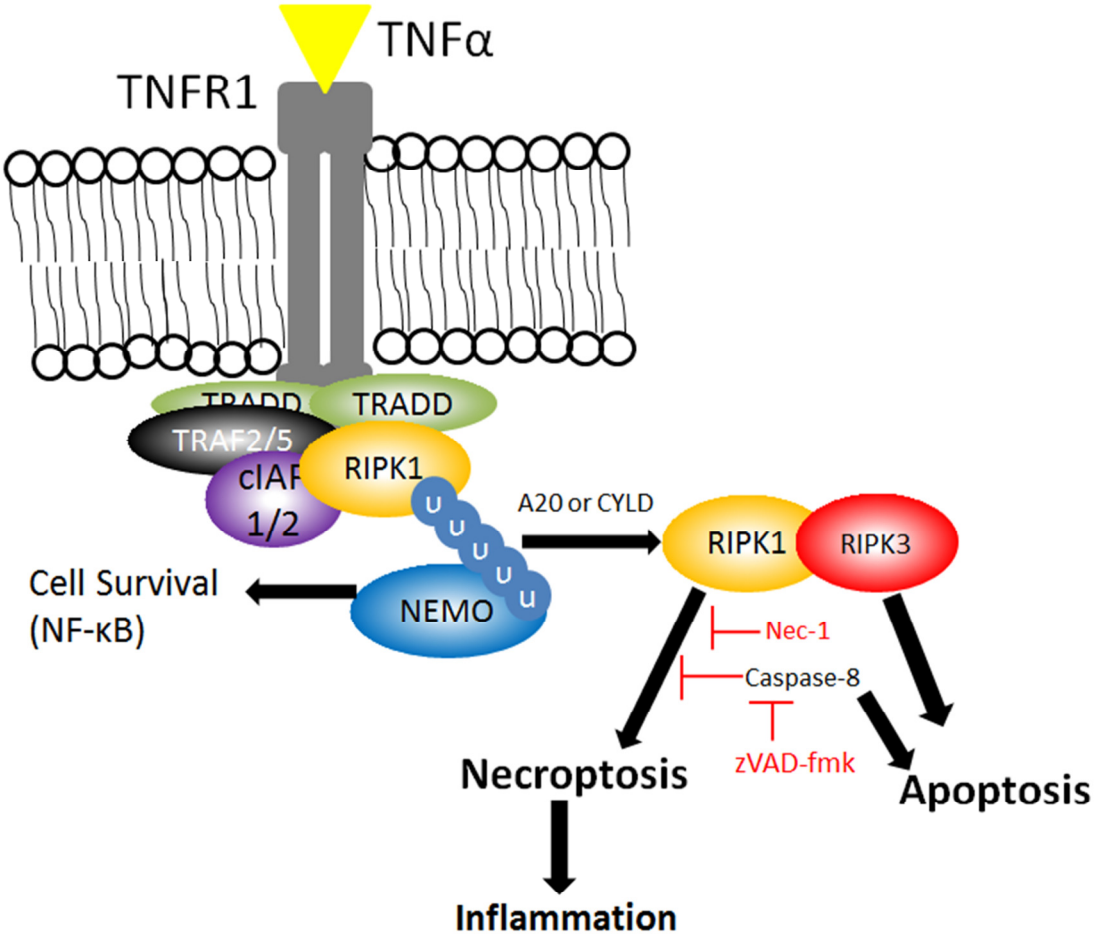

TNFα
TNFR1
TRADD
TRADD
TRAF2/5
cIAP
1/2
RIPK1
A20 or CYLD
RIPK1
RIPK3
Cell Survival
(NF-κB)
NEMO
Nec-1
Caspase-8
zVAD-fmk
Necroptosis
Apoptosis
Inflammation

Figure 2 – The mechanism of TNFα mediated necroptosis

TNFα initiates necroptosis through binding of TNFR1. Following this, an intracellular complex forms on the innermembrane domain of the TNFR1 receptor, including polyubiquitilylated RIPK1. Binding of NEMO to the ubiquitin tail of RIPK1 results in a downstream pathway activating NFκB. Alternatively, enzymes A20 or CYLD deubiuinate RIPK1, which associates with RIPK3 to form the necrosome. Caspase 8, if present, will cleave the necrosome and initiate caspase-dependent apoptosis. If caspase 8 is absent or inhibited, the necrosome will autophosphorylate and activate downstream enzymes, initiating necroptosis.

1.7 Inflammatory implications of cell death

Apoptotic death prevents the release of cell contents and can minimize inflammation, whereas necrosis results in the release of pro-inflammatory CDAMPs, including high mobility group box 1 (HMGB1), heat shock protein (HSP), RNA, DNA, fibronectin, and uric acid[74, 75]. These released danger molecules promote inflammatory responses through interactions with TLRs and other innate receptors[74-76]. In transplantation, TLR engagement has been found to accelerate vascular allograft rejection[77-79], making proinflammatory CDAMPs such as HMGB1, which are only released following cell lysis, a powerful marker for necroptosis[75]. Indeed, necroptosis has been implicated in a variety of diseases resulting in CDAMP release including cerulean-induced pancreatitis[60, 80], cerebral cortex infarction[63], chronic intestinal inflammation[81], systematic inflammatory response syndrome[82], myocardial infarction[65, 83] and IRI in the kidney[84, 85] and heart[86, 87]. Distinguishing these mechanisms of cell death could be crucial in transplantation as necroptosis is phenotypically similar to necrosis and may play a central role in allograft rejection by enhancing the release of proinflammatory danger molecules and promoting inflammatory injury in the graft.

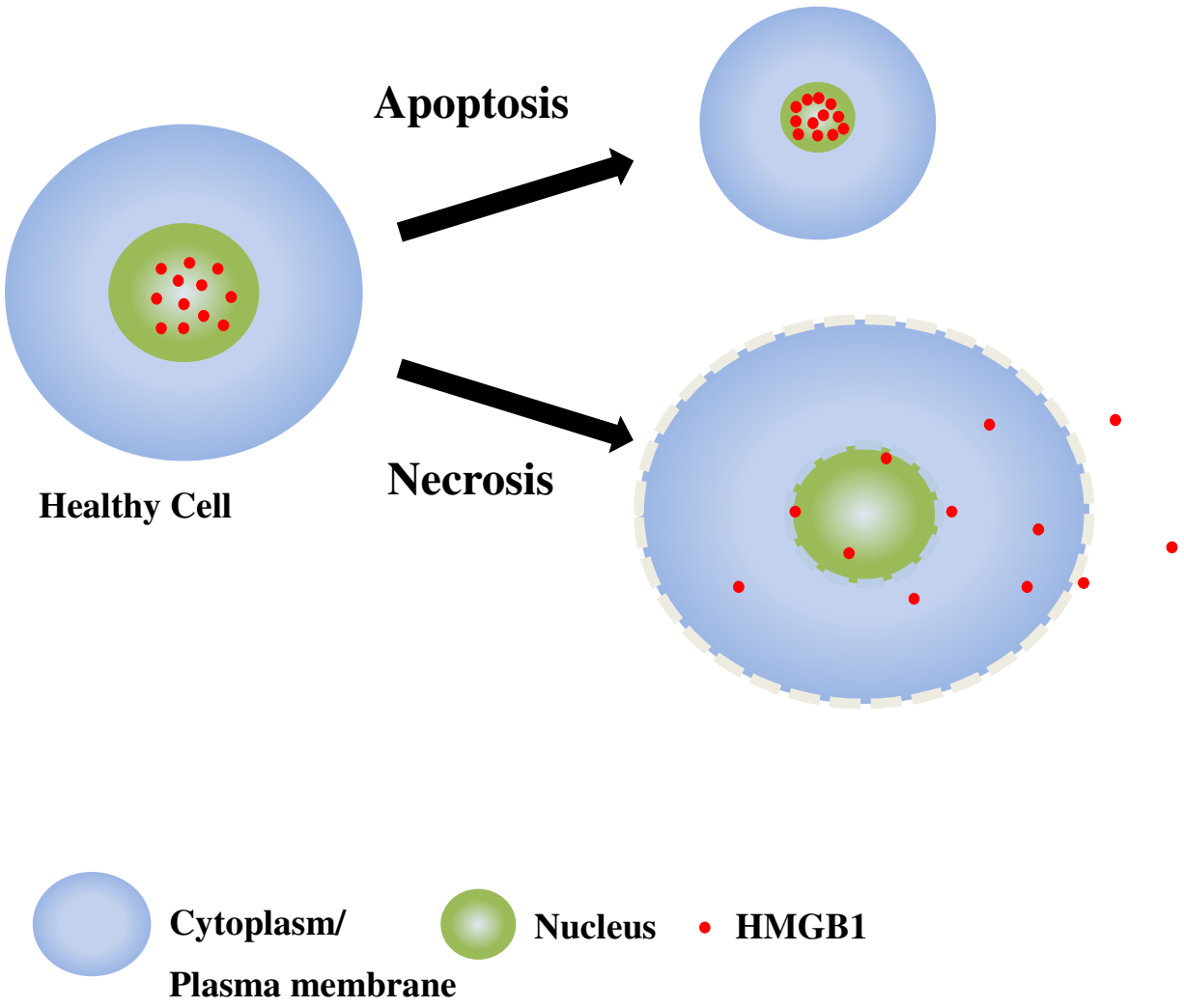


Figure 3 – HMGB1 release following necrotic cell death

HMGB1, a nuclear protein involved in chromatin architecture, is a potent immunogenic CDAMP. During apoptosis, HMGB1 is oxidized and sequestered to the nucleus where it is rendered inactive. However, during necrosis or necroptosis, active HMGB1 is released from the cell where it will interact with TLRs and RAGE receptors on immune cells, initiating an immune response.

1.8 Hypothesis

We hypothesize that the pattern of cell death in the graft following cardiac transplantation affects graft survivability as necrotic or necroptotic death will lead to exacerbated inflammation and accelerated graft rejection.

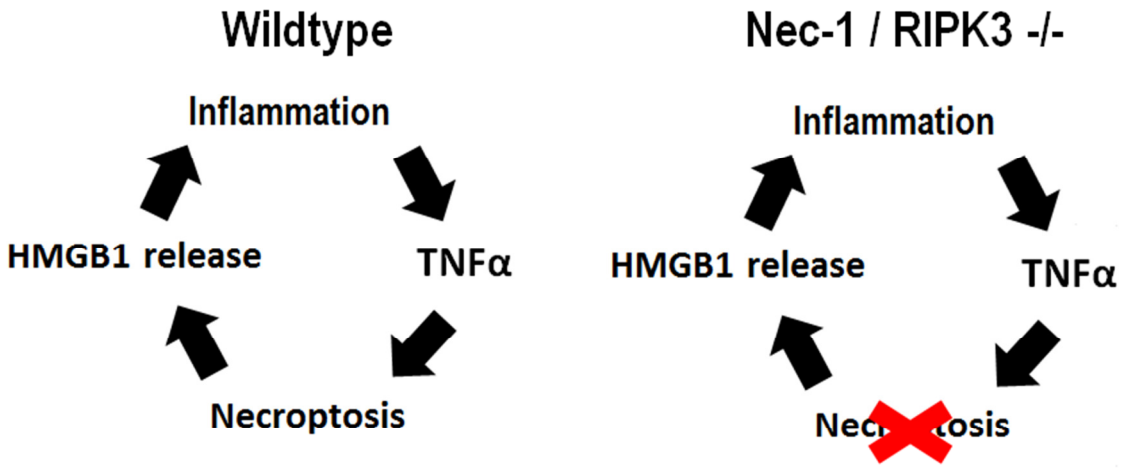


Figure 4 – Necrostatin-1 or RIPK3 knockout will prevent exacerbation of inflammation

Following cardiac transplantation, inflammation will lead to graft immune cell infiltration and the release of TNFα. In wild type mice, heart cells will be susceptible to RIPK1/RIPK3 mediated necroptosis, which leads to the release of the immunogenic CDAMP HMGB1. This in turn leads to the activation of more host immune cells and further inflammation. Treatment with Nec-1 or knockout of RIPK3 prevents cells from undergoing RIPK1/RIPK3 mediated necroptosis, breaking the inflammatory cycle.

1.9 Specific aims

1. To determine the effects of apoptosis and necroptosis on MVECs.

2. To show that the inhibition of RIPK1 and RIPK3 function in MVECs results in decreased necroptotic death and subsequent danger molecule HMGB1 release.

3. To determine if RIPK3 deficiency in donor heart grafts inhibits necroptosis and subsequently prolongs graft survival.

Chapter 2

2 Materials and Methods

2.1 Animals

C57BL/6 (H-2b), BALB/c (H-2d) (Jackson Laboratories, Bar Harbor, ME), and B6 RIPK3$^{-/-}$ mice (H-2b) (Genentech)[88] were maintained in the animal facility at the Western University. All experimental procedures were approved by the Western University Animal Care Committee.

2.2 Microvascular endothelial cell (MVEC) culture

MVECs from mice hearts were isolated as previously described[89] using anti-CD31 coated Dynal magnet beads (Invitrogen). MVECs were immortalized through transfection of origin-defective SV40 DNA[90]. Transfection was done using the calcium-phosphate co-precipitation method (Invitrogen). Cells were grown in complete EGM-2 MV containing 5% FBS, 0.04% hydrocortisone, 0.4% hFGF-b, 0.1% VEGF, 0.1% R3-IFG-1, 0.1% Ascorbic acid, 0.1% hEGF and 0.1% GA-1000 (Lonza). MVEC phenotype was confirmed by staining with anti-CD31, CD102 and CD105 (eBioscience). Cells were passaged a maximum of 10 times before being discarded.

2.3 Heart transplantation

BALB/c mice (H-2^{d}) were transplanted with heterotopic hearts from either C57BL/6 or B6 RIPK3$^{-/-}$ (H-2^{b}) mice. Ischemic time was limited to 15 minutes during all transplantations. The donor heart was anastomosed via the donor aorta to the recipient abdominal aorta and donor pulmonary artery to recipient inferior vena cava using microsurgical techniques. The donor vena cava and pulmonary vein were sutured shut.

Following reperfusion, the heart grafts resumed spontaneous contraction. Recipients were treated daily with 1mg/kg sirolimus (LC Laboratories, Woburn, MA) from day 0 until postoperative day 9[91] or CD154 antibody (MR1) (200ug) one preoperative day before transplantation. The strength and quality of cardiac muscle contractions were monitored by palpation of the abdomen. Graft rejection (cessation of pulsation) was monitored daily after transplantation and confirmed by pathological analysis.

2.4 In situ ethidium homodimer perfusion

Recipient animals were anesthetized with the mixture ketamine/xylazine according to the approved animal protocol. The abdominal cavity was opened and the heterotopic heart was isolated from surrounding tissue. A blood sample was taken from the inferior vena cava above the left renal vein at this time. The inferior vena cava and dorsal aorta were then fully occluded just above and below the heterotopic heart using a microsurgical vascular clamp. The pulmonary artery of the donor heart was then cut to allow the perfusion solution to drain. A solution of 5 μM ethidium homodimer (Invitrogen) in saline was perfused into the aorta at 1 ml/min for 5 minutes[92]. Successful perfusion was observed by flushing of blood from the descending coronary arteries, expansion of the heterotopic heart and exit of perfusion solution from the pulmonary artery incision. Following this, saline was perfused at 1ml/min for 10 mins to flush excess and unbound ethidium homodimer. The heart was then removed and cut through the transverse plane before being put in formalin or liquid nitrogen.

Tissues were analyzed and quantified for area and fluorescent intensity by a fluorescent microscope (NIS Elements 3.0, Nikon). Areas positive for necrosis were measured by ethidium homodimer labelled red fluorescence.

2.5 Western blot

Supernatants were collected from equal number cell seeded wells for western blot analysis. Protein was isolated from heart tissue using cytoplasmic lysis buffer (10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 0.1%NP40, 1mM DTT, 0.5mM PMSF) for non-nucleic protein analysis. Protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad) at 595nm on a spectrophotometer (Beckman Coulter). Sample buffer (2ME, glycerol, bromophenol blue, Tris-HCl) was added to the protein and was separated by gel electrophoresis by 4% resolving gel and 10% running gel. Protein was transferred to a nitrocellulose membrane using the iBlot dry transfer system (Invitrogen). Blots were blocked with 5% milk (Carnation) in Tris buffered saline and Tween 20 (TBS-T) and were incubated with polyclonal rabbit anti-RIPK3 (Abcam), rabbit anti-HMGB1 (Abcam), or mouse anti-β-actin (Sigma) in 2.5% milk TBS-T. Protein was visualized using secondary anti-rabbit IgG (Sigma) antibody with conjugated horseradish peroxidase and chemiluminescent HRP substrate (Millipore). Protein concentration was semi-quantitated by densitometry (Alpha View) and normalized using β-actin.

2.6 RNA isolation and real-time PCR

Total RNA was extracted from tissue or cells by Trizol extraction (Invitrogen). cDNA was generated from RNA using Superscript II (Invitrogen). Primers used for real time PCR include: RIPK3: 5'-GGGACCTCAAGCCCTCTAAC-3' and 5'-GATCCCTGATCCTGACCCTGA-3'. β-Actin: 5'-CCA GCC TTC CTT CCT GGG TA and 3'-CTA GAA GCA TTT GCG GTG CA. Real-time quantitative PCR was performed on standardized quantities of cDNA using the SYBR qPCR Master Mix kit (BioRad).

Gene amplification was performed and detected using the Mx3005 system (Stratagene). β-actin amplification was used as the endogenous control. The normalized delta threshold cycle value and relative expression levels ($2^{-\Delta\Delta Ct}$) were calculated according to the manufacturer's protocol.

2.7 Cell death assay

MVECs were grown to monolayer and treated with 100μg/ml of recombinant human TNFα (Peprotech) and 60μg/ml recombinant mouse IFNγ (Peprotech), 60 μM zVAD-fmk (BD Bioscience) and/or 10 μM necrostatin-1(Calbiochem) in serum free media for 48h. MVECs were trypsinized (Gibco) and washed with PBS. Cell death was detected with propidium iodide (P.I.) or annexin V labeling (BD Bioscience) and analyzed by flow cytometry (Beckman Coulter).

2.8 Histology and immunohistochemistry

Tissue sections were stored in 5% formalin (Sigma) and paraffin fixed and stained with hematoxylin and eosin (H&E). Slides were scored 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)[91, 93]. Criteria for allograft injury included endothelial cell damage, infarction, lymphocyte infiltrate, fibrosis, thrombosis, hemorrhage, myocyte damage and neutrophil infiltrate.

RIPK3 and HMGB1 expression in the heart graft was detected by anti-RIPK3 (Abcam) or anti-HMGB1 (Abcam) by immunohistochemistry according to a standard protocol. Apoptosis in tissue was determined by TUNEL staining (Calbiochem).

2.9 Statistical analysis

Data was compared using Student's t-test for unpaired values. Graft survival was analyzed by log-rank testing (Mantel-Cox). Data was presented as mean ± standard deviation (SD) and p values < 0.05 were considered to be significantly different.

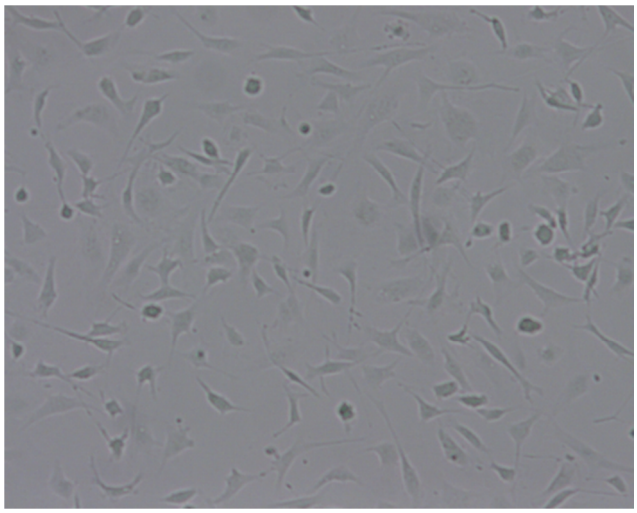
Chapter 3

3 Results

3.1 Isolation and characterization of MVECs

MVECs were developed from C57BL/6 hearts as described in the methods. The phenotype of MVECs was confirmed by morphology (Figure 5A) and flow cytometry through CD31[94], CD102[95], and CD105[96] positivity (Figure 5B).

A



B

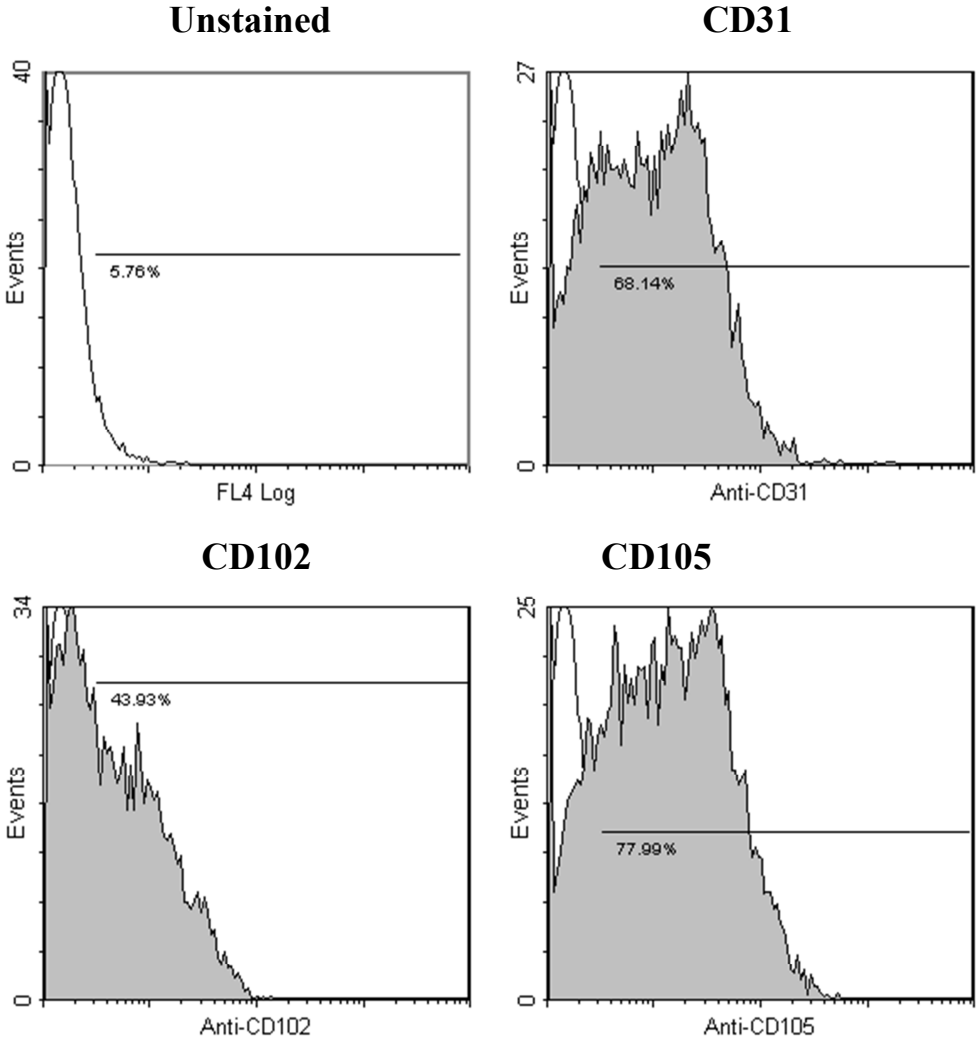

Unstained
CD31
CD102
CD105
Events
FL4 Log
Anti-CD31
Anti-CD102
Anti-CD105
5.76%
68.14%
43.93%
77.99%
0
40
27
34
25

Figure 5 – Isolation and characterization of MVECs

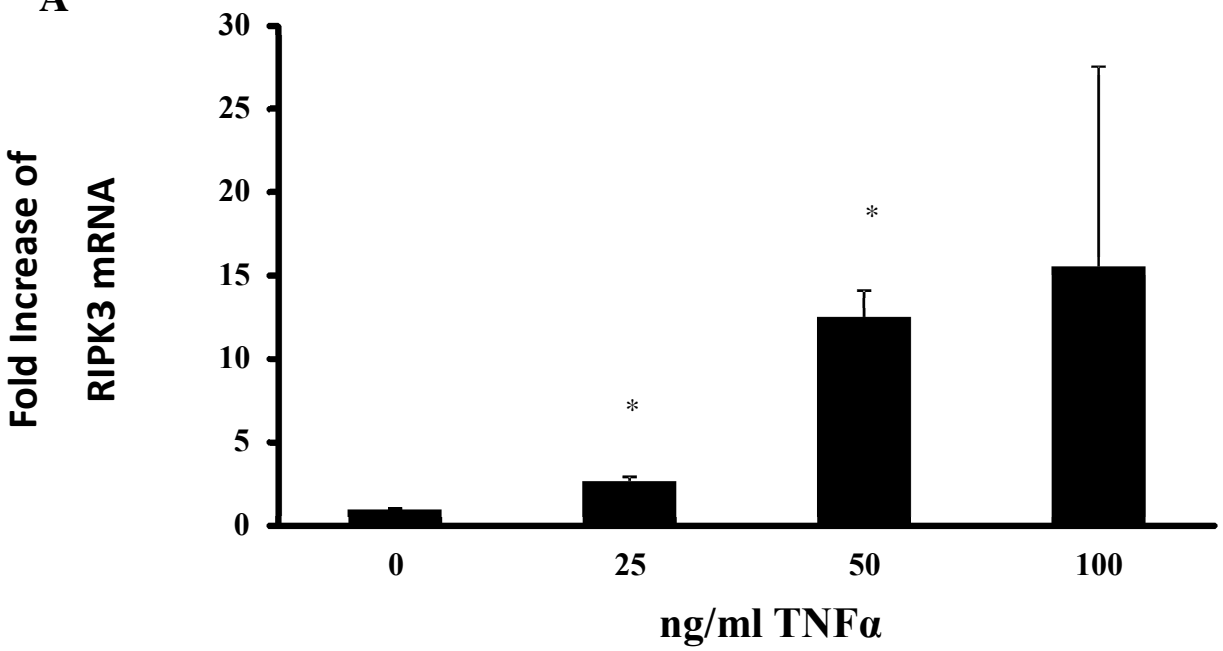
A) MVECs were isolated using CD31 coated beads and immortalized with origin deficient SV40 DNA. Cells were grown in a monolayer analysed using confocal microscopy at 400X magnification. B) Cells were also analysed for the presence of the endothelial markers CD31, CD102 and CD105 by flow cytometry analysis.

3.2 RIPK3 and TNFR1 expression in MBECs are regulated by proinflammatory cytokines

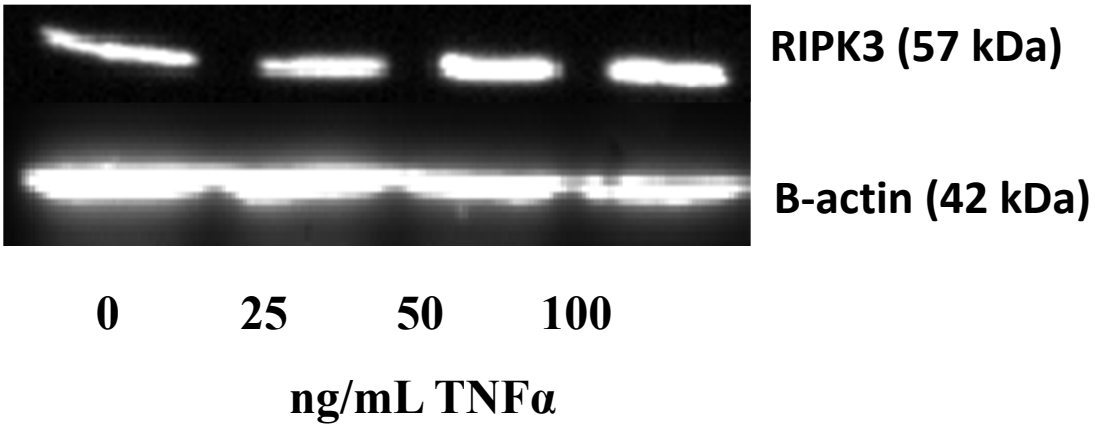
We first determined whether TNFR1 and RIPK3 expression can be regulated by proinflammatory cytokines in MVECs as TNFα mediated apoptotic or necroptotic cell death requires expression of death receptor TNFR1 and RIPK3. Expression of RIPK3 in MVECs was analyzed by qPCR after treatment with TNFα or IFNγ. Our data indicates that treatment with TNFα up-regulates RIPK3 mRNA expression in a dose dependant manner (Figure 6A). Significant differences were seen at treatments of 25ng/ml (mean$\pm$SD: 2.7 $\pm$ 0.2 fold change, n=3, $p<0.01$) and 50 ng/ml of TNFα (12.5 $\pm$ 1.6 fold change, n=3, $p<0.05$) compared to untreated MVECs. Expression of RIPK3 in MVECs was confirmed by western blot analysis (Figure 6B).

Surface expression of TNFR1 on MVECs was determined by FACS analysis. As shown in Figure 6C, expression levels of TNFR1 on MVECs can be modulated by TNFα or IFNγ with maximum upregulation at 60ng/ml TNFα or IFNγ. Hence, proinflammatory cytokines can upregulate both TNFR1 and RIPK3 expression in MVECs.

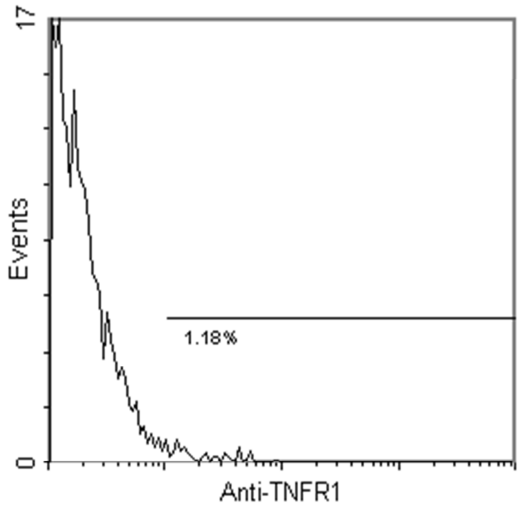
A


Fold Increase of RIPK3 mRNA
30
25
20
15
10
5
0
*
*
0
25
50
100
ng/ml TNFα

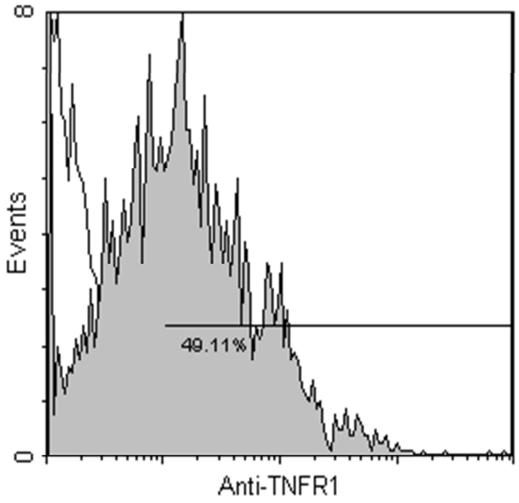
B


RIPK3 (57 kDa)
B-actin (42 kDa)
0
25
50
100
ng/mL TNFα

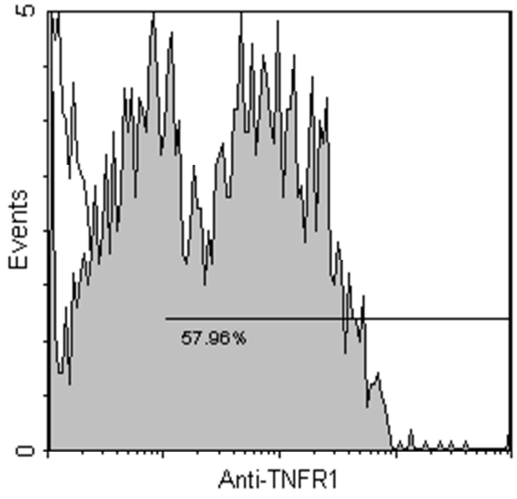
C



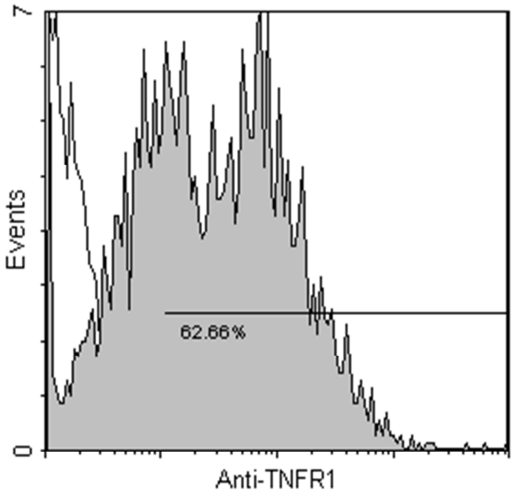
Unstained



Untreated



60ng/ml TNFα



60 ng/ml IFNγ

Figure 6 - Expression of RIPK3 and TNFR1 in microvascular endothelial cells are upregulated by proinflammatory cytokines

MVECs were isolated as described in the methods. A) MVECs were treated with 0, 25, 50 or 100 ng/ml TNFα for 48 hours in serum free EBM media. RIPK3 expressions were measured by real time qPCR as described in the methods. B-actin was used as control (n=3, *: $p < 0.05$). B) RIPK3 protein was detected by western blot by anti-RIPK3 (Abcam) after 48 hour treatment with TNFα. C) Cells were treated with either 60 ng/ml TNFα or 60ng/ml IFNγ. Surface expression of TNFR1 on MVECs was measured using anti-TNFR1-PE (eBioscience) and analyzed by flow cytometry.

3.3 TNFα induces necroptosis in L929 cells under anti-apoptotic conditions

As previously described, L929 mouse fibroblasts are extremely susceptible to necroptotic death[49]. Before attempting to induce necroptosis in MVECs, L929 cells were treated with TNFα, zVAD-fmk and necrostatin and cell death was determined by PI positivity. TNFα can signal through either TNFR1 or TNFR2, initiating either cell death or cell survival response, respectively[97, 98]. We therefore used human TNFα (hTNFα) to maximize TNFR1-mediated cell death as hTNFα specifically initiates TNFR1, rather than TNFR2, signalling on murine cells[99]. As seen in Figure 7, hTNFα (80ng/ml) + IFNγ (30ng/ml) treatment increased PI positivity, not Annexin V positivity, compared with untreated cells. The addition of (4uM) Nec-1 was able recover this PI positivity. Conversely, the addition of (10uM) zVAD-fmk increased cell death substantially, which was again recovered partially by Nec-1.

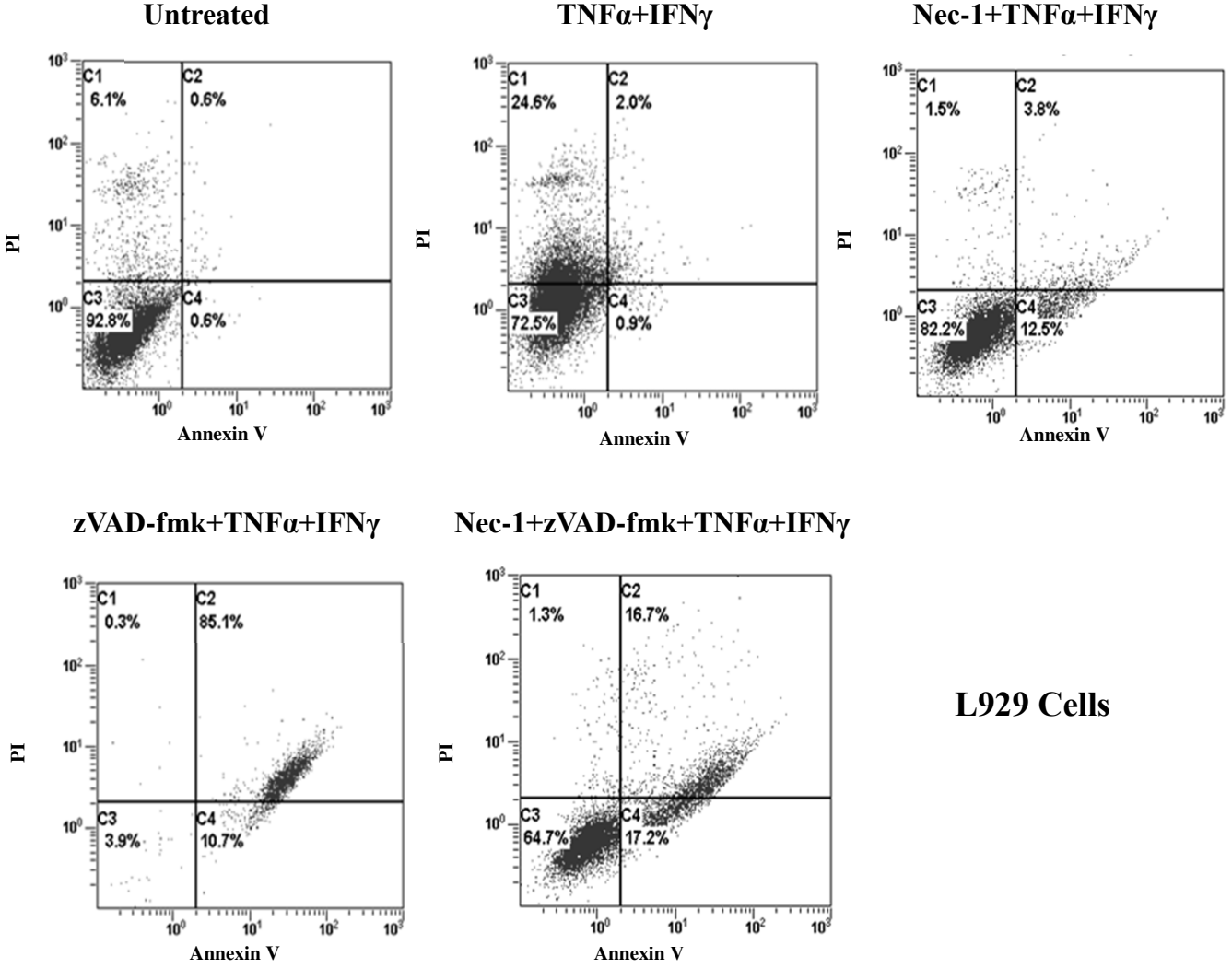


Figure 7 – L929 cells undergo necroptotic cell death after TNFα treatment

L929 cells were treated with 80ng/ml hTNFα and 30 ng/ml IFNγ in the presence or absence of 4uM Nec-1 and 10uM zVAD-fmk for 24 hours and cell death was determined using Annexin V and PI staining on FLOW cytometry analysis.

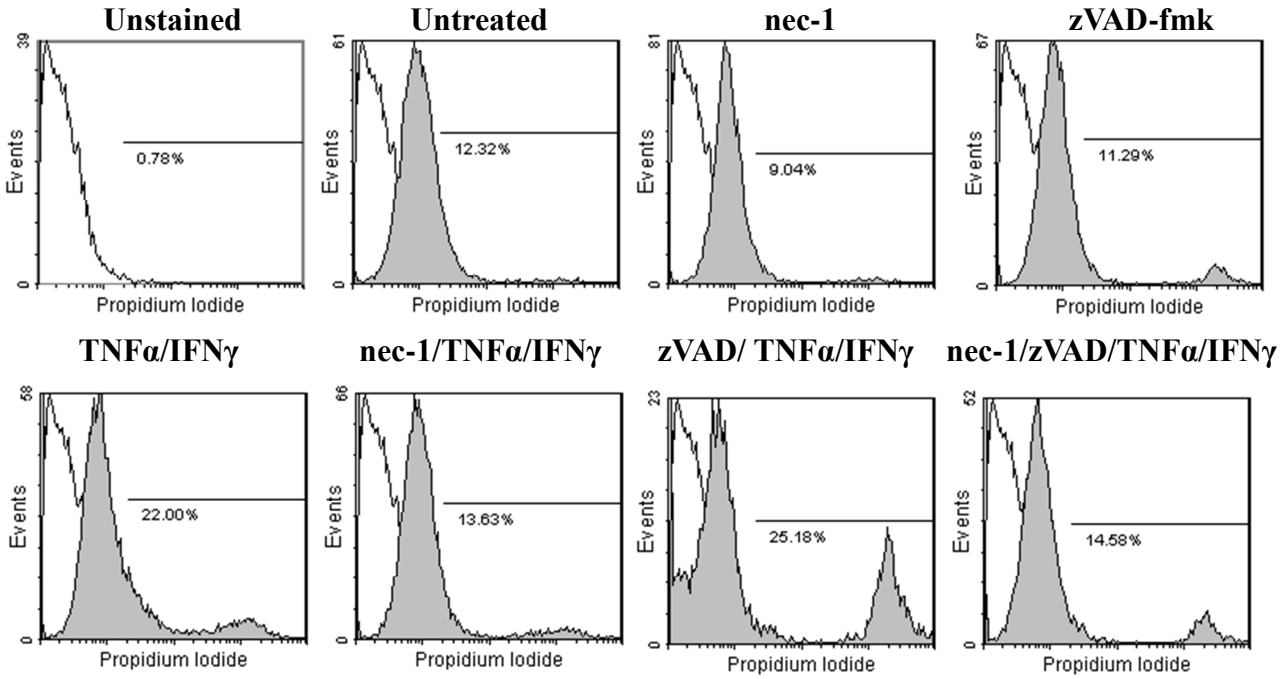
3.4 MVECs with RIPK1 inhibition are resistant to necroptotic death

We next measured TNFα-induced necroptotic death in MVECs following treatment with TNFα. TNFα can signal through either TNFR1 or TNFR2, initiating either a cell death or cell survival response, respectively[97, 98]. Again, hTNFα was used to induce cell death. As shown in Figure 8A&B, we observed that MVECs treated with hTNFα (100ng/mL) + IFNγ (60ng/mL) increased cell death compared with untreated cells (PI positivity: 19.3 $\pm$ 2.1% in TNFα treated vs 9.4 $\pm$ 1.6% in untreated cells, n=3, p=0.01). In order to modulate necroptotic death, the RIPK1 inhibitor necrostatin-1 (Nec-1) (10uM), and pan-caspase inhibitor zVAD-fmk (60uM) were used. Addition of Nec-1 to hTNFα treatment reduced cell death to 12.5 $\pm$ 5.1% PI positive (Figure 8A&B, n=3, p=0.09). However, zVAD-fmk+hTNFα treatment increased PI positivity to 25.9 $\pm$ 2.7% (n=3, p=0.001), suggesting that increased PI positive cell death occurs only after the inhibition of caspases. Furthermore, zVAD-fmk induced cell death could be reversed by addition of the RIPK1 inhibitor Nec-1 (15.6 $\pm$ 2.0%, n=3, p=0.02, Figure 8A&B), providing further evidence that a RIPK-mediated cell-death response occurs when caspase-mediated apoptotic pathways are blocked. These results suggest that MVECs are able to undergo TNFα induced cell death, which can be modulated by the inhibition of RIPK1.

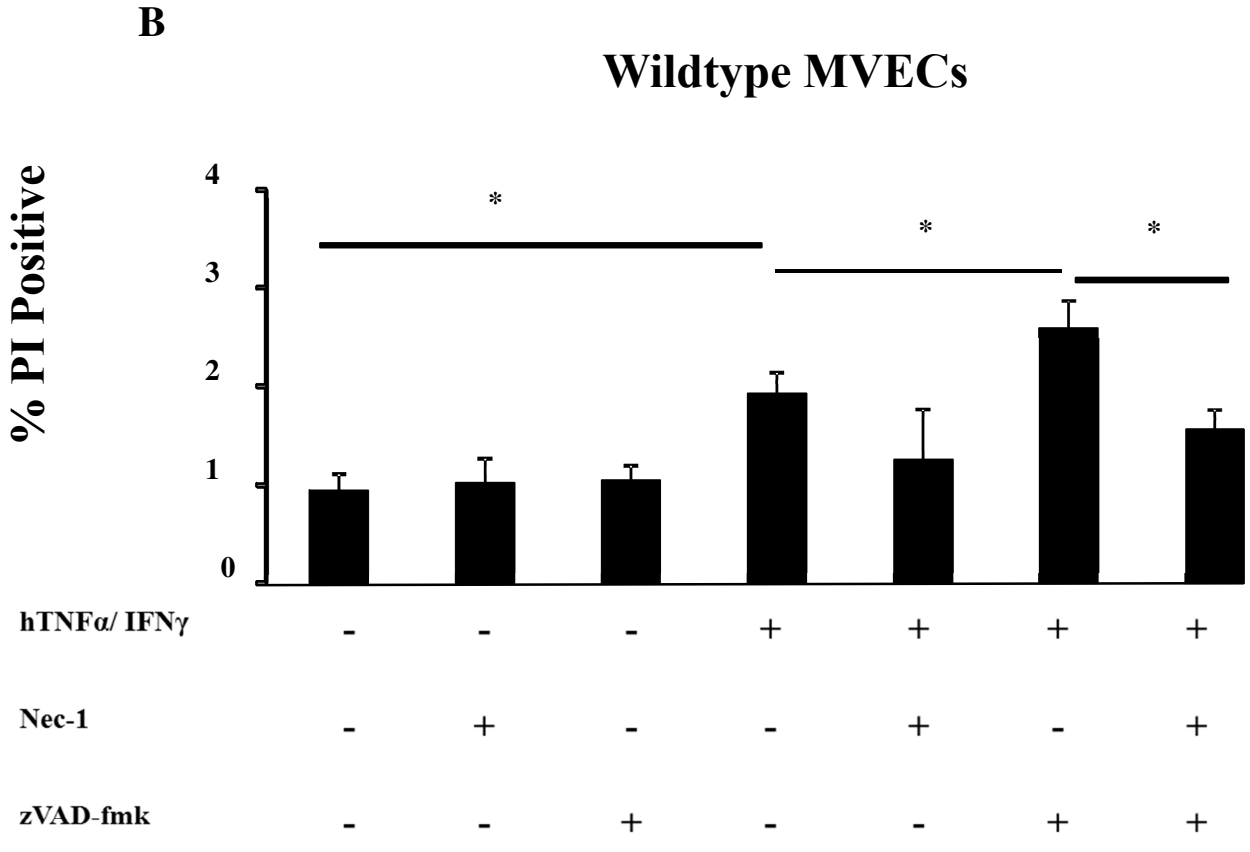
To further illustrate that the RIPK proteins regulate necroptosis and HMGB1 release, RIPK3 deficient MVECs were isolated from RIPK3$^{-/-}$ mice and treated with hTNFα and zVAD-fmk. PI positivity did not increase significantly following hTNFα treatment alone (6.5 $\pm$ 3.0%, vs 7.3 $\pm$ 2.3% in untreated cells, n=3, p=0.39) or after hTNFα treatment with

zVAD-fmk (7.5 $\pm$ 1.1%, n=3, p=0.27, Figure 8C), suggesting that TNFR1 signaling cannot induce necroptosis following the deletion of RIPK3.

A


Unstained
Untreated
nec-1
zVAD-fmk
TNFα/IFNγ
nec-1/TNFα/IFNγ
zVAD/ TNFα/IFNγ
nec-1/zVAD/TNFα/IFNγ
Events
Propidium Iodide
0.78%
12.32%
9.04%
11.29%
22.00%
13.63%
25.18%
14.58%

B


Wildtype MVECs
% PI Positive
0
1
2
3
4
*
*
*
hTNFα/ IFNγ
Nec-1
zVAD-fmk

C

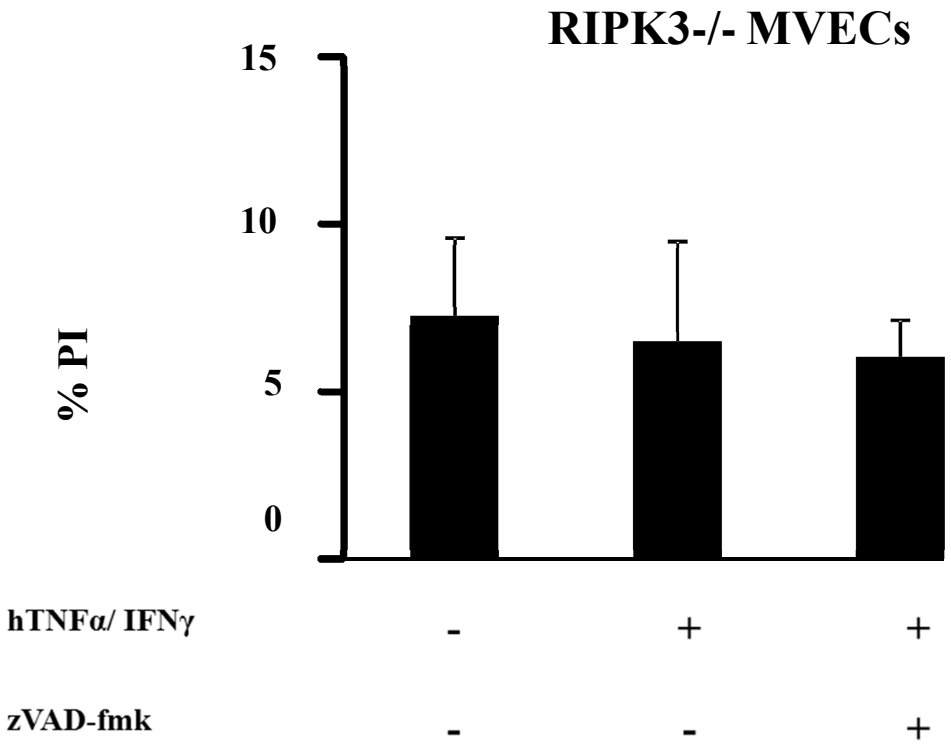


Figure 8 - RIPK1 and RIPK3 regulates necroptosis in MVECs

MVECs from C57BL/6 wild type mice or RIPK3$^{-/-}$ mice were plated in equal numbers (10^5) and treated with 100ng/ml TNFα, 60uM zVAD-fmk or 10uM Nec-1 in serum free media. A) C57BL/6 MVECs death was measured by PI positivity after 48 hours using flow cytometry analysis. Histograms are representative of three experiments. B) Quantitated average of three independent cell death experiments from C57BL/6 MVECs (n=3, *: $p < 0.05$, **: $p < 0.01$). C). Quantitated average of three independent cell death experiments from RIPK3$^{-/-}$ MVECs (n=3, $p > 0.05$).

3.5 SMAC mimetics exacerbate TNFα mediated necroptosis

Upon signaling TNFR1, TNFα can initiate either a cell survival or cell death response[61]. In an attempt to abrogate the pro-survival pathway following TNFR1 signaling, cIAP inhibiting SMAC mimetics were used to prevent up-regulation of NF-κB genes through the prevention of protein translation[100]. As seen in Figure 9, treatment with SMAC mimetic (100nM) + hTNFα (100ng/mL) increased the PI quadrants after just 6 hours. Addition of Nec-1 attenuated PI positive cell death, however, zVAD-fmk (10uM) was not able to exacerbate PI positivity, likely due to the low concentration added.

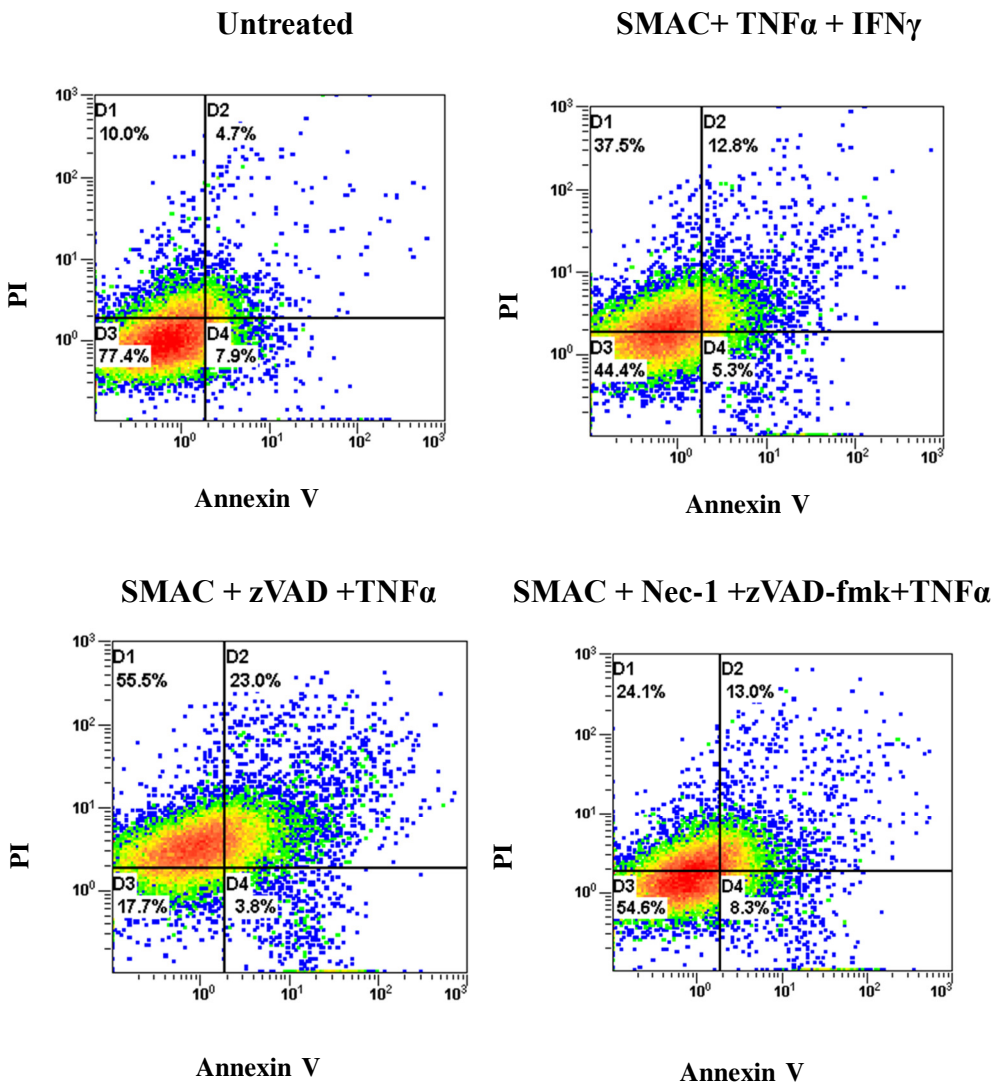


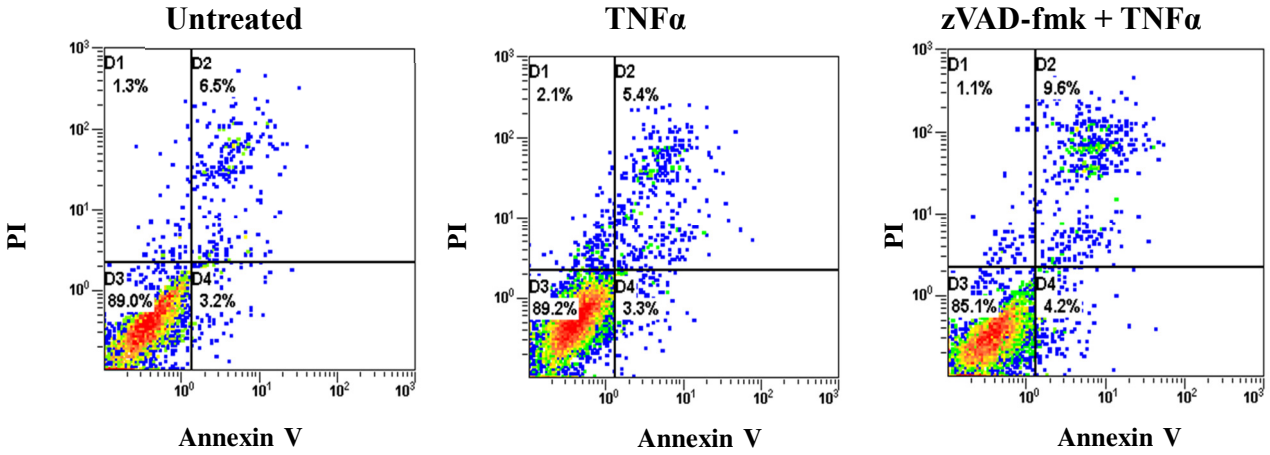
Figure 9 – SMAC mimetic exacerbates TNFα mediated necroptosis

Wild type MVECs were plated in equal numbers (10^5) and treated with SMAC Mimetic (100nM), hTNFα (60ng/mL) in the presence and absence of Nec-1 (10uM) and zVAD-fmk (50uM) and cell death was analysed using Annexin V and PI labeled flow cytometry analysis.

3.6 RIPK3 null MVECs are resistant to apoptotic and necroptotic death

Since we were previously unsuccessful in initiating a cell death response in RIPK3$^{-/-}$ MVECs, we suspected that the removal of the necroptotic pathway may be preferentially initiating apoptosis, rather than necrosis after TNFα treatment. To test this, cells were treated with TNFα in the presence and absence of zVAD-fmk and stained with both Annexin V and PI. As seen in Figure 10A, RIPK3$^{-/-}$ cells treated with 100ng/mL hTNFα did not undergo significant increases in Annexin V positive or PI positive cell death, even after treatment with zVAD-fmk (60uM). However, super-physiological addition of 600ng/mL hTNFα induced significant Annexin V positivity with no change in PI positivity, indicating an apoptotic response. This response could subsequently be recovered with the addition of zVAD-fmk (60uM), as seen in Figure 10B. These data indicate that RIPK3 null MVECs are indeed resistant to TNFα mediated necroptosis and preferentially undergo apoptosis after treatment with super-physiological amounts of hTNFα, which can be recovered with zVAD-fmk, a caspase-8 inhibitor.

A


Untreated
TNFα
zVAD-fmk + TNFα
PI
Annexin V
D1 1.3%
D2 6.5%
D3 89.0%
D4 3.2%
D1 2.1%
D2 5.4%
D3 89.2%
D4 3.3%
D1 1.1%
D2 9.6%
D3 85.1%
D4 4.2%

B

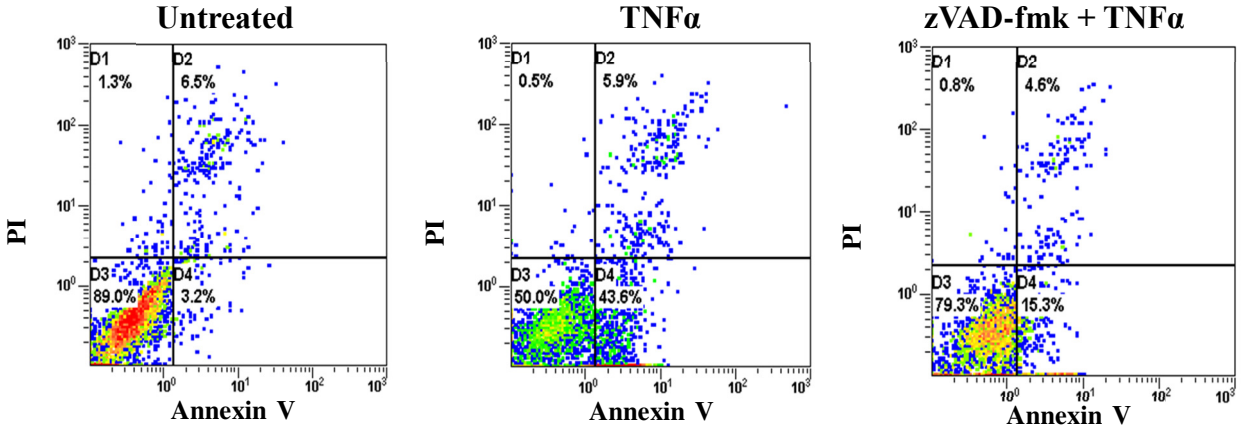

Untreated
TNFα
zVAD-fmk + TNFα
PI
Annexin V
D1 1.3%
D2 6.5%
D3 89.0%
D4 3.2%
D1 0.5%
D2 5.9%
D3 50.0%
D4 43.6%
D1 0.8%
D2 4.6%
D3 79.3%
D4 15.3%

Figure 10 – RIPK3$^{-/-}$ MVECs are resistant to apoptotic and nectoptotic cell death

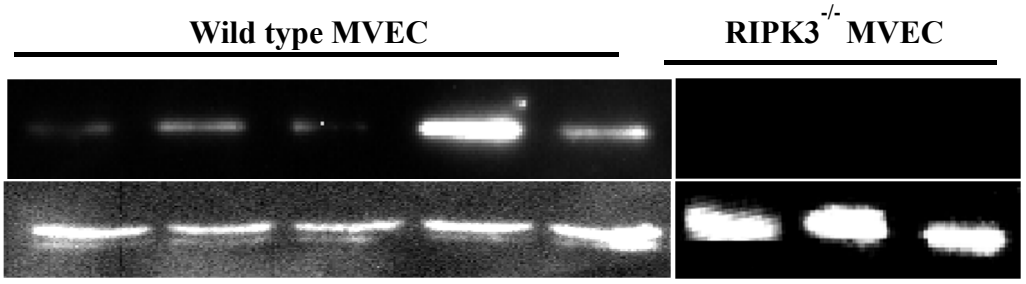
MVECs from RIPK3$^{-/-}$ wild type mice were plated in equal numbers (10^5) and treated with A) 100ng/ml TNFα, 60uM zVAD-fmk or 10uM Nec-1 in serum free media or B) 600ng/ml TNFα, 60uM zVAD-fmk or 10uM Nec-1 in serum free media and cell death was measured by Annexin V and PI positivity after 48 hours using flow cytometry analysis.

3.7 RIPK1 inhibition or RIPK3 deficiency prevents to danger molecule HMGB1 release in MVECs

Cells undergoing necrosis, not apoptosis or late apoptosis, release proinflammatory CDAMPs such as HMGB1[74, 101]. We therefore measured cell-released HMGB1 in the supernatant of MVECs treated with hTNFα (100ng/mL) + IFNγ (60ng/mL) in the presence and absence of Nec-1 (10uM) or zVAD-fmk (60uM) using western blot analysis to confirm our findings in PI labelling cell death assays to verify the presence of necrotic cells. As expected, release of HMGB1 in TNFα treated MVECs correlated with the necroptotic pattern of cell death. Following treatment with zVAD-fmk and TNFα, HMGB1 release in MVEC supernatant increased significantly (band density: 8.33$\pm$3.61 vs. 1 in untreated, n=4, p=0.02, Figure 11A&B). Addition of Nec-1 was able to attenuate HMGB1 release under anti-apoptotic conditions (intensity index 2.43$\pm$1.22, n=4, p=0.03, Figure 11A&B). These results indicate that HMGB1 release follows RIPK1 mediated necroptotic MVEC cell death.

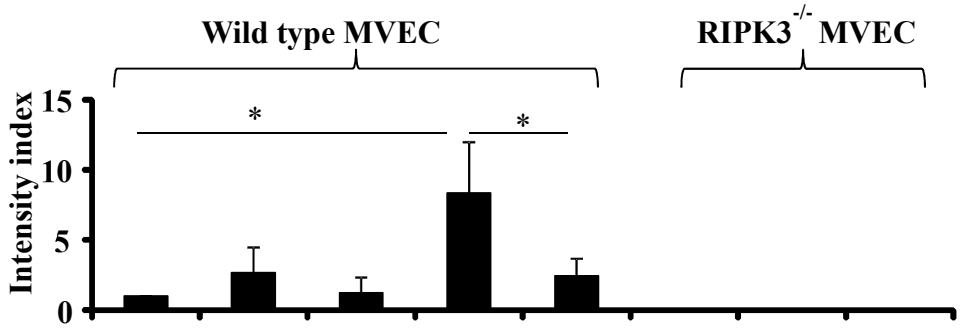
Similarly, there were no significant levels of HMGB1 detected in the supernatant of untreated RIPK3$^{-/-}$ cells and this remained unchanged following TNFα and zVAD-fmk treatment (Figure 11A). Therefore, RIPK3 deficiency, similar to RIPK1 inhibition, in MVECs also prevents TNFα induced-necroptotic death and HMGB1 release.

A



hTNFα	-	+	+	+	+	-	+	+
Nec-1	-	-	+	-	+	-	-	-
zVAD-fmk	-	-	-	+	+	-	-	+

B



hTNFα	-	+	+	+	+	-	+	+
Nec-1	-	-	+	-	+	-	-	-
zVAD-fmk	-	-	-	+	+	-	-	+

Figure 11 – RIPK1 and RIPK3 regulate HMGB1 release in MVECs

MVECs from C57BL/6 wild type mice or RIPK3$^{-/-}$ mice were plated in equal numbers (10^5) and treated with 100 ng/ml TNFα, 60mM zVAD-fmk or 10mM Nec-1 in serum free media for 48 hours. (**A**) Supernatants collected from treated C57BL/6 MVECs and RIPK3$^{-/-}$ MVECs were analyzed for HMGB1 release by western blot. Equal volumes of supernatants were loaded for each treatment. As loading controls, remained C57BL/6 MVECs and RIPK3$^{-/-}$ MVECs were collected and the cytoplasmic lysates were used for detected the unreleased HMGB1 inside MVECs. (**B**) Western blot results were quantitated by densitometry analysis (Alpha View). Untreated MVECs were use as a reference. Quantitated average of three independent cell death experiments (n=3, *: $p < 0.05$).

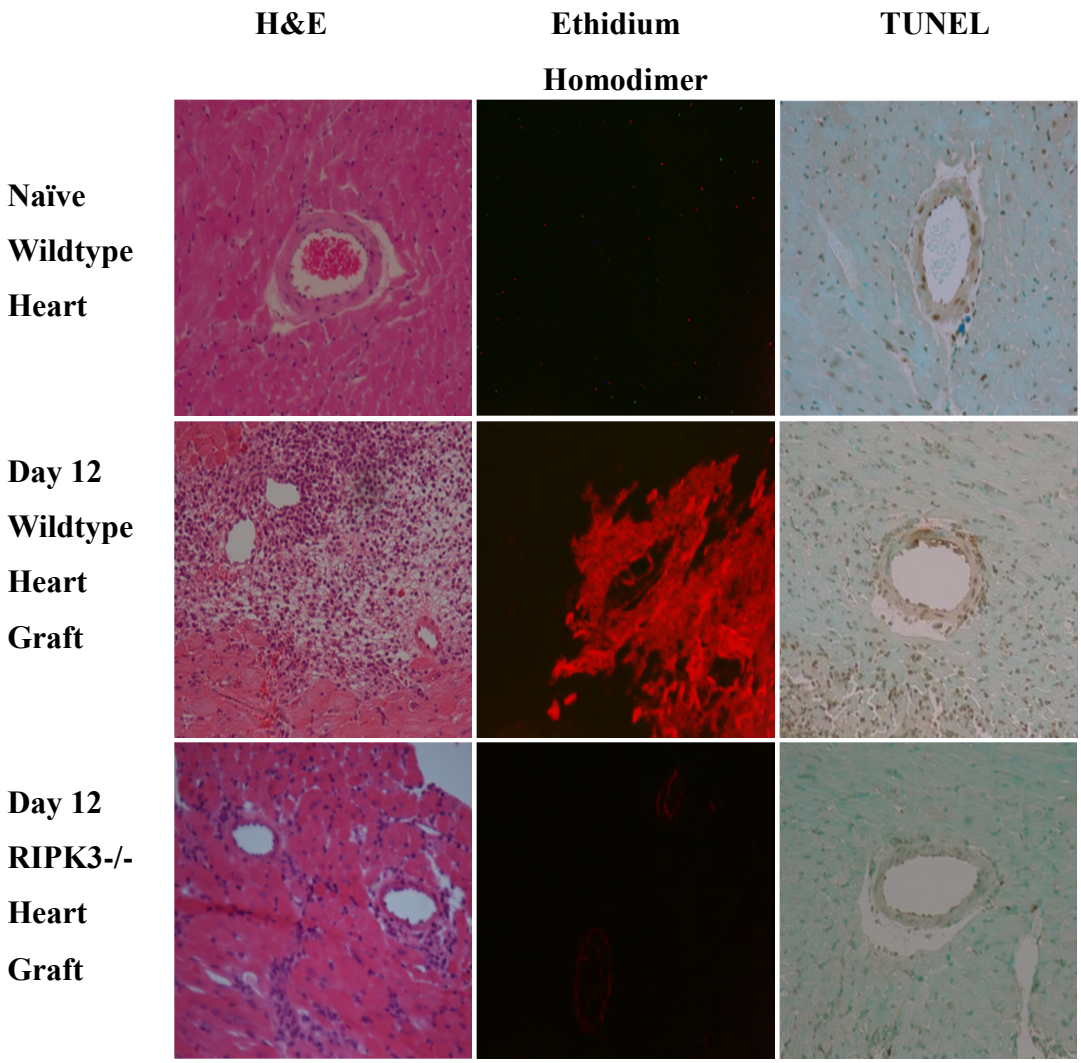
3.8 RIPK3 regulates heart graft necrosis and HMGB1 release after transplantation

RIPK1 and RIPK3 are integral for the formation of the necrosome which leads to downstream necroptosis[50, 60]. However, knockout of RIPK1 in mice results in newborn lethality and therefore no model involving RIPK1 knockout exists *in vivo*[102]. To further investigate the effect of RIPK signalling in donor heart grafts, heterotopic heart transplantations were performed using wildtype C57BL/6 or B6 RIPK3$^{-/-}$ mice as donors and BALB/c mice as recipients. Recipient mice were given brief sirolimus treatment from day 0-9 to prolong heart survival[91] and were sacrificed 12 days after transplantation. Upon sacrifice, heart grafts were collected for analysis of early tissue necrosis and apoptosis. I*n situ* ethidium homodimer perfusion was performed as described in the methods to detect necrotic cells[92]. Heart grafts were examined for ethidium homodimer labelled areas (red) for necrosis using fluorescence microscopy.

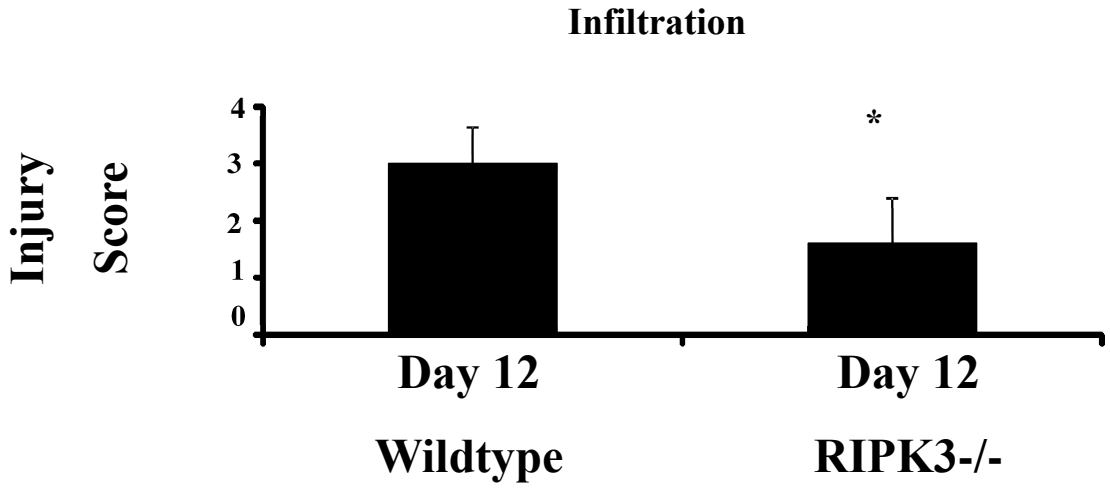
As seen in Figure 12A&B, H&E staining of wildtype and RIPK3$^{-/-}$ grafts showed increased lymphocyte infiltration localized primarily around the vessels in wildtype grafts compared with RIPK3$^{-/-}$ grafts (severity score: 3 $\pm$ 0.6 in wildtype vs. 1.6 $\pm$ 0.8 in RIPK3$^{-/-}$ heart, n=5, p=0.01). Ethidium homodimer staining revealed that wildtype heart grafts had significantly higher levels of necrosis localized around the vessels of the heart compared with RIPK3$^{-/-}$ grafts (intensity index: 157.1 $\pm$ 106.2 in wildtype vs. 12.1 $\pm$ 9.5 in RIPK3$^{-/-}$ heart, n=3, p=0.03) (Figure 12A&C), where staining was confined primarily to the endothelial barrier. TUNEL staining was used to detect apoptotic cells *in situ* and showed no significant differences between wildtype and RIPK3$^{-/-}$ heart grafts (5.5 $\pm$ 5.7

in wildtype vs 1.6 $\pm$ 2.4 in RIPK3$^{-/-}$, n=3, p=0.09) (Figure 12A&E), suggesting that RIPK3 deficiency did not alter apoptosis in the graft compared with wild type mice. RIPK3 deficiency in RIPK3$^{-/-}$ naïve hearts was confirmed by western blot analysis (Figure 12D). In summary, these data suggests that RIPK3 deficiency protects heart grafts from necrotic death and endothelial damage post transplantation.

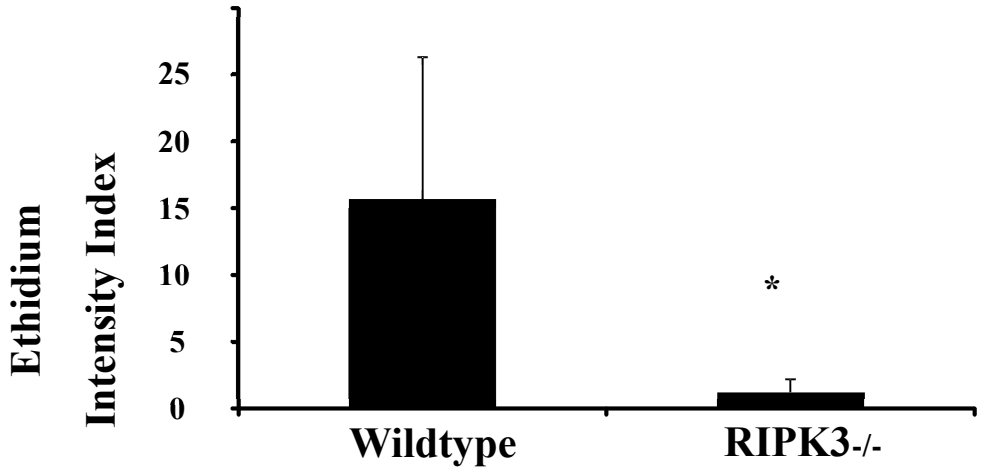
A


H&E
Ethidium Homodimer
TUNEL
Naïve Wildtype Heart
Day 12 Wildtype Heart Graft
Day 12 RIPK3-/- Heart Graft

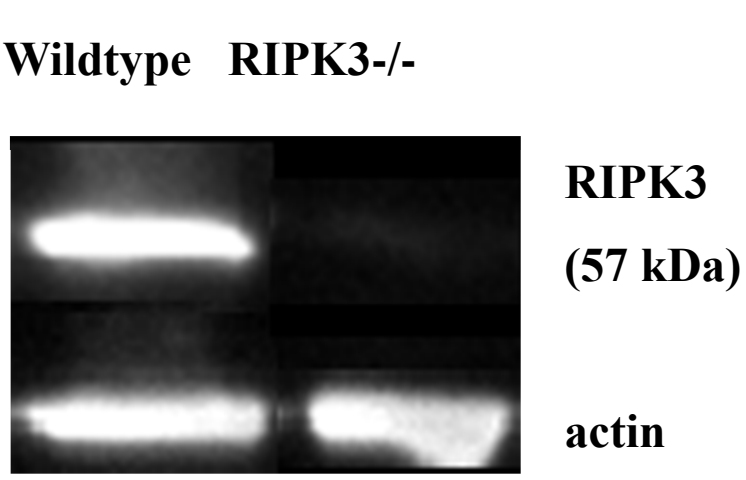
B


Infiltration
Injury Score
4
3
2
1
0
*
Day 12
Wildtype
Day 12
RIPK3-/-

C


Ethidium Intensity Index
25
20
15
10
5
0
*
Wildtype
RIPK3-/-

D


Wildtype
RIPK3-/-
RIPK3
(57 kDa)
actin

E

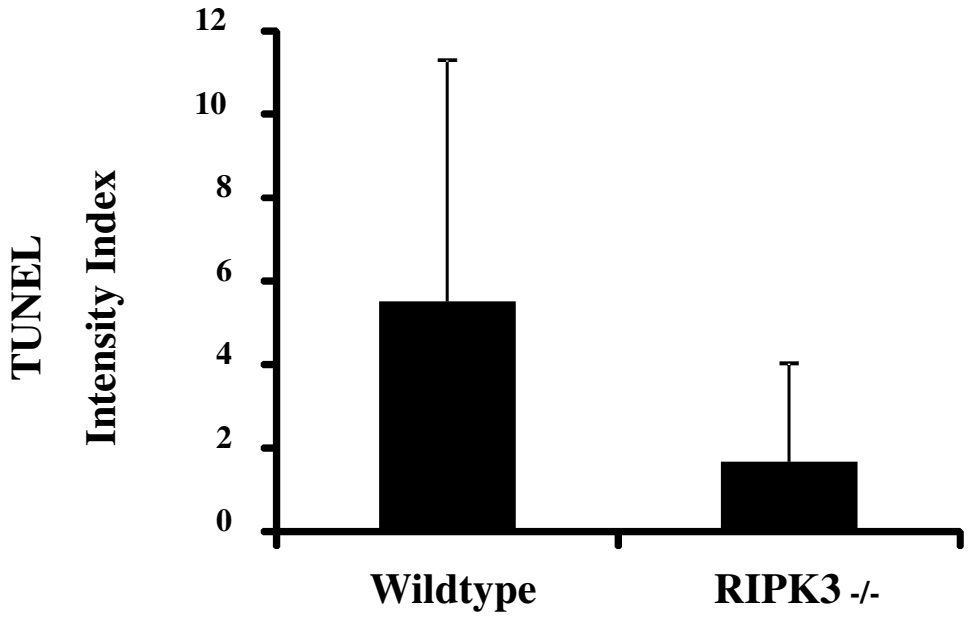


Figure 12 – RIPK3 deficiency reduces necrosis and graft infiltration following heart transplantation

Wildtype C57BL/6 or B6 RIPK3$^{-/-}$ donor hearts were heterotopically transplanted into BALB/c recipients. Sirolimus (1mg/kg) was given from operative day 0 to post-operative day 9 and heart grafts were collected at post-operative day 12. A) Graft tissue obtained was formalin fixed at the time of sacrifice and stained by H&E, ethidium homodimer or TUNEL assay. Data is representative of five transplanted grafts. Areas of greatest injury are shown in both wildtype and RIPK3$^{-/-}$ grafts. B) Blinded quantitative pathological scoring of cellular infiltration in wildtype and RIPK3$^{-/-}$ grafts. Slides were scored on a scale from 0-4 as described in the methods (n=5, *: $p < 0.05$). C) Quantification of ethidium homodimer fluorescence staining for necrosis in wildtype and RIPK3$^{-/-}$ heart grafts 12 days following transplantation (n=3, *: $p < 0.05$). D) Quantification of TUNEL staining for apoptosis in wildtype and RIPK3$^{-/-}$ heart grafts 12 days following transplantation (n=3, ns: $p > 0.05$). E) Western blot analysis of wildtype and RIPK3$^{-/-}$ heart grafts for expression of RIPK3 protein.

3.9 HMGB1 release is attenuated in RIPK3 null allografts following cardiac transplantation

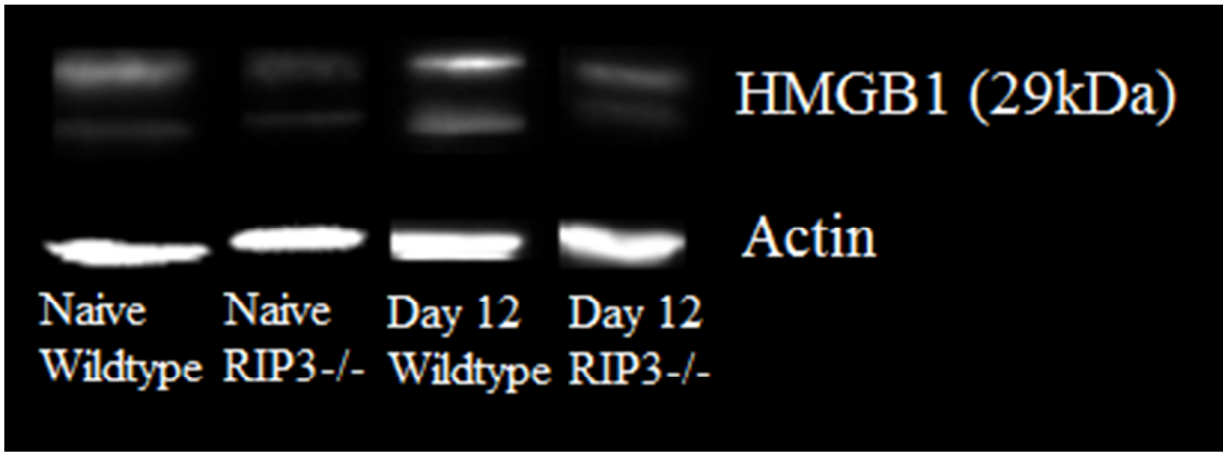
As mentioned previously, necrosis results in the release of proinflammatory HMGB1[74, 101]. Interestingly, HMGB1 released following necrotic cell death can exist in an immunogenically active form, whereas cells undergoing apoptosis can release a tolerance promoting (oxidized) form[103]. We therefore measured HMGB1 in heart graft lysates isolated by cytoplasmic lysis buffer at day 12 following transplantation. Western blot analysis revealed that only the active form of HMGB1 increased in wildtype grafts, but not in RIPK3$^{-/-}$ grafts (Intensity index: 0.99 $\pm$ 0.22 in wildtype vs. 0.64 $\pm$ 0.19 in RIPK3$^{-/-}$, n=5, p=0.03, Figure 13A&B). Interestingly, the (lower) oxidized band of HMGB1 remained statistically similar between wildtype and RIPK3$^{-/-}$ grafts (0.34 $\pm$ 0.19 in wildtype vs. 0.35 $\pm$ 0.13 in RIPK3$^{-/-}$, n=5, p=0.12, Figure 13A&B).

Immunohistochemistry for HMGB1 revealed increased HMGB1 release from the nucleus of cardiomyocytes surrounding the vessels (red arrows) in wildtype grafts compared with RIPK3$^{-/-}$ grafts (Figure 13C). Released HMGB1 can be seen diffusely in the cytoplasm of injured cells. Taken together, these data further support the notion that deficiency of RIPK3 provides a protective role against necroptotic death and subsequent HMGB1 release in heart grafts.

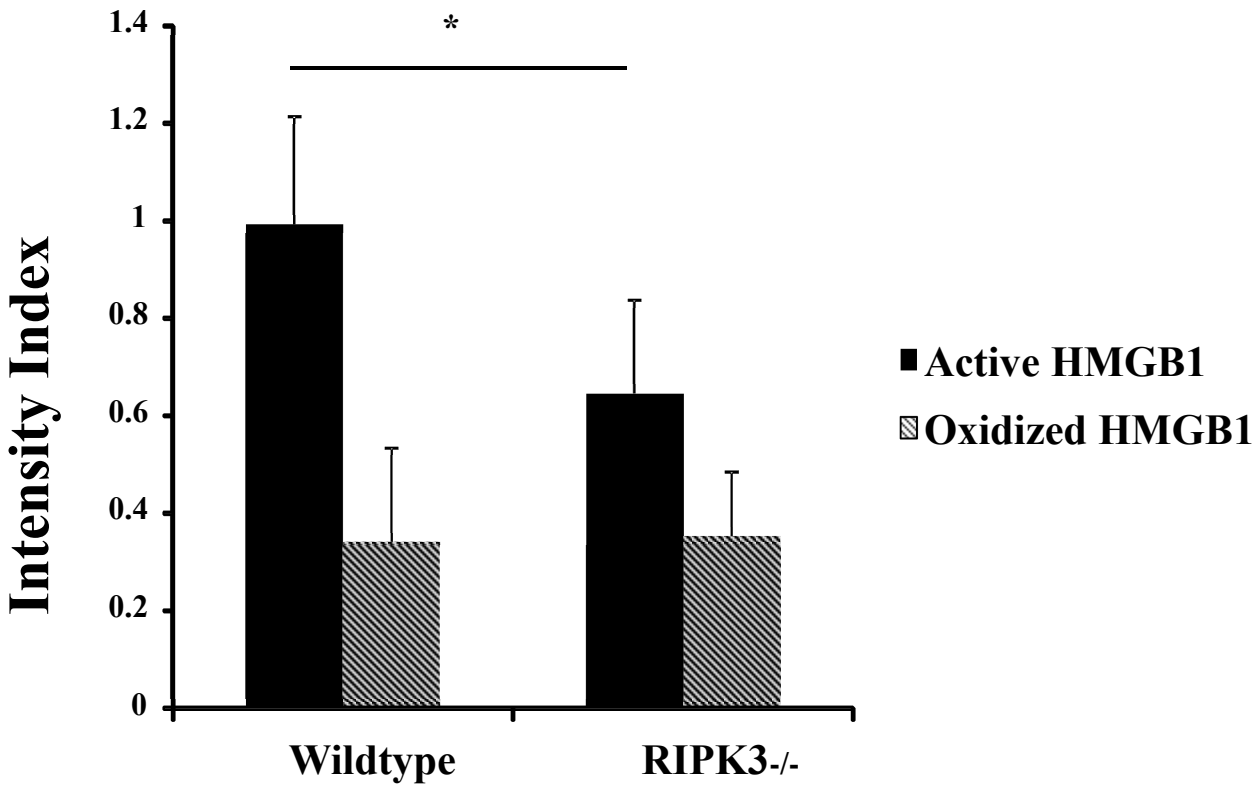
Lastly, we measured the chemokine expression levels in wildtype and RIPK3 null grafts 12 days following transplantation, as shown in Figure 13D. Compared with naïve,

untreated C57BL/6 hearts, wildtype grafts expressed higher transcript levels of CXCL10 (26.5 $\pm$ 27.6 fold increase in wildtype vs 13.2 $\pm$ 13.2 in RIPK3$^{-/-}$, n=5, p=0.2), CCL3 (30.9 $\pm$ 53.7 fold increase in wildtype vs 9.5 $\pm$ 8.5 in RIPK3$^{-/-}$, n=5, p=0.24), RANTES(8.1 $\pm$ 14.9 fold increase in wildtype vs 1.2 $\pm$ 2.0 in RIPK3$^{-/-}$, n=5, p=0.21) and MCP-1 (31.3 $\pm$ 55.1 fold increase in wildtype vs 1.63 $\pm$ 1.8 in RIPK3$^{-/-}$, n=5, p=0.17) than RIPK3 null grafts. However, these differences were non-significant.

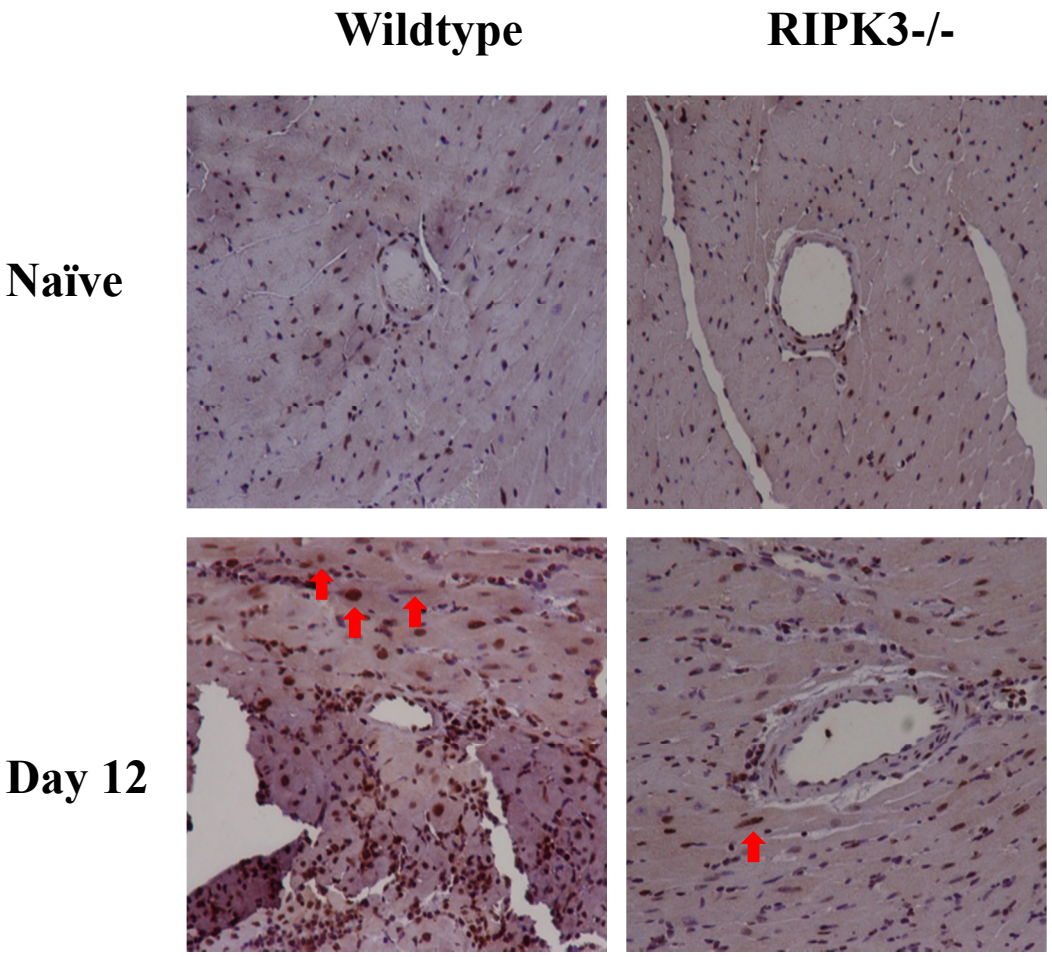
A


HMGB1 (29kDa)
Actin
Naive Wildtype
Naive RIP3-/-
Day 12 Wildtype
Day 12 RIP3-/-

B


*
Intensity Index
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Wildtype
RIPK3-/-
Active HMGB1
Oxidized HMGB1

C


Wildtype
RIPK3-/-
Naïve
Day 12

D

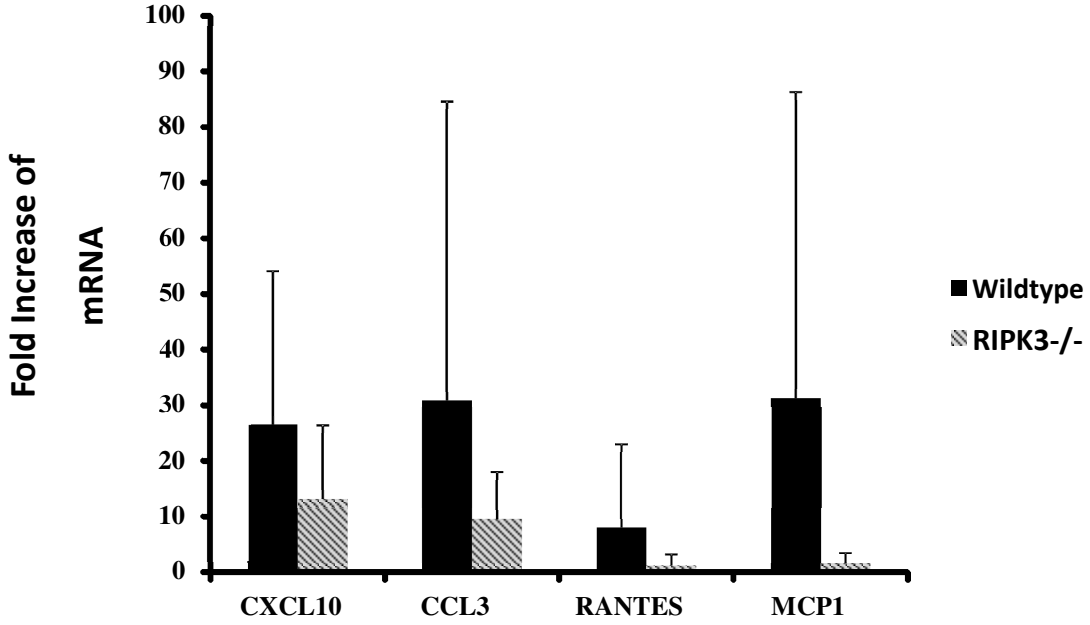

Fold Increase of mRNA
100
90
80
70
60
50
40
30
20
10
0
CXCL10
CCL3
RANTES
MCP1
Wildtype
RIPK3-/-

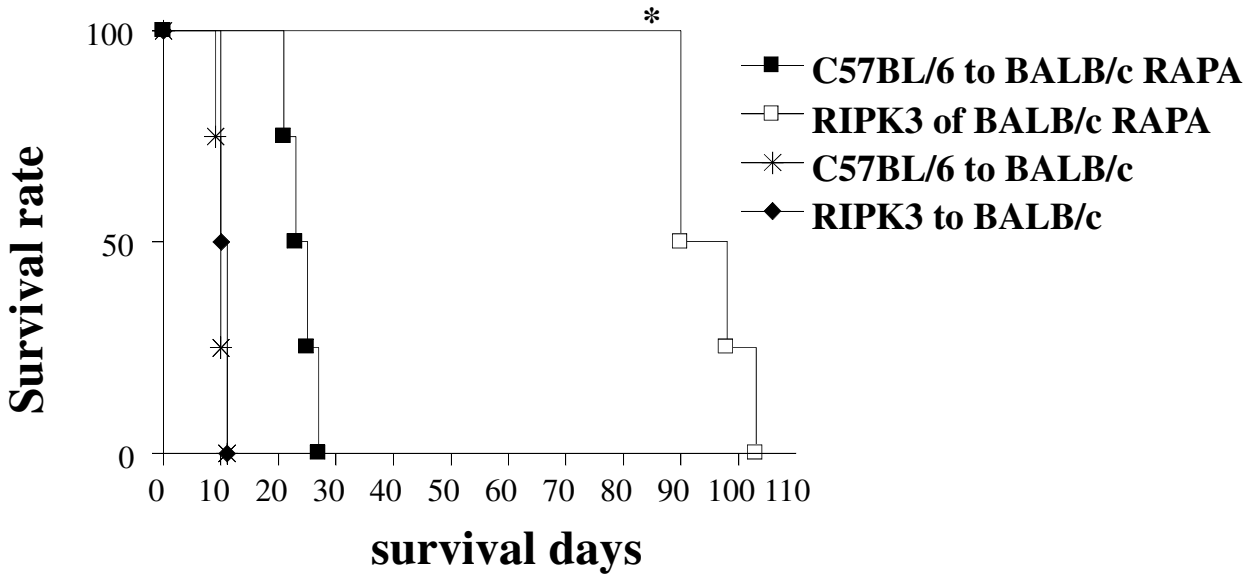
Figure 13 – RIPK3 deficiency inhibits danger molecule HMGB1 release and inflammatory responses in the heart graft

Protein from heart grafts collected 12 days following heart transplantation as described in Figure 12 were isolated by cytoplasmic lysis buffer before being examined for HMGB1. A) Western blot analysis of HMGB1 in naïve and transplanted wildtype and RIPK3$^{-/-}$ hearts. Separate bands represent the amount of immunogenic (upper band) or oxidized forms (lower band) of HMGB1. Data shown is representative of five transplanted grafts. B) Densitometry analysis of immunogenic and oxidized HMGB1 (n=5, *: $p<0.05$). β-actin was used as a loading control and quantitation reference. C) Paraffin fixed sections were stained for total HMGB1in both wild type and RIPK3-/- hearts. Areas of HMGB1 being released into the cytoplasm and in nuclei undergoing harryorrhexis are indicated by arrows. D) cDNA was isolated from heart grafts collected 12 days following heart transplantation and analysed by RT-qpPCR for chemokine expression. Values are representative of five separate experiments.

3.10 RIPK3 deficiency in heart allografts prolongs graft survival after transplantation following sirolimus treatment

To determine if RIPK1/3 mediated necroptotic death could influence cardiac transplant survival, we performed heterotopic transplantation of wildtype C57BL/6 or B6 RIPK3$^{-/-}$ donor hearts into fully MHC mismatched recipient BALB/c mice. As shown in Figure 14A, without immunosuppressive treatment, RIPK3 null heart grafts have similar survival times compared to wildtype grafts (10$\pm$0.8 days in wildtype vs. 10.3$\pm$0.5 days in RIPK3$^{-/-}$, n=4). However, treatment with sirolimus (1mg/kg) from surgical day 0 to day 9 extended the survival time of wildtype grafts to 24 $\pm$ 2.6 days and RIPK3$^{-/-}$ grafts to 95 $\pm$ 5.8 days (n=4, P=0.011). As shown in Figure 14B, naïve C57BL/6 hearts do not appear histologically different from naïve RIPK3$^{-/-}$ hearts, but wildtype heart grafts show severe lymphocyte infiltration and vasculopathy at rejection day compared with RIPK3$^{-/-}$ grafts collected on day 40. RIPK3 was also detected in the endothelium and cardiac myocytes of naïve wild type hearts, but not RIPK3$^{-/-}$ hearts, by immunohistochemistry. This data indicates the absence of RIPK3 in heart grafts can greatly prolong graft survival, suggesting that RIPK3 mediated necroptotic death has a deleterious effect on heart graft survival.

A


*
100
50
0
Survival rate
0 10 20 30 40 50 60 70 80 90 100 110
survival days
C57BL/6 to BALB/c RAPA
RIPK3 of BALB/c RAPA
C57BL/6 to BALB/c
RIPK3 to BALB/c

B

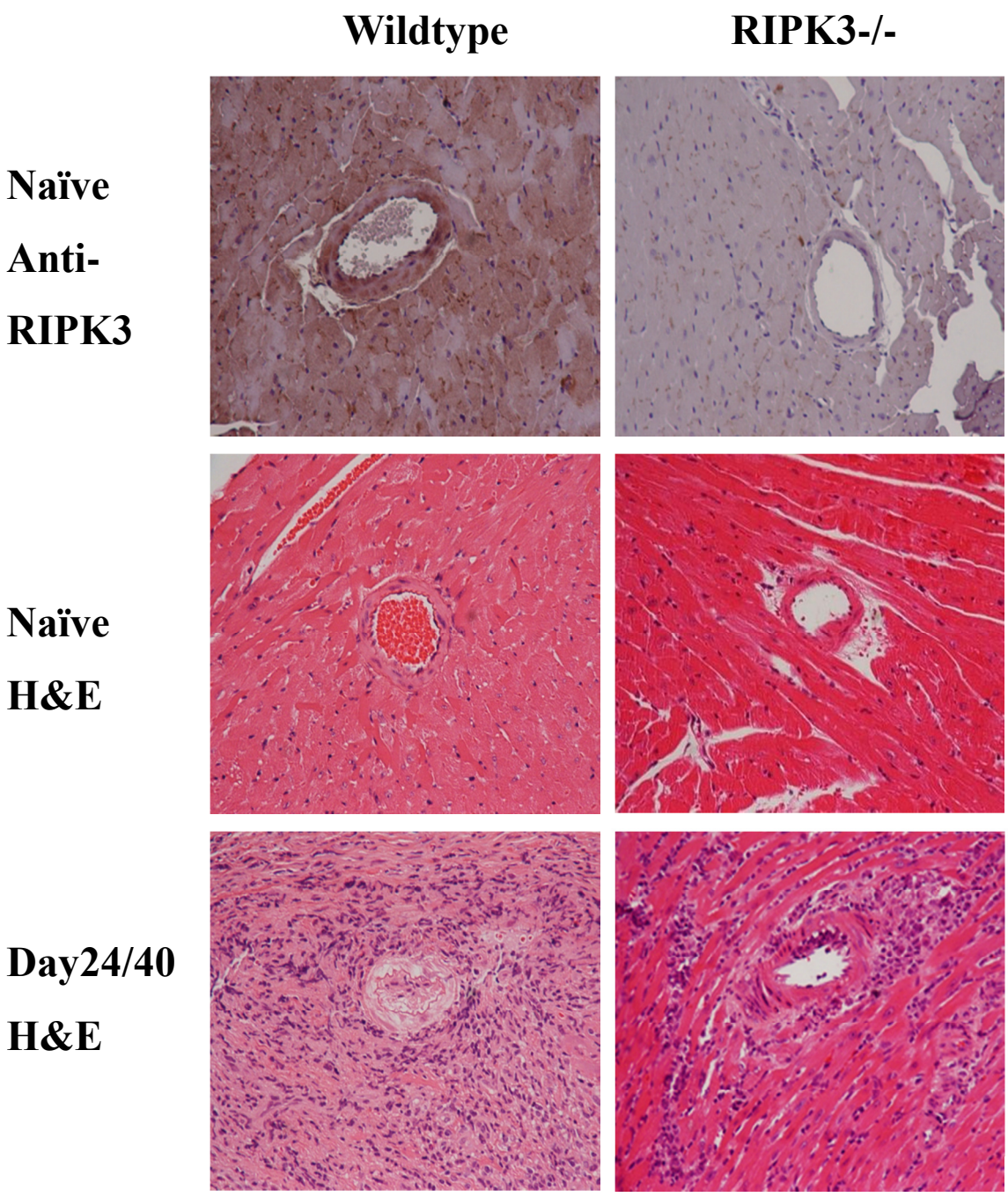


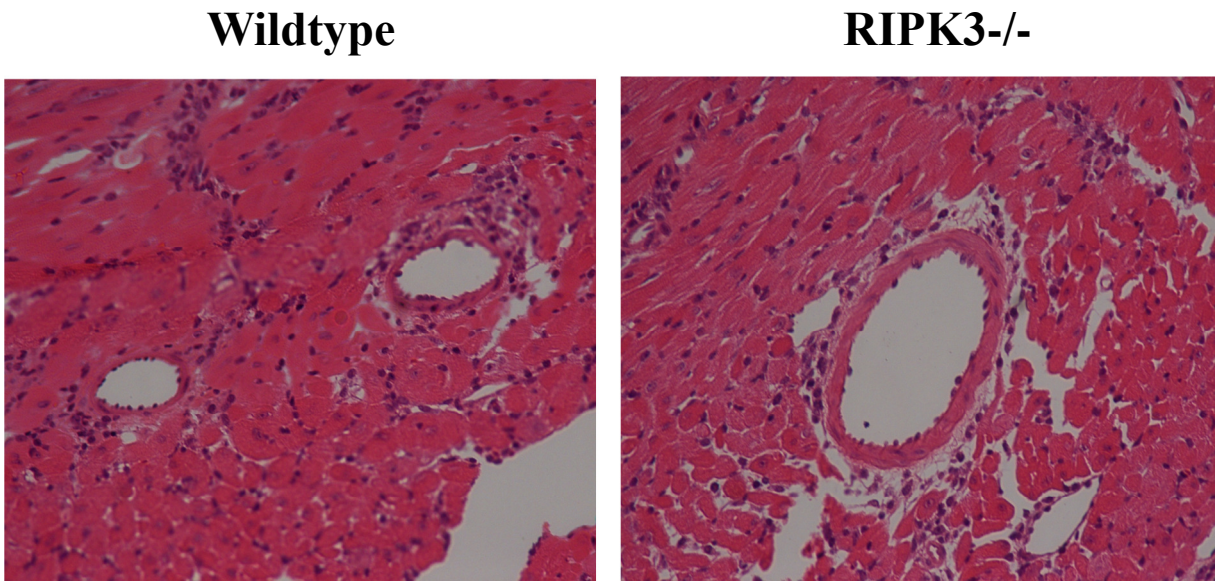
Figure 14 – RIPK3 deletion in donor grafts induced long term graft survival

Wildtype C57BL/6 or B6 RIPK3$^{-/-}$ donor hearts were transplanted into BALB/c recipients. Sirolimus (1mg/kg) was given daily from operative day 0 to post-operative day 9. Graft survival was monitored daily by palpitation. Significant drop or cessation of pulsation is considered as graft rejection. A) Survival curve of wildtype C57BL/6 and RIPK3$^{-/-}$ donor heart grafts in BALB/c mice was obtained from two repeated experiments with and without sirolimus treatment (n=4, *: $p < 0.05$, Log rank test). B) RIPK3 detection in heart grafts and H&E comparison of wildtype and RIPK3$^{-/-}$ grafts. Heart grafts were collected from wild type recipients 24 days after transplantation and RIPK3$^{-/-}$ graft recipients 40 days after transplantation and fixed by formalin before H&E staining or immunohistochemistry analysis by anti-RIPK3 (Abcam).

3.11 CD40 costimulatory blockade does not extend RIPK3 null graft survival following cardiac transplantation

Cardiac allograft transplantation was again performed using wildtype and RIPK3$^{-/-}$ donor grafts with a single treatment of MR1 (200ug) one day before transplantation. In both wildtype and RIPK3$^{-/-}$ grafts, rejection began at post-operative day 20 and is shown histologically in Figure 15A. Pathological scoring during rejection revealed no significant differences in endothelial damage, necrosis, infiltrate or myocyte damage (n=2) between wildtype and RIPK3$^{-/-}$ grafts, as shown in Figure 15B.

A


Wildtype
RIPK3-/-

B

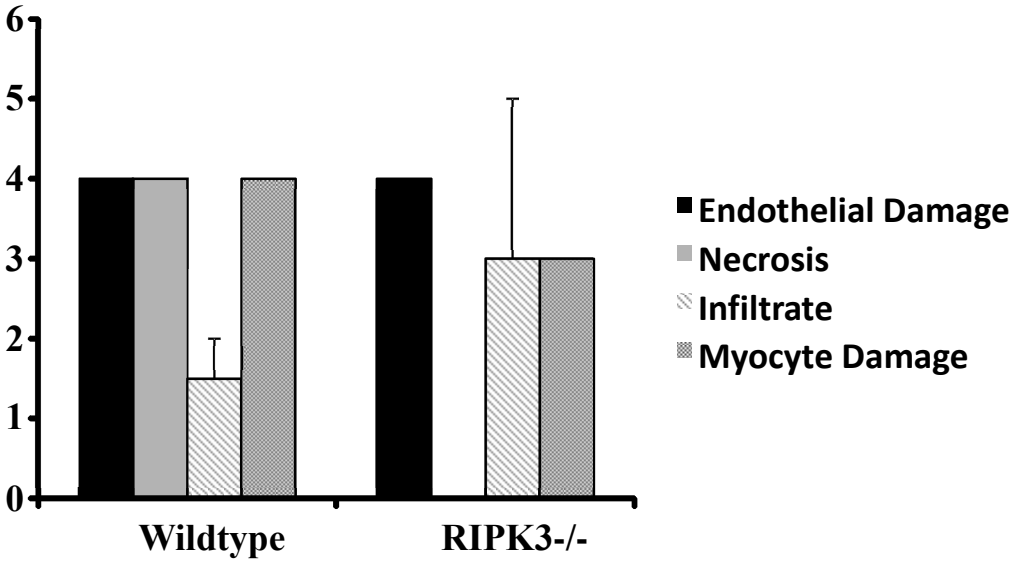

6
5
4
3
2
1
0
Wildtype
RIPK3-/-
Endothelial Damage
Necrosis
Infiltrate
Myocyte Damage

Figure 15 – RIPK3 deletion does not protect heart grafts following CD40 costimulatory blockade

Wildtype C57BL/6 or B6 RIPK3$^{-/-}$ donor hearts were transplanted into BALB/c recipients. Anti-CD154 (200ug) was given daily from operative day 0 to post-operative day 9. Graft survival was monitored daily by palpitation. Significant drop or cessation of pulsation is considered as graft rejection. A) Wild type and RIPK3$^{-/-}$ grafts were examined using H&E staining and confocal microscopy (400X) following rejection after day 20. B) Blinded pathological scoring of rejected grafts for endothelial damage, necrosis, infiltrate and myocyte damage ($n=2$, $p > 0.05$).

Chapter 4

4 Discussion

4.1 Summary of this study

In this study, we isolated, immortalized and characterized a new murine MVEC cell line for *in vitro* experimental use (Figure 5). We have shown at the mRNA and protein level that RIPK3 can be upregulated by TNFα and that TNFR1 can be upregulated by both TNFα and/or IFNγ in MVECs (Figure 6). Necroptotic death and subsequent danger molecule HMGB1 release were also observed in wildtype MVECs under anti-apoptotic conditions, which could be significantly attenuated following pharmacological RIPK1 inhibition or RIPK3 knockout (Figures 8 and 11). We also observed that RIPK3 null MVECs underwent apoptosis following super-physiological treatment of TNFα, which could be partially recovered by caspase inhibition (Figure 10). Following cardiac allograft transplantation, RIPK3 null donor grafts survived significantly longer following sirolimus treatment than wild type donor grafts (Figure 14). Following transplantation, RIPK3 null grafts showed significantly less host immune infiltration and necrosis compared with wild type grafts while maintaining similar levels of apoptosis (Figure 12). Lastly, protein levels of danger molecule HMGB1 were attenuated in RIPK3 null allografts shortly following transplantation (Figure 13). The ability of immunosuppression to provide rejection protection is dependent on the cell death pattern of the graft following transplantation. We suggest that targeting proteins RIPK1 or RIPK3 in heart grafts may be beneficial in future transplantation therapies.

4.2 Inflammatory injury, cellular rejection and cell death

In clinical cardiac transplantation, injury and inflammation can result in delay or loss of graft function and reduced survival rates following transplantation. Mechanisms of injury, such as IRI, commence early in the transplantation procedure, while others continue throughout the life of the graft, such as chronic cellular rejection and GVD. These mechanisms of injury initiate different forms of cell death in graft parenchymal and endothelial cells, including apoptosis, autophagy and necrosis, which may influence the outcome of the graft through inflammatory sequelae.

Cardiac transplantation invariably results in damage to the transplanted organ beginning with organ procurement, storage, surgical manipulation, ischemic time and reperfusion injury. These necessary procedures can often influence long-term graft survival through early up-regulation of alloantigens and cell adhesion molecules, leading to accelerated allorecognition[104]. IRI can result in apoptotic, autophagic and necrotic cell death, subsequently initiating host immune responses and the promotion of antibody and cell mediated rejection[86, 105-107]. These various forms of cell death potentially have important implications on the severity of the immune responses following transplantation and can greatly influence early and chronic survival of the graft.

Following IRI, cardiac grafts are susceptible to ACR, which constitutes one of the most frequent causes of profound graft tissue damage within the first year after transplantation. Acute injury is often an adaptive immune response initiated following the maturation of

recipient CD4+ helper T cells, CD8+ cytotoxic T cells and B lymphocytes. These cell types can attack the graft through direct or indirect allorecognition pathways.

Chronic cardiac allograft rejection presents as GVD, a severe, diffuse vascular intimal hyperplasia with eventual stenosis of the vascular wall resulting in compromised vascular flow and ischemia. It is unclear whether the incidence of ACR can affect long term graft survival by accelerating GVD through early allorecognition. Many studies have positively correlated ACR and GVD[108-110], however, others have failed to find a direct correlation between biopsy-confirmed acute rejection and GVD[111-113]. Instead, GVD seems to result from any cellular insult including IRI[9, 12-14], indirect allorecognition by CD4+ T cells[17] and cytokine release[15, 16].

In clinical transplantation, the short-term success rate of allografts has steadily improved over the decades while long-term graft attrition has remained constant[1, 114-116]. Improvements in short-term transplant success can be attributed to improved immunosuppressive management of acute rejection episodes, organ preservation, donor selection, biopsy management and techniques, and diagnosis and management of infection. However, despite our increased knowledge of immunobiology and improved immunosuppressive therapies, long-term functional survival times of cardiac allografts remains stagnant and thus transplantation has remained a treatment, rather than a cure, for devastating cardiac events. In this study, we attempt to augment cell death in the donor graft through inhibition or deletion of the RIPK proteins, which are critical in the

initiation of necroptotis, a cell death pathway that enhances rejection initiating immune responses.

Apoptotic cell death has been extensively studied in cancer, infectious disease, autoimmune disease and transplantation[117, 118]. Apoptosis leads to the formation of annexin-V positive apoptotic bodies that sequester cellular contents and are rapidly eliminated via phagocytosis by other cells with minimal inflammation[117, 118]. This process is initiated following the activation of caspases, a series of highly regulated endogenous cysteine proteases. Some caspases are pro-inflammatory, such as caspase-1, which localizes in inflammasomes to process IL-1β and IL18[119], while others require death receptor engagement. The death receptor pathway is induced by TNFR super-family members (TNFR1, Fas, TRAIL) and requires the activation of caspase 8 to initiate apoptosis. Traditionally, apoptosis was thought to be a neutral factor in transplantation due to its non-immunogenic properties. However, recent studies have proved otherwise. Endothelial cell apoptosis has been shown to be an immunoregulatory process that is important in GVD related intimal remodelling[120, 121], while the release of "find me" and "eat me" signals by apoptotic cells has been shown to recruit macrophages and monocytes[122-124]. In addition, apoptotic cells not cleared by the immune system may undergo secondary necrosis with subsequent plasma membrane permeation and release of CDAMPS, similar to necrosis or necroptosis[117].

Necrosis was traditionally regarded as an unregulated means of cell death as a result of unexpected cellular stresses. Recently, it has been observed that the death receptors

TNFR1 or Fas, when engaged, can elicit necrosis as well as apoptosis[64, 125, 126], suggesting necrosis can also be regulated by signal transduction pathways. This regulated form of caspase-dependent necrosis has now been termed necroptosis and is controlled by RIPK1 and RIPK3[59, 69, 80, 126]. Necroptosis is characterized by the same morphological features as unregulated necrotic death, including early loss of plasma membrane integrity and release of cytoplasmic contents[127]. These contents, such as immunogenic CDAMPS, can bind to immune cell receptors and initiate powerful immune responses[75].

In this study, necroptosis was induced *in vitro* using TNFα. TNFα is a proinflammatory cytokine produced by graft infiltrating cells, endothelial cells, and cardiac myocytes. TNFα quickly increases in cardiac grafts after transplantation[43, 45] and has been shown to be an important contributor of heart graft rejection[44-46, 48, 128]. In addition, TNFα blockade or TNFR deficiency has been shown to prolong cardiac graft survival in several animal models[44, 46, 47]. TNFα induces death through TNFR1 but promotes pro-inflammatory responses through TNFR2[129].

Interestingly, myocardium in human donor heart grafts can modulate TNFR expression following rejection related injury by radically down-regulating TNFR1 and up-regulating TNFR2 surface expression following heart transplantation[48]. Conversely, human and murine endothelial cells do not undergo radical changes in TNFR1 expression patterns before and post transplantation[48] and therefore are susceptible to TNFα induced cell death. As the first barrier between graft and host, MVEC's undergoing early necroptotic

death may promote cardiac graft inflammation and subsequently augment rejection responses. Here we have shown that inhibition of caspase function enhanced necrotic cell death and the release of HMGB1 in endothelial cells and inhibition of RIPK1 or RIPK3 function attenuated necroptotic death (Figure 8 A-C) and subsequent danger molecule HMGB1 release (Figure 11A&B). RIPK3 deficiency in donor heart allografts prevented endothelial necrotic death, HMGB1 release, and substantially prolonged graft survival after short-term sirolimus treatment. This study suggests that RIPK1/3-mediated necroptosis contributes to inflammatory injury in cardiac allografts and that RIPK1 and RIPK3 are important therapeutic targets to induce long term graft survival.

4.3 The pattern of cell death can be differentiated using pharmacological inhibitors or genetic modification

In recent years, our understanding of cell death has expanded beyond apoptosis, autophagy and necrosis and now includes regulated necrosis which comprises RIPK mediated necroptosis, pyronecrosis, excitotoxicity, pyroptosis, parthanatos, NETosis, entosis, ferroptosis, anoikis, lysosomal membrane permeabilization, Wallerian degeneration and heat-stroke-induced regulated necrosis[130]. Consequently, various forms of cell death detection have arisen to differentiate between cell death forms. These methods include quality of DNA fragmentation, morphology, cell viability, caspase activation, PARP1 cleavage, and labeling with Annexin or PI, among others. However, these methods alone are often non-definitive and often require conjunction with inflammatory markers and the use of pathway-specific pharmalogical inhibitors, RNA

knockdown or animal models with genetic deletion to confirm a specific cell death pathway[75, 131].

In this study, PI labeling was used *in vitro* to identify necroptotic cell death, a method previously used in many studies[49, 50, 60, 65, 86, 132, 133], while others have used annexin-V labeling to detect necroptotic death [69, 133-135]. PI is an intercalating agent and binds to DNA inside the nucleus of cells. This requires the loss of plasma membrane integrity as PI cannot permeate healthy cell membranes. Annexin V binds phosphatidylserine, making it a useful marker for detecting apoptosis following the apoptotic plasma membrane translocation of phospholipids[136]. Sawai and colleagues, using a human monocyte leukemia cell line, U-937, have previously shown that necroptotic cells can label as annexin V positive / PI negative, annexin V negative / PI positive, or annexin V positive / PI positive[137]. However, their data only shows PI positive labeling in anti-apoptotic conditions after zVAD-fmk treatment, an important pro-necroptotic condition. Furthermore, their conclusion that necroptotic cells could label as annexin V positive / PI negative was based on the observation that Nec-1 could recover annexin V positive / PI negative cell death. Although it is known that PI positivity can result from secondary necrosis following apoptosis[137], PI labeling while augmenting cell death using zVAD-fmk, Nec-1 and RIPK3$^{-/-}$ MVECs in conjunction with the release of the inflammatory marker HMGB1 is sufficient evidence of necroptosis.

Necroptosis becomes the predominant form of cell death when caspase 8-mediated apoptotic pathways are blocked and can be reverted to apoptosis in the presence of RIPK inhibition[64, 138, 139]. Hence, caspase-8 is not only an inducer of apoptosis, but also an inhibitor of necroptosis as it has been observed that caspase-8 deficiency leads to unregulated necroptosis in the yolk sack vessels of developing embryos, resulting in early embryonic lethality[140-142]. Therefore, blocking apoptotic regulators, particularly caspase 8, may not benefit heart graft survival as it may enhance necroptosis and worsen inflammatory injury in the graft. Alternatively, targeting RIPK proteins would inhibit necroptosis and thus minimize graft injury. Vercammen and colleagues showed the profound effects of zVAD-fmk in L929 fibroblasts on necroptotic death. In their experiments, the half-maximal dose of TNFα needed to induce necroptosis was decreased by a factor of 1000 in the presence of zVAD-fmk and exogenous TNFα over TNFα alone[50]. Since these findings were published, zVAD-fmk has been used in several studies to induce necroptosis[54, 63, 69, 84, 135, 143]. This mechanism was discovered to be prominent in mouse L929 fibroblasts[49] and was confirmed in this study (Figure 7).

4.4 HMGB1 as a marker for necroptosis and inflammation

Necrosis results in inflammatory sequelae mediated by the release of cell contents including HMGB1, heat-shock proteins, histones, uric acid, RNA, and DNA, all of which can trigger inflammatory reactions[74-76]. HMGB1, which augments expression of chemokines and cytokines that attract and activate diverse immune cells through binding on its receptors TLR-2 or -4, has been shown to participate in early heart injury and further augments adaptive immune responses during graft rejection[75, 76, 144]. Importantly,

HMGB1 is mainly released from necrotic, but not apoptotic or late apoptotic cells, which results in pro-inflammatory responses[74]. In our study, HMGB1 was used to confirm necroptotic cell death in conjunction with PI labeling, which may bind late apoptotic and secondary necrotic cells. Indeed, we found that necroptosis-induced HMGB1 release was substantially inhibited in RIPK3 deficient MVECs or by inhibiting RIPK1 with Nec-1 (Figure 11A&B). This finding was further supported by the results of our *in vivo* study where the release of HMGB1was inhibited in RIPK3$^{-/-}$ heart grafts compared with wild type heart grafts (Figure 13A&B). However, a challenge arose in finding a method of quantifying necrotic cell death *in vivo* to correlate cell death and HMGB1 release in heart grafts. Previous studies have relied on microscopy and pathological scoring, which are susceptible to high degrees of inter-observer variability. To address this, we modified a protocol of ethidium homodimer perfusion into a mouse kidney[92] for application in a heterotopic heart. Ethidium homodimer, similar to PI, binds DNA after cell membrane rupture. In the heterotopic heart, ethidium homodimer staining of the endotheilium and cardiomyocytes indicated the level of vascular damage, leakiness and myocyte necrosis. Consequently, we found that ethidium homodimer staining and lymphocyte infiltration were significantly greater in wild type mice 12 days after transplantation than RIPK3$^{-/-}$ heart grafts (Figure 12A-C). In addition, immunohistochemistry in heart graft sections revealed increased HMGB1 staining in cellular nuclei undergoing karyorrhexis and in the cytoplasm of cardiomyocytes (Figure13C). Finally, compared with wildtype donor grafts, RIPK3 deficiency in donor heart grafts induced long term graft survival following short-term sirolimus treatment (Figure 14A). These data suggest that RIPK3-mediated necroptosis contributes to the release of the danger molecule HMGB1 and subsequently

enhances inflammatory injury in heart allografts. Hence, RIPK1/3-mediated necroptosis and subsequent inflammatory responses play a reciprocal role for heart graft rejection. Inhibiting the feedback loop of TNFα, RIPK, HMGB1 and infiltrating immune cells may prevent graft injury and promote long term graft survival.

4.5 HMGB1 subtypes influence immune responses

Previous research suggests that different forms of HMGB1 can be released during cell death[103, 145]. The active form of HMGB1 released from necrotic cells has an immunogenic function through TLR interaction, whereas cells undergoing apoptosis release a tolerogenic form of HMGB1whose stimulatory function is neutralized after oxidization[103]. More recently, a third form of HMGB1, all-thiol-HMGB1, has been shown to exist and acts as a chemoattractant to recruit leukocytes. In our study, wild type heart grafts 12 days after transplantation have much higher levels of the active form of HMGB1 than RIPK3$^{-/-}$ grafts (Figure 13A&B). Binding of this form of HMGB1 to TLR-4 and RAGE receptors promotes T cell, macrophage and DC maturation, exacerbating cellular mediated graft rejection. There were no differences in levels of the oxidized form of HMGB1 between wild type and RIPK3 null heart grafts, indicating that apoptosis in heart grafts was not altered by RIPK3 deficiency. This finding was supported by heart tissue TUNEL staining, which showed no significant differences in apoptotic area in heart grafts compared wild type and RIPK3$^{-/-}$ mice (Figure 13A&E). However, the presence of oxidized HMGB1 in heart grafts contrasted the HMGB1 profile in MVECs *in vitro*, where only the immunogenic form of HMGB1 was detected by western blot

following MVEC necroptosis. In this instance, the oxidized form of HMGB1 seen *in vivo* may have originated from host infiltrating cells[146] or other heart cells. The third form of HMGB1 has yet to be identified via western blot but likely contributed to graft rejection via a chemotactic funciton[145]. Additionally, no significant changes were seen in the expression of chemokines between wild type and RIPK3$^{-/-}$ grafts. Nonetheless, both our *in vitro* and *in vivo* results further support the notion that necroptosis results in the release of danger molecule HMGB1 and augments graft rejection in transplantation. In contrast, apoptosis will result in decreased danger molecule release and minimize cell death-induced inflammatory responses in heart graft. Hence, programmed apoptotic cell death, rather than necroptotic cell death, may be beneficial for heart graft survival.

4.6 Cardiac allograft survival and immunosuppressive management

In clinical transplantation, proper immunosuppressive management is critical in preventing both acute and chronic rejection. Several immunosuppressive regimens currently exist in the clinic, many of which inhibit T cell survival, activation and clonal expansion. This outcome is achieved by targeting molecular pathways involved in T cell recognition of foreign antigens, costimulatory pathway activation and the production of IL-2. Clinically, drugs such as tacrolimus, sirolimus and thymoglobulin are used to prevent T cell proliferation, while costimulatory blockades have been successful in pre-clinical trials but have not yet been used clinically.

In this study, wild type C57BL/6 and RIPK3$^{-/-}$ heterotopic donor cardiac grafts were transplanted into recipient Balb/c mice followed by short-term sirolimus treatment. Using this model, we observed significant improvements in graft survival in both wild type and RIPK3$^{-/-}$ donor hearts compared with untreated grafts. Furthermore, we observed significant increases in graft survival time, cellular necrosis and lymphocyte inflammation in RIPK3$^{-/-}$ grafts compared with sirolimus treated wild type grafts (Figure 14A&B). This data provides evidence that the efficacy of immunosuppression is dependent on the pattern of cell death in the graft and the removal of immunogenic necroptosis benefits heart graft survival.

However, it is possible that the deletion of RIPK3 in donor grafts may affect other cell death pathways, such as apoptosis and autophagy. Indeed, we observed significant resistance of RIPK3$^{-/-}$ MVECs to apoptosis following hTNFα treatment (Figure 10). Furthermore, augmentation of cell death pathways may be further influenced by the chosen method of immunosuppression. Previous groups have reported the induction of cellular autophagy following sirolimus treatment in many mouse models[147-149]. The induction of autophagy by sirolimus could potentially benefit long-term graph survival, sparing the graft from apoptotic and immunogenic necroptotic death. Therefore, the beneficial effect of sirolimus in heart cells needs further investigation, despite only treating with sirolimus for 9 days after transplantation. Alternatively, we transplanted wild type and RIPK3$^{-/-}$ hearts into Balb/c recipients using anti-CD154 costimulatory blockade to inhibit T cell responses. A sub-therapeutic dose of 200ug anti-CD154 antibody was used since Zhang and colleagues have previously shown a 250ug single-

dose treatment of anti-CD154 induces a 40% acceptance rate after 100 days in wild type grafts[150]. However, RIPK3-/- grafts treated with 200ug of anti-CD154 showed no differences in survival or lymphocyte infiltration (Figure 15A&B). Future studies should use a 250ug dose to compare the 40% wild type acceptance rate to that of RIPK3$^{-/-}$ grafts.

These results indicate that the method of immunosuppression may be important during graft-targeted therapy. However, retrospective consideration of the tolerance inducing mechanism of our RIPK3$^{-/-}$ model versus that of anti-CD154 therapy leads us to believe that the tolerance inducing mechanism in each model is mutually exclusive. As mentioned previously, the CD40/CD154 interaction has important implications in plasma cell antibody type switching and absence of CD40 or inhibition of CD154 results in hyper-IgM syndrome[28]. As RIPK3 mediated necroptosis is initiated by TNFα or Fas binding to cellular receptors, disruption of immunoglobulin class switching should have no effect on the survival of RIPK3$^{-/-}$ grafts. Although CD40/CD154 is known to affect T cell activation, the mechanism is indirect via the upregulation of CD80 or CD86 on APCs and may not greatly affect TNFα production by CD4+ T cells. Furthermore, anti-CD154 treatment alone is known to induce only acute rejection protection, and not chronic rejection protection, in heart allograft models[30, 151]. Instead, anti-CD154 treatment is recommended to be used in conjunction with sirolimus to promote long-term tolerance and prevent chronic rejection[152]. In either model, robust cellular and humoral immune responses quickly cause cell death within the graft. Immunosuppressive therapies currently prevent early graft rejection, but chronic rejection still can occur through the slow and steady destruction of heart cells over time. Therefore, the RIPK1/RIPK3

mediated death pathway is more relevant in chronic, rather than acute, rejection. Future studies using treatment with CTLA4Ig, which directly blocks CD28 activation on T cells, may have be a more effective method of demonstrating the advantage of RIPK3$^{-/-}$ grafts. Nonetheless, anti-CD154 and CTLA4Ig treatment have not been successfully transferred into clinical therapy. This study may provide a sound rationale that using clinically relevant drugs such as sirolimus, tacrolimus, or thymoglobulin in conjunction with donor-specific anti-cell death therapy for future patient treatment.

4.7 Conclusion

In this study we have demonstrated that RIPK3 and TNFR1 expression on MVECs can be regulated by TNFα and IFNγ. We have shown for the first time in MVECs that caspase 8 plays a protective role against RIPK1 and RIPK3 mediated necroptosis as blockade of caspase 8 exacerbates, while inhibition of RIPK1 or RIPK3 abrogates, necroptotic death. We have also shown that danger molecule HMGB1 release correlates with necroptotic cell death in MVECs. Furthermore, following heterotopic heart transplantation, deletion of RIPK3 in donor hearts ameliorates necrotic death, inflammation and HMGB1 release in the donor graft, leading to prolonged survival. Inhibition of RIPK1 or RIPK3 in transplant patients may therefore protect the donor heart graft from necroptosis, decreasing graft immune infiltration and the release of proinflammatory danger signals that lead to chronic allograft injury.

4.8 Future direction

We recommend that future studies explore the clinical importance of necroptosis in transplantation through the use of clinically relevant immunosuppressive therapies such that this treatment may be brought from bench to bedside. This includes developing methods of graft specific targeting of proteins RIPK1 and RIPK3, perhaps during donor-to-recipient transfer, and determining the effect of RIPK1/RIPK3 inhibition on other forms of cell death following immunosuppression.

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Curriculum Vitae

Name: Alexander Pavlosky

Post-Secondary Education

2007 Ontario Secondary School Diploma
- Central Secondary School in London, Ontario

2011 Science and Business (Honours BSc)
- University of Waterloo

2014 Master of Science (MSc)
- Schulich School of Medicine and Dentistry, University of Western Ontario

Professional Positions

2007-2008 Owner – AP Marketing and Research

- Contracted by Siemens Healthcare, Canada
- Collected and compiled patient teaching cases involving Molecular Imaging Technology (SPECT/CT)
- Cases used for marketing and education
- Cases were submitted, accepted, and published online by the Molecular Imaging University, Chicago, Illinois

2011 Tutor
- Tutored students taking nursing pathology at Western University, Ontario
- Hosted individual and group teaching sessions (3-5

students)
- Organised and hosted mid-term and final exam review sessions (up to 70 students)

Academic Distinctions/ Honours:

2004-2007	Ontario Secondary School Honour Role
2007	Graduated as an Ontario Scholar
2007	University of Waterloo Faculty of Science Entrance Scholarship
2007-2011	University of Waterloo Dean's Honour List
2011	Western Graduate Research Scholarship, Western University, Ontario
2011	Schulich Graduate Scholarship, Schulich School of Medicine and Dentistry, Western University, Ontario
2012	Poster Presentation Award for a Graduate Students, Department of Pathology, Western University, Ontario
2012	Western Graduate Research Scholarship, Western University, Ontario
2012	Schulich Graduate Scholarship, Schulich School of Medicine and Dentistry, Western University, Ontario

2013 "Best Basic/Clinical Science Collaborative Poster Presentation", Department of Pathology, Western University, Ontario. Awarded for best multi-departmental and group collaboration

2014 "Young Innovator Award", Cutting Edge of Transplantation 2014, Chandler, Arizona USD500

2014 Research FITS for top 5 basic science abstracts, Canadian Society of Transplantation Annual Meeting, Montreal, Quebec

2014 "Young Investigator Award", World Transplant Congress Annual Meeting, San Francisco, California

Presentations

2012 "The Role of Receptor Activating Protein 3 in Cardiac Allograft Transplantation", London Health Research Day, London Ontario

2012 "The Role of Receptor Activating Protein 3 in Cardiac Allograft Transplantation", Department of Pathology Research Day, London, Ontario

2012 "The Role of Receptor Activating Protein 3 in Cardiac Allograft Transplantation", Department of Medicine Research Day, London Health Sciences Center, London, Ontario

2013	Top 12 basic science abstracts for oral plenary presentation. "RIPK3 regulates microvascular endothelial cell necroptosis and cardiac allograft rejection", Canadian Society of Transplantation Annual Meeting, Lake Louise, Alberta
2013	"RIPK3 regulates microvascular endothelial cell necroptosis and cardiac allograft rejection" Selected for poster presentation at the American Society of Transplantation Annual Meeting, Seattle, WA
2013	"RIPK3 regulates microvascular endothelial cell necroptosis and cardiac allograft rejection", London Health Research Day, London, Ontario
2013	"RIPK3 regulates microvascular endothelial cell necroptosis and cardiac allograft rejection", Department of Pathology Research Day, London, Ontario
2013	"RIPK3 regulates microvascular endothelial cell necroptosis and cardiac allograft rejection", Department of Medicine Research Day, London Health Sciences Center, London, Ontario
2014	"RIPK3 regulates microvascular endothelial cell necroptosis and cardiac allograft rejection", Cutting Edge of Transplantion 2014, Chandler, Arizona

2014	"RIPK3 regulates microvascular endothelial cell necroptosis and cardiac allograft rejection", Canadian Society of Transplantation Annual Meeting 2014, Montreal, Quebec
2014	"RIPK3 regulates microvascular endothelial cell necroptosis and cardiac allograft rejection", World Transplant Congress, San Francisco, California

Research / Publications

2014	Lau A et al. *RIPK3 mediated necroptosis promotes donor kidney inflammatory injury and reduces allograft survival*, American Journal of Transplantation.
In Press, 2014	Pavlosky A et al. *RIPK3 regulates microvascular endothelial cell necroptosis and cardiac allograft rejection*, American Journal of Transplantation.
In Press, 2014	Lau et al. *SPI-6 inhibits granzyme B mediated injury of renal tubular cells and promotes renal allograft survival*, Transplantation.