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## CD4+ T-cell Mediated Microvascular Endothelial Cell Death and Chronic Cardiac Allograft Rejection Involves Necroptosis

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Graduate Program in Pathology  
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science  
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«CD4<sup>+</sup> T-cell Mediated Microvascular Endothelial Cell Death and Chronic Cardiac  
Allograft Rejection Involves Necroptosis»

(Thesis format: Monograph)

by

Cecilia Kwok

Graduate Program in Pathology

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

The School of Graduate and Postdoctoral Studies  
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## Abstract

Heart transplantation is the only viable option for patients with end-stage heart failure. Despite advances in immunosuppressive therapies, the rate of transplanted graft loss remains substantial. Graft loss is primarily due to tissue damage mediated by immune responses. Cell death and organ rejection can occur as an active molecular process through apoptotic and necrotic pathways. We now recognize that cell death may also ensue through a newly described form of programmed necrotic cell death, termed necroptosis that involves receptor-interacting protein kinase (RIPK) 1/3. In this study, I aim to establish the role of RIPK3 in T cell-mediated chronic cardiac allograft rejection using the single MHC class II mismatch [C57BL/6 (H-2<sup>b</sup>; B6) or B6.129R1-RIPK3<sup>tm1Vmd</sup> (H-2<sup>b</sup>; RIPK3<sup>-/-</sup>) to B6.C-H-2<sup>Bm12</sup> (H2-Ab1<sup>bm12</sup>; bm12)] transplantation model.

My studies show that allo-reactive CD4<sup>+</sup> T-cells produce tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and express Fas ligand (FasL). My results also show that CD4<sup>+</sup> T-cell-mediated heart graft rejection is reduced in RIPK3 deficient donor grafts with reduced cellular infiltration and vasculopathy. TNF- $\alpha$ -mediated necroptosis was triggered *in vitro* with caspase 8 inhibition in B6 but not in RIPK3<sup>-/-</sup> endothelial cells. RIPK3<sup>-/-</sup> endothelial cells were resistant to CD4<sup>+</sup> T-cell induced cell death via mechanisms involving granzyme B and FasL.

In conclusion, cytotoxic CD4<sup>+</sup> T-cell-mediated endothelial cell death is dependent on TNF- $\alpha$ , and may be regulated by FasL and granzyme B. Loss of RIPK3 attenuates allo-immune responses, however, injury is not eliminated in a single MHC class II mismatch chronic rejection model.

## Keywords

Cardiac transplantation, RIPK3, necroptosis, CD4<sup>+</sup> T-cells, microvascular endothelial cells

## Co-Authorship Statement

The following individuals contributed to this thesis.

Xu-Yan Huang	Generation of stable cell lines
Ziqin Yin	Animal care
Weihua Liu	Immunostaining
Jifu Jiang	Animal surgeries and post-op care
Dameng Liang	Animal surgeries and post-op care
Alex Pavlosky	Data interpretation
Aaron Haig	Histological analyses
Anthony M. Jevnikar	Study design, data interpretation, critical evaluation of thesis; grant funding, biosafety and ethics approvals
Zhu-Xu Zhang	Study design, data interpretation, critical evaluation of thesis; grant funding, biosafety and ethics approvals



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# Table of Contents

Abstract.....	ii
Co-Authorship Statement .....	iii
Acknowledgments .....	iv
Table of Contents.....	v
List of Figures.....	viii
List of Appendices.....	ix
List of Abbreviations .....	x
Chapter 1.....	1
1 Introduction.....	1
1.1 Cardiac allograft transplantation .....	2
1.1.1 Epidemiology.....	2
1.1.2 Guideline Considerations for Heart Transplantation.....	2
1.1.3 Graft rejection after transplantation.....	3
1.1.4 Current immunosuppression therapies .....	5
1.2 Transplant Immunology .....	6
1.2.1 Innate and adaptive immunity .....	7
1.2.2 Mechanism of antigen-induced T-cell activation .....	7
1.2.3 Subsets of T-cells involved in chronic allograft rejection.....	11
1.3 Mechanisms of cell death .....	15
1.3.1 Apoptosis and necrosis .....	15
1.3.2 Regulated necrosis: necroptosis.....	17
1.3.3 Molecular Pathway after Death Receptor Signaling .....	17
1.3.4 Granule-mediated cell death.....	21
1.4 Rationale and Hypothesis .....	23

1.4.1	Rationale.....	23
1.4.2	Hypothesis .....	23
1.4.3	Objectives .....	23
Chapter 2.....		24
2	Materials and Methods .....	24
2.1	Animals.....	24
2.2	Microvascular Endothelial Cell Culture.....	24
2.3	T-Cell purification and MLR stimulation.....	24
2.4	Antibodies.....	25
2.5	Heterotopic heart transplantation and post-operative monitoring .....	25
2.6	Histology and Immunohistochemistry.....	25
2.7	Cell death measurement.....	26
2.7.1	Cytokine-induced MVEC death .....	26
2.7.2	CD4 <sup>+</sup> T-cell-induced MVEC death .....	27
2.8	Immunoblot Analyses.....	27
2.9	Statistical Analyses.....	28
Chapter 3.....		29
3	Results.....	29
3.1	RIPK3 deficiency protect donor heart graft from T-cell-mediated rejection <i>in vivo</i> .....	29
3.1.1	Graft Survival .....	29
3.1.2	Measures of Graft Rejection.....	29
3.2	Allo-reactive CD4 <sup>+</sup> T cells express TNF- $\alpha$ , IFN- $\gamma$ , FasL, granzyme B, and perforin .....	32
3.3	RIPK3 deficiency in endothelial cells is protective against <i>in vitro</i> TNF- $\alpha$ -mediated death and release of cDAMPs.....	34
3.5	CD4 <sup>+</sup> T-cell induce apoptosis and necroptosis in target MVEC .....	39

3.6 CD4 <sup>+</sup> T-cell induced death are mediated by both FasL-Fas and granzyme B/perforin interactions.....	44
3.7 Summary of Results.....	48
Chapter 4.....	49
4 Discussion.....	49
4.1 Heart Transplantation Model.....	49
4.2 Mechanisms of CD4 <sup>+</sup> T-cell-mediated EC death .....	51
4.3 Conclusions .....	53
4.4 Future Directions .....	53
References .....	55
Appendices .....	77
Curriculum Vitae .....	78

## List of Figures

Figure 1. Activation of CD4 <sup>+</sup> T-cells require induction and costimulatory molecules .....	10
Figure 2. Death receptor signaling lead to apoptosis and necroptosis.....	20
Figure 3. Cytotoxic CD4 <sup>+</sup> T-cell interactions with an endothelial cell. ....	22
Figure 4. Heart grafts deficient in RIPK3 attenuates graft rejection. ....	31
Figure 5. Allo-activated CD4 <sup>+</sup> T-cells have cytokine producing and cytotoxic phenotype. ....	33
Figure 6. RIPK3 deficient MVEC are protected from cytokine-induced cell death. ....	37
Figure 7. RIPK3 deficient MVEC are resistant to cytokine-induced release of cDAMPs.....	38
Figure 8. Characterizing surface proteins of mouse interferon-gamma treated MVEC.....	40
Figure 9. CD4 <sup>+</sup> T-cells lyse B6 MVEC <i>in vitro</i> . ....	42
Figure 10. B6 MVEC are more sensitive than RIPK3 <sup>-/-</sup> MVEC to alloactivated CD4 <sup>+</sup> T-cell-induced death <i>in vitro</i> . ....	43
Figure 11. Allo-activated CD4 <sup>+</sup> T-cells use Fas-FasL and granzyme B as cytolytic mechanisms against MVEC.....	47

## List of Appendices

Appendix A: Animal Protocol (2007-096-10) Approval .....	77
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## List of Abbreviations

ACR	Acute cellular rejection
AMR	Antibody mediated rejection
Apaf-1	Apoptotic protease activating factor-1
APC	Antigen presenting cell
BID	BH3 interacting-domain death agonist
cDAMPs	Cellular danger associated molecular patterns
cFLIP	Cellular FLICE-inhibitory protein
cIAP	Cellular inhibitors of apoptosis
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CYLD	Cylindromatosis
DC	Dendritic cell
DTH	Delayed Type Hypersensitivity
EC	Endothelial cell
FADD	Fas-associated death domain
FLICE	FADD-like IL-1 $\beta$ -converting enzyme
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1
HSP	Heat shock protein

ICAD	Inhibitor of caspase-activated DNase
ICAM-1	Intracellular adhesion molecule-1
ICOS-L	Inducible costimulator ligand
IFN- $\gamma$	Interferon gamma
IL-1	Interleukin-1
IRI	Ischemia reperfusion injury
ISHLT	International society of heart and lung transplantation
LFA	Lymphocyte function-associated antigen
MAPK	Mitogen-activated protein kinase
mAB	Monoclonal antibody
MLKL	Mixed lineage kinase domain-like protein
MLR	Mixed lymphocyte reaction
MHC	Major histocompatibility complex
MVEC	Microvascular endothelial cell
NEMO	NF- $\kappa$ B essential modulator
PCD	Programmed cell death
PD-L1	Programmed death-ligand 1
RIPK	Receptor interacting protein kinase
ROS	Reactive oxygen species
SMC	Smooth muscle cell



TCR	T-cell receptor
TLR	Toll-like receptor
Th	T-helper cell
ThCTL	Cytotoxic T-helper cell
Treg	T-regulatory cell
Tmem	T-memory cell
TNF- $\alpha$	Tumour necrosis factor alpha
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor associated factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very Late Antigen-4

## Chapter 1

### 1 Introduction

Heart transplantation is the standard treatment for heart failure. Despite recent advances in immunosuppression and improvement in survival of acute cardiac allograft rejection, development of cardiac allograft vasculopathy and chronic rejection remains one of the top causes of death for heart transplant recipients.<sup>1</sup> Chronic allograft vasculopathy (CAV) affects 8%, 30%, and 50% of patients surviving to 1, 5, and 10 years respectively.<sup>1</sup> Furthermore, organ donation rates in Canada are lower than other countries and more than 4000 Canadians are on the wait list for organ donation.<sup>2</sup> In addition, the number of donated organs are plateauing in recent years.<sup>1,3</sup> To compound the issue, CAV is one of the most common reasons for retransplantation.<sup>1</sup> Therefore, strategies are needed to lengthen donor graft survival and improve clinical outcomes.

## 1.1 Cardiac allograft transplantation

### 1.1.1 Epidemiology

Globally, 4,196 adult and pediatric heart transplants were performed in 2012 and reported to The Registry of the International Society for Heart and Lung Transplantation (ISHLT). As of 2014, the Organ Procurement and Transplantation Network reported that 233 heart transplants were conducted in the United States<sup>4</sup> and in 2012, the Canadian Institute for Health Information reported that 129 heart transplant procedures took place in Canada.<sup>5</sup> The number of reported heart transplants has remained stable for several years since 2004 and now appears to be slowly increasing, particularly in North America and Europe.<sup>1</sup> The ISHLT reported that of a total of 104,000 heart transplant patients worldwide, the median survival was 10.9 years during 1993–2003.<sup>6</sup> The leading causes of death for all transplant recipients in early years after their transplants are graft failure, infection, and multiple organ failure.<sup>1</sup> Infections often develop as a result of aggressive immunotherapeutic medicines weakening the immune system. At 3-5 years, malignancy, cardiac allograft vasculopathy (CAV), and renal failure are the leading causes of death.<sup>1</sup>

### 1.1.2 Guideline Considerations for Heart Transplantation

Time on the wait list, geographic proximity to the donor and transplant center, urgency, and immune sensitization are important when considering the allocation of a donor heart to a specific patient. Typically, women who have had more than one child or individuals who have had multiple blood transfusions are allo-sensitized with anti-human leukocyte antigen (HLA) antibodies that would lead to a higher chance of a positive cross-match with donors. Guidelines produced by the Canadian Cardiovascular Society<sup>7</sup> and the Heart Failure Society of America<sup>8</sup> suggest that patients with severe heart failure, refractory angina, or ventricular arrhythmias that cannot be controlled with pharmacological methods, mechanical device or alternative surgery, to be evaluated for cardiac transplantation. However, patients with systemic disease (i.e. active malignancy,<sup>9</sup> infection,<sup>10</sup> or pulmonary hypertension) are absolute contraindications for heart transplantation.<sup>11</sup> Recently, the Canadian Society of Transplantation and the Canadian

National Transplant Research Program increased risk donor working group published recommendations on the increased risk of infectious organs for donation.<sup>12</sup>

### 1.1.3 Graft rejection after transplantation

Primary graft failure accounts for significant mortality in the first 30 days after transplantation, is greatly affected by donor-related factors, and is mostly due to under-immunosuppression.<sup>13</sup> Depending on when rejection occurs, transplant recipients suffer from three types of rejection: hyperacute, acute, and chronic. Hyperacute rejection happens within minutes to hours of the transplantation and is rare because of recent advances (i.e. HLA typing) to avoid donor-recipient cross-matching and thus will not be further discussed.

#### 1.1.3.1 Acute cardiac allograft rejection

Acute rejection can be classified as either cellular (ACR) or humoral (antibody-mediated; AMR) rejection and usually occurs early after transplantation. Within the first three years after transplantation, cellular and antibody-mediated acute rejection accounts for approximately 10% of deaths.<sup>1</sup> In the first year post-transplant, 19% of all heart transplant patients reported in the ISHLT registry experienced at least one acute rejection episode that required treatment within the first year post-transplantation.<sup>14</sup>

ACR is mainly caused by inflammation and myocardial injury induced by T-cells<sup>15,16</sup> while humoral immunity (AMR) involves vascular rejection where antibodies activate the complement system which leads to vessel damage and graft failure.<sup>17</sup> Severity of ACR and AMR are based on histopathologic and immunopathologic findings from biopsies graded according to ISHLT guidelines.<sup>18-20</sup> It is proposed that inflammation and endothelial cell damage as a result of acute rejection is a risk factor for CAV and appears to increase the risk by almost tenfold.<sup>21</sup>

#### 1.1.3.2 Chronic cardiac allograft rejection

Chronic cardiac allograft rejection manifests as a type of coronary atherosclerosis or CAV. It is among the leading cause of death post-transplant and is responsible for 32% of all patient deaths five years post-transplantation.<sup>1</sup> CAV is characterized by the

proliferation of vascular smooth muscle cells in the donor heart, concentric intimal thickening, and diffuse coronary artery lumen narrowing.<sup>22</sup> The elastic lamina is usually intact but may be disrupted in severe cases.<sup>21</sup> This is in contrast to the focal, eccentric proliferation of coronary vessel intima with disruption of the elastic lamina and presence of fatty streaks and calcium deposition that is present in coronary artery disease.<sup>23</sup> As CAV development is usually a diffuse process, conventional revascularization procedures are usually not possible. The arteries and arterioles of the donor heart are mainly affected, leading to progressive graft dysfunction and can result in cardiac arrhythmia, ventricular dysfunction, ischemia, graft failure, and death. Sudden death may occur possibly due to rapidly-developing pump failure.<sup>24</sup> Angina is usually not a symptom, as the heart is denervated. CAV is routinely detected by coronary angiography and the only treatment is retransplantation.

Although the pathogenesis of CAV is not completely understood, immunological responses affected by non-immunological events such as hyperlipidemia and insulin risk factors are believed to be involved.<sup>25</sup> Some of these immunological responses include ischemia–reperfusion injury (IRI),<sup>26-29</sup> episodes of acute rejection,<sup>30-34</sup> histocompatibility mismatch, viral infection, donor brain death, and chronic inflammation. Direct activation of recipient CD4<sup>+</sup> T-cells by donor allograft major histocompatibility complex [MHC; or HLA in humans] class II or CD8<sup>+</sup> T cells by MHC class I lead to further production of cytokines, which worsen injury.<sup>21</sup>

Vascular disruption is a complex process and may involve innate immunity, B-cells and CD4<sup>+</sup> T-cells in the grafted tissue.<sup>35</sup> Briefly, endothelial cells (EC) bearing foreign MHC Class II are targeted by the host immune system and subsequent EC damage further leads to elaboration and release of cytokines and chemokines.<sup>36,37</sup> These activate innate immune cells including macrophages,<sup>35</sup> NK cells,<sup>38</sup> and neutrophils.<sup>39</sup> In addition, macrophages secrete interleukin (IL)-1, IL-6, platelet-derived growth factor (PDGF) and TNF- $\alpha$  in response to interferon- $\gamma$  (IFN- $\gamma$ ). IFN- $\gamma$  is necessary for CAV development but not for parenchymal rejection.<sup>36</sup> IL-1 and TNF- $\alpha$  further promote surrounding cells to secrete IL-1, promoting smooth muscle cell (SMC) migration and proliferation.<sup>36,40-42</sup> Released high-mobility group box-1 (HMGB1)<sup>43</sup> and IL-2 signal to CD4<sup>+</sup> T-cells to

migrate into the lesion that further stimulates B-cell proliferation. T-cells play the integral role of increased extracellular matrix (ECM) deposition, which ultimately results in severe intimal thickening and graft dysfunction.

#### 1.1.4 Current immunosuppression therapies

Immunosuppression is used to treat graft rejection while steps are taken to minimize risks of drug toxicities, malignancy, and infections. Immunosuppressive protocols can be classified as induction therapy (aggressive immunosuppression early after transplantation to prevent hyper-acute and acute rejection), or maintenance therapy (life-long drug protocol to prevent chronic rejection).

##### 1.1.4.1 Induction Therapy

Approximately 47% of all patients who received a heart transplant in 2012 received induction immunosuppression.<sup>14</sup> Yet, the advantage of induction therapy is debatable. A recent meta-analysis reviewed 22 randomized controlled trials and reported no significant differences in outcomes (mortality, CAV, infection, malignancy and renal function) in recipients after heart transplantation.<sup>44</sup> Hence, more resources are needed to explore the potential benefits of induction therapy.

During the early postoperative period, antibodies targeting T-cells are used to provide augmented immunosuppression when risk of graft rejection is highest. It follows that powerful immunosuppression soon after transplantation can prevent early rejection events. Induction anti-bodies can be polyclonal and monoclonal which mediate T-cell depletion in peripheral blood, lymph nodes, and spleen by complement-dependent lysis and apoptosis. Induction immunosuppression currently prescribed to patients after heart transplant are anti-thymocyte globulins,<sup>45</sup> anti-CD52,<sup>46</sup> and IL-2 receptor antagonists.<sup>47</sup>

### 1.1.4.2 Maintenance Therapy

Most maintenance immunosuppressive protocols consist of a combination of three drugs: calcineurin-inhibitor, an antimetabolite agent, and corticosteroids. Calcineurin-inhibitors (cyclosporine or tacrolimus) are effective at inhibiting calcineurin and reduce T-cell activation and proliferation by acting on transcription factors involved with production of IL-2, TNF- $\alpha$ , IFN- $\gamma$ , IFN $\beta$  and granulocyte-macrophage colony stimulating factor. However, calcineurin inhibitors have undesirable nephrotoxic side effects. Antimetabolites are antiproliferative agents (sirolimus, mycophenolate mofetil or azathioprine) that inhibit the production of nucleic acids, also reducing the proliferation of T and B-cells. Mammalian target of rapamycin (mTOR) inhibitors (everolimus and sirolimus), proliferation signal inhibitors, may also be used to inhibit T and B-cell proliferation and differentiation in selected patients to slow progression of renal insufficiency and CAV. However, its use is limited due to the high incidence of adverse effects such as delayed wound healing.<sup>48</sup> Corticosteroids are nonspecific anti-inflammatory drugs that primarily inhibit two transcription factors [activator protein-1 and nuclear factor- $\kappa$ B (NF $\kappa$ B)] in lymphocytes<sup>49</sup> and are used in tapering doses as they are associated with numerous adverse effects. These side effects include induction of diabetes mellitus, hyperlipidemia, hypertension, myopathy, osteoporosis, and predisposition towards opportunistic infections.

## 1.2 Transplant Immunology

Both the innate and adaptive immune systems are involved in a dynamic and complex interplay that begins with endothelial injury and inflammation, which further perpetuates CAV development. Understanding of mechanisms of T-cell stimulation, activation and proliferation and their interactions with target cells are crucial to the concepts of graft rejection. Furthermore, EC are the “first barrier cells” and play a critical role as targets of T-cell-mediated rejection and it has been suggested that alloantigen-dependent mechanisms act to intensify initial non-immune damage to EC.<sup>50</sup>

### 1.2.1 Innate and adaptive immunity

The innate immune system is composed of cellular and non-cellular elements, which respond immediately and nonspecifically to microbes at the site of infection. The cellular components of innate immunity consist of neutrophils, macrophages, dendritic cells (DC), and natural killer (NK) cells.<sup>51</sup> The molecular components include toll-like receptors (TLR), complement proteins, chemokines, and cytokines among others.<sup>52-54</sup> As a result of transplant injury, innate components (complement activation and TLR stimulation)<sup>55</sup> are triggered, stimulate adaptive immunity and contribute to delayed graft functions. In contrast, the adaptive immune system is composed of antigen-specific immune response against a recognized foreign antibody and result in memory. The adaptive system is further divided into humoral immunity and cell-mediated immunity, which consist of antibody secreting B-cells and cytotoxic/helper T-cells respectively. When stimulated, these cells will activate and proliferate, to eliminate the foreign antigen. The specificity and strength of the adaptive immune response are the most dangerous factors to affect the transplanted organ.

### 1.2.2 Mechanism of antigen-induced T-cell activation

T lymphocytes recognize antigens via direct and indirect pathways. The direct pathway involves recognition of intact foreign MHC antigens on the surface of donor antigen presenting cells (APC), whereas, the indirect pathway involves processed antigens presented as peptides bound to MHC class II molecules on the surface of host APC.

Upon foreign antigen recognition, T-cells activate, proliferate, and differentiate in response and become effector cells. The process begins with T-cell recognition of a foreign peptide on APCs. APC can be divided into two classes; professional and non-professional. Professional APC (i.e. dendritic cells, B cells, macrophages) continuously presents foreign peptides on MHC Class II that is recognized by CD4<sup>+</sup> T-cells. Non-professional APC (i.e. any nucleated cell) are able to present material on MHC Class I that is recognized by CD8<sup>+</sup> T cells. Most somatic cells do not express MHC Class II under normal physiological conditions. However, during inflammation MHC Class II are induced, such is the case of human and porcine microvascular ECs.<sup>56</sup> Professional APC



endocytose foreign, exogenous material into early endosomes, which, when acidified, activates proteases to cleave into small peptides and are loaded onto MHC class II and shuttled to the cell surface. CD4<sup>+</sup> T-cells with surface heterodimeric T-cell receptor (TCR) complex specific to those MHC Class II molecules can bind to the APC and result in T-cell activation and proliferation.

T-cell activation by antigens require two signals: (1) initial binding of the TCR complex with the alloantigen-bound MHC molecule on the surface of APC; (2) binding of other costimulatory molecules (signaling or adhesion molecules). If costimulatory signals are not present, the T-cell either differentiates into regulatory T-cell, is deleted, or becomes anergic, where they fail to respond to their specific antigen and are ineffective at mounting a response (tolerance). The activation of a CD4<sup>+</sup> T-cell is summarized in Figure 1.

#### 1.2.2.1 Signal 1: Induction

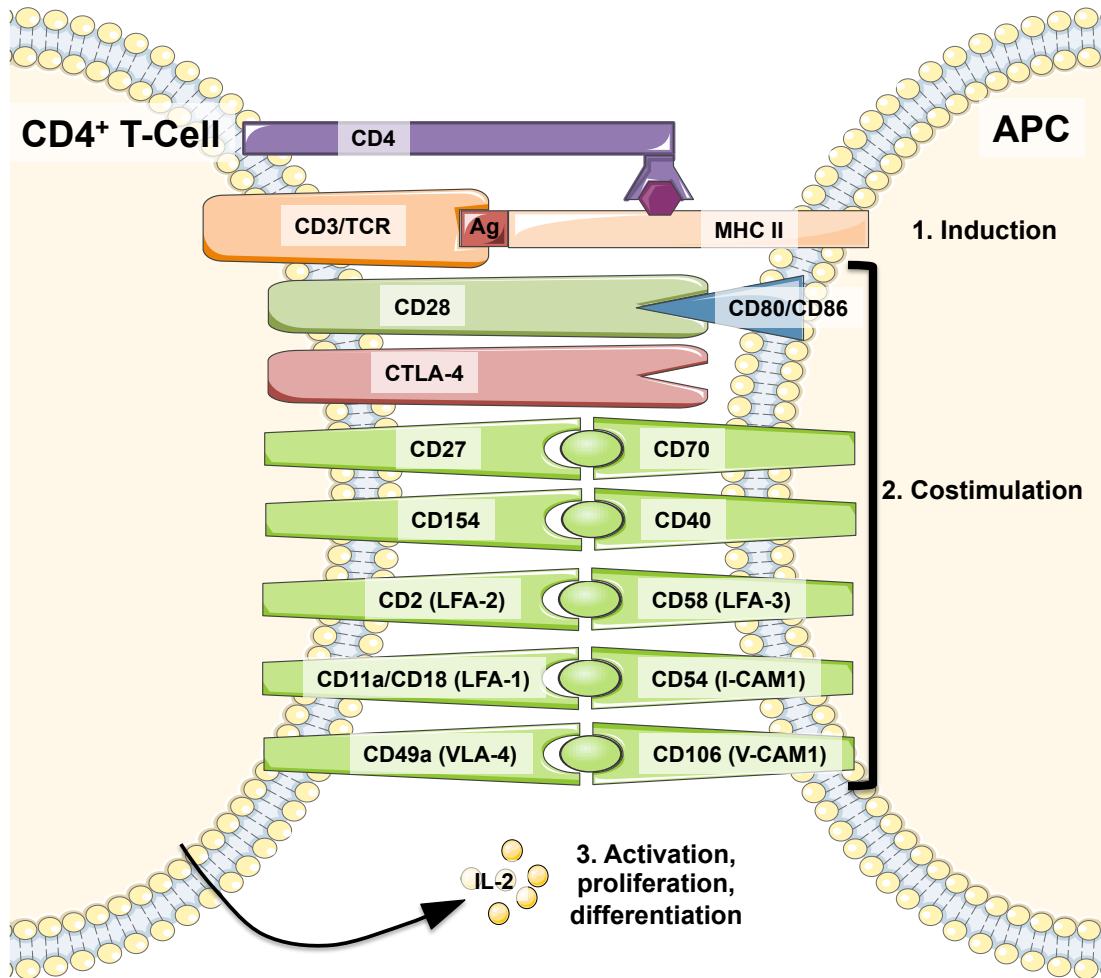
Upon recognizing the specific antigen, the first signal is transmitted when the TCR-CD3 complex binds strongly to the peptide-MHC complex on the APC surface. The CD4<sup>+</sup> molecule on the Th cell (co-receptor of the TCR complex) also binds to the MHC molecule. Interaction of LFA-1 on the T cell and ICAM on the APC brings the cells closer together, facilitating TCR kinase activity and activation of CD3 via phosphorylation.

#### 1.2.2.2 Signal 2: Costimulation

The second signal is critical to T-cell activation and gives it effector abilities to respond to antigens. The two most important signals are provided by CD28 binding to CD80 and CD86 (B7-1, B7-2) and CD154 (CD40-ligand) binding to CD40.<sup>57,58</sup> CD154 also activates APC, which in turn provides signals (i.e. B7 molecules) that costimulate T-cells. Other stimulatory signals include CD11a/CD18 (LFA-1), CD2 (LFA-2), CD49a (VLA-4), and CD27 of the T-cell that bind to CD54, CD58 (LFA-3), CD106 (VCAM-1), and CD70 on the APC<sup>56</sup> respectively (Figure 1). CD28 is the only constitutively expressed costimulation surface molecule and B7 molecules are the only major costimulatory molecule in mice.<sup>59</sup>

CTLA-4 is an inhibitory ligand that can also bind to CD80 and CD86 on APCs to prevent costimulation and inflammation.<sup>60,61</sup> Recently, immunosuppressive agents that use CTLA-4-Ig fusion protein to interrupt CTL-A-CD80, CD86 (and CD28) interactions has been promising at reducing immune inflammation *in vivo*.<sup>60</sup>

Successful costimulation of the T cells result in IL-2 production and subsequent T-cell proliferation and differentiation into an effector cell. Primed T-cells follow chemokines and migrate to sites of inflammation. Recognition of foreign antigen on the MHC complex lead to effector cell release of proinflammatory cytokines (IFN- $\gamma$ ,<sup>36</sup> IL-17,<sup>62</sup> IL-4,<sup>63</sup> IL-18)<sup>64</sup> and also produce granzyme B, perforin or FasL and that lead to death of the APC, infected, and/or allogeneic cell.<sup>65</sup> This results in development of highly specific memory that upon subsequent encounters with a given antigen, the T-cell is re-activated to prevent reinfection.



**Figure 1. Activation of CD4<sup>+</sup> T-cells require induction and costimulatory molecules**

Recognition of antigen mounted on the MHC Class II by the TCR-CD3 complex is the first induction signal of T-cell stimulation. The CD4<sup>+</sup> molecule on the Th cell (co-receptor of the TCR complex) also binds to the MHC molecule. Costimulatory molecules on the Th cell CD28, CD27, CD154, CD2 (LFA-2), CD11a/CD18 (LFA-1), CD49a (VLA-4) interact with CD80/CD86, CD70, CD40, CD58 (LFA-3), CD54 (I-CAM1) and CD106 (V-CAM1) on the APC respectively. CTLA-4 is an inhibitory ligand that can also bind to CD80/CD86, preventing co-stimulation.

### 1.2.3 Subsets of T-cells involved in chronic allograft rejection

Pivotal experiments done with the carotid transplantation model in various strains of mice showed that CAV development is independent of CD8<sup>+</sup> T-cells and NK cells, but rather, involves interactions between CD4<sup>+</sup> T-cells, B cells, and macrophages<sup>66-68</sup>. Furthermore, a reduction in SMC proliferation and migration observed in mice that lacked CD4<sup>+</sup> T-cells was most probably due to an interruption in the inflammation cytokine signaling cascade. Therefore, critical signaling that promotes B-cell proliferation, antibody production, macrophage activation,<sup>35,69</sup> and secretion of TNF- $\alpha$  or IFN- $\gamma$ <sup>36,70</sup> is hindered, resulting in a dampened intimal proliferative response.

However, others reported that CD8<sup>+</sup> T-cell depletion prevents development of intimal proliferation in swine and suggested that MHC class I antigens may play an important role in the early pathogenesis of CAV.<sup>71</sup> Further, class I-disparate, cyclosporine A-treated hosts became tolerant to heart grafts from the same donors and survived for a long period.<sup>72</sup> These findings are inconsistent with models of mouse and rat heart allograft transplantation, and may be due to delays in immune response to antibody depletion of CD8<sup>+</sup> cells or because of the fact that swine and humans constitutively express MHC Class II but mice and rats do not. Using murine recipients deficient in CD8<sup>+</sup> T-cells, Fischbein and colleagues found that CAV was greatly reduced though not abrogated and suggested that CD4<sup>+</sup> and CD8<sup>+</sup> T-cells work in concert; such that CD8<sup>+</sup> lymphocyte allo-activation is dependent on activated CD4<sup>+</sup> lymphocytes and their participation in the development of intimal lesions is dependent on CD4<sup>+</sup> allo-activation.<sup>42,73</sup> CD8<sup>+</sup> T-cells can be activated by exogenous antigens via cross-presentation by dendritic cells<sup>74</sup> and participate in chronic rejection either directly by cytolytic effects against target cells or indirectly by releasing IFN- $\gamma$ ,<sup>75</sup> yet on its own do not secrete enough IL-2 and IFN- $\gamma$  to induce chronic rejection. Further, it was proposed that since CD8<sup>+</sup> T-cells could be stimulated via other costimulatory ligands (ICOS, ICAM-1, 4-1BB), costimulatory blockade for CD28 and CD40 ligand might not be a sufficient immunosuppressive therapy on their own.<sup>73</sup>

Nevertheless, it is indisputable that both direct and indirect recognition by CD4<sup>+</sup> T-cells play a main role in eliciting a hostile immunogenic response to foreign antigens.

### 1.2.3.1 Cytotoxic CD4<sup>+</sup> T-cells are developed from chronic antigen stimulation

MHC Class II restricted effector CD4<sup>+</sup> T-cells play a central role in immunological homeostasis. This is primarily achieved by providing critical activation or inhibitory signals to other immune cells to induce or dampen immunological responses. During activation, CD4<sup>+</sup> cells are directed by the cytokines in the microenvironment into specific T helper (Th) cells. It is well established that naïve CD4<sup>+</sup> T-cells can mature into several distinctive subtypes including Th1 cells (transcription factor: T-bet) which produce IFN- $\gamma$  and are pivotal in driving cellular reactions; Th2 cells (transcription factor: GATA-3) which secrete IL-4 and are responsible for mediating humoral/antibody responses; Th17 cells (transcription factor: ROR $\gamma$ t) which secrete IL-17 and activate neutrophils; Foxp3 expressing CD4<sup>+</sup> T-cells (also known as regulatory cells or Treg) which have the unique role of dampening the immune response by restricting clonal expansion of effector CD4<sup>+</sup> T-cells.

In addition, developments in viral immunity studies have suggested that there is a small subset of CD4<sup>+</sup> T-cells that may attain cytolytic abilities and can directly kill MHC Class II expressing infected or allogeneic cells.<sup>76</sup> These cytotoxic CD4<sup>+</sup> T-cells (ThCTL) have been identified in humans after chronic viral infections including human cytomegalovirus,<sup>77-79</sup> hepatitis,<sup>80</sup> Epstein-Barr Virus,<sup>81</sup> human immunodeficiency virus,<sup>82,83</sup> influenza,<sup>84</sup> and in mice after infection with mouse small pox virus,<sup>85</sup> lymphocytic choriomeningitis virus,<sup>86</sup> and gamma herpes virus.<sup>87</sup> Furthermore, numbers of ThCTL are found to be increased in humans with autoimmune disorders and vascular diseases.<sup>88,89</sup> It is interesting to note that ThCTL functions are mostly restricted to MHC Class II bearing APCs and non-antigen presenting cells in humans<sup>77,90</sup> and mice.<sup>91</sup> ThCTL have also been shown to induce endothelial cell death following cytomegalovirus infection.<sup>90</sup>

The exact purpose of ThCTL is unknown, however, it has been postulated that they can play an immunogenic role in chronic inflammatory conditions such as auto-immune diseases, vascular, and inflammatory bowel disease,<sup>88,92,93</sup> as numbers of cytotoxic CD4<sup>+</sup> T cells are elevated. It has also been suggested that it is a compensation for virally-inhibited MHC Class I expression and their ability to evade CD8<sup>+</sup> cells<sup>94</sup> as well as age-dependent decline of CD8<sup>+</sup> CTL activity.<sup>76</sup>

ThCTL are described to be “Th1-like”, potent secretors of IFN- $\gamma$ , TNF- $\alpha$ , and IL-2.<sup>76</sup> ThCTL are currently identified by the surface expression of markers of degranulation [CD107a (LAMP1) and CD107b (LAMP2)], and co-expression of perforin and granzyme B. They lose costimulatory marker CD28, which is consistent with all differentiated T-cells, however, phenotype of ThCTL is generally varied depending on the organ in which they function.<sup>76</sup> Mechanisms of ThCTL-mediated cell toxicity are similar to CD8<sup>+</sup> CTLs and include Fas-FasL-mediated apoptosis, granule (granzyme and perforin)–mediated apoptosis, and non-contact forms of cell death such as TNF-mediated apoptosis. It is reported that ThCTL mainly use granules as its effector mechanism,<sup>95</sup> however, unlike CTL CD8<sup>+</sup> cells that can express perforin/granzyme with just IL-2 stimulation, ThCTL require activation through TCR to induce perforin expression,<sup>96,97</sup> and up-regulation of granzyme B.<sup>98</sup>

It is also well established that CD4<sup>+</sup> T-cells can exhibit cytotoxicity in acute rejection<sup>99-103</sup> by direct recognition of MHC Class II-expressing allograft ECs.<sup>102,103</sup> Predominantly, the TNF family member surface protein FasL is used to induce apoptosis upon binding with its corresponding Fas (CD95) on target cells.<sup>104,105</sup> Studies show that CD4<sup>+</sup> T-cells can use perforin as a cytotoxic mechanism following infection *in vivo*.<sup>79,84,92,106-109</sup> and *in vitro*.<sup>110</sup> Previous reports suggested that T-cell mediated endothelial death is via mechanisms of granzyme B and perforin.<sup>111-113</sup> TNF- $\alpha$  and IFN- $\gamma$  contribute significantly to vasculopathy as they can influence expression of molecules involved in antigen processing. Examples include costimulatory molecules of the B7 family (ICOS-L,<sup>114</sup> PD-L1)<sup>115</sup>, costimulatory molecules of the TNF receptor superfamily (CD40)<sup>116</sup> and adhesion molecules (E-selectin, VCAM-1, and ICAM-1). IFN- $\gamma$  stimulates and increases surface MHC Class I and II, activates macrophages, and regulates proliferation of T

lymphocytes.<sup>36,117-119</sup> IFN- $\gamma$  can also act on transplanted arteries to induce CAV by potentiating PDGF induced mitogenesis.<sup>75</sup> Upregulation of adhesion molecules promotes lymphocyte adhesion and entry through the endothelium;<sup>120,121</sup> and may also contribute to memory T cell activation by costimulation of stabilizing adhesion.<sup>122</sup> Therefore, ThCTL play a critical role in the context of cardiac allograft rejection.

### 1.2.3.2 Endothelial cells present antigens to activate T-cells

At the blood-tissue interface, EC are in a position that allows their direct contact with T-cells and involvement in producing and responding to immune responses. In transplantation, donor ECs express foreign MHC molecules, which can be recognized by the host immune system. Endothelial injury is an important initiating event to transplant vascular disease,<sup>123</sup> and the term *endothelialitis* was introduced in 1990 to describe cytotoxic T-lymphocyte-mediated endothelial injury.<sup>124</sup> Choi and colleagues described ECs as “sentinels, presenting antigen so as to initiate a secondary immune response.”<sup>122</sup>

Cardiac allografts may be acutely rejected via direct recognition of foreign MHC class I molecules by CD8<sup>+</sup> T-cells, which have also been found to indirectly target skin graft peptides displayed by host ECs.<sup>125</sup> There is considerable debate on the specific cell types that initiates antigen presentation to T-cells. Using the H-2M mouse model that cannot load antigen on its MHC Class II, Ardehelli and colleagues showed that abrogation of indirect recognition did not improve CAV, and concluded that direct recognition is sufficient to drive CAV.<sup>126</sup> However, as most donor-derived hematopoietic APCs migrate out of the organ soon after transplantation as the Passenger Leukocyte Theory described, it has been suggested that ECs<sup>127</sup> play an active role in antigen presentation resulting in chronic stimulation of the recipient immune system in both mice<sup>125,128-130</sup> and humans.<sup>131</sup> This response is similar to that of Delayed Type Hypersensitivity (DTH), but while DTH usually subsides as the antigen clears, foreign antigen of the graft is constitutively presented to T-cells and chronic DTH persists, resulting in cytokine-induced fibrosis.

However, others have shown that donor hematopoietic cells (and not ECs) are responsible for direct allo-recognition by CD4<sup>+</sup> T-cells.<sup>132</sup> There is also evidence of

preferential recruitment of CD8<sup>+</sup> T-cells specific for an antigenic peptide in the tissues which implicates ECs as providers of the homing signal.<sup>133</sup> While human vascular ECs constitutively express MHC class I and II, MHC class II is inducible in mice by activating cytokines such as IFN- $\gamma$ .<sup>134-141</sup> The proinflammatory cytokine, tumor necrosis factor alpha (TNF- $\alpha$ ) released by macrophages and T-cells can also augment ECs inflammatory profile via NF $\kappa$ B activation and upregulation in VCAM-1, changing the vessel's ability to recruit and activate smooth muscle cells.<sup>142</sup> An increase in TNF-receptor 1 (TNFR1), the major receptor for soluble TNF- $\alpha$  are observed on ECs after transplantation and lack of donor TNFR1/2 also leads to attenuated CAV.<sup>142</sup>

Much evidence implicates endothelial cell injury as an important initiating event in CAV: Anti-endothelial antibodies and anti-HLA antibodies increase the risk of CAV,<sup>143,144</sup> and the development of anti-endothelial antibodies correlate with increased rate of coronary artery disease after cardiac transplantation.<sup>145</sup> Increased Fas expression on ECs also increase apoptosis and initiates arteriosclerosis,<sup>146</sup> and can also act as targets of granzyme B<sup>147</sup> and perforin.<sup>111</sup> Further evidence of EC and T-cell interactions include EC expression of costimulatory molecules; CD40 in both human and mouse ECs,<sup>148,149</sup> B7-2 on cultured cardiac ECs,<sup>150</sup> B7-1 and B7-2 expression on cultured brain EC.<sup>148,151</sup> Activated EC may also express CD154<sup>152-154</sup> and act as costimulators of T-cells as they interact with CD40. Whether ECs actively stimulate T-cells *in vivo* still needs to be determined. Nonetheless, both direct<sup>126</sup> and indirect antigen presentation lead to chronic cardiac rejection, and survival of donor graft EC are crucial to the health of the heart graft.

## 1.3 Mechanisms of cell death

### 1.3.1 Apoptosis and necrosis

Cell death has historically been understood to occur through two main processes: programmed cell death (PCD), or apoptosis, and unregulated cell death, or necrosis. First reported by Kerr, Wyllie and Currie in 1972,<sup>155</sup> apoptosis was described as a type of controlled cellular event important in development and metabolic processes. Morphologically, it is characterized by cell shrinkage, chromosomal condensation,



nuclear fragmentation, membrane blebbing, and formation of apoptotic bodies. Apoptotic bodies emit signals such as soluble lysophosphatidylcholine and phosphatidylserine,<sup>156,157</sup> which allow their uptake by macrophages resulting in resolution of the injury and avoidance of inflammatory reactions. For example, Kidney Injury Molecule-1 (KIM-1) is a phosphatidyl serine receptor that is expressed on kidney epithelial cells and recognizes apoptotic cells and induces phagocytosis to limit further inflammation.<sup>158</sup> Apoptotic bodies that are not systematically cleared can lose membrane integrity and undergo secondary necrosis, releasing cellular damage-associated molecular patterns (cDAMPs), which can then initiate an immune response. There are two types of apoptotic pathways that have been characterized: the intrinsic mitochondria-mediated pathway<sup>159</sup> and the extrinsic death-receptor-mediated pathway.<sup>160</sup>

In contrast, necrosis has historically been regarded as passive or accidental cell death as a result of nonspecific stress factors such as extreme heat, freeze-thawing, or osmotic shock. Upon membrane rupture, immunogenic cDAMPs are released. cDAMPs are a family of molecules that perform non-inflammatory roles when intracellular and include factors such as heat shock proteins HSP70, HSP90, and GP96, histones, high mobility group protein B1 (HMGB1), RNA, DNA fragments, monosodiumurate microcrystals, IL-1a, uric acid, mitochondrial fragments, and ATP.<sup>161</sup> Local tissue injury during transplantation may lead to the passive release of cDAMPs, which, when released, are immunogenic and act to initiate adaptive immune responses.<sup>162</sup> cDAMPs are involved in acute allograft rejection,<sup>162</sup> while skin allografts deficient for HSP70 also show prolonged graft survival.<sup>163</sup> cDAMPs stimulate pattern-recognition receptors (PRR), which are sensors of infection and coordinate inflammatory response. PRRs include Toll-like receptors (TLR), RIG-I-like receptors (RLR), nucleotide binding domain and leucine-rich repeat containing molecules (NLR), and C-type lectin receptors (CLR). These same PRRs recognize pathogen-associated molecular patterns (PAMP), which suggest similarities between inflammatory responses for endogenous vs. exogenous material clearance.

Thus, in most cases, apoptosis is mostly regarded as a tolerogenic and anti-inflammatory type of cell death while necrosis is a trigger of inflammation and damage.

### 1.3.2 Regulated necrosis: necroptosis

Evidence in the last decade has challenged the paradigm of apoptosis as the only form of PCD.<sup>164</sup> Findings suggest that necrosis can also be considered as a type of PCD, as cells with a necrotic appearance can contribute to embryonic development and tissue homeostasis.<sup>165,166</sup> Furthermore, programmed necrosis can be induced by specific ligands binding to membrane receptors, and can also be regulated by genetic, epigenetic, and pharmacological factors.<sup>165-168</sup> This form of programmed necrosis is termed necroptosis. Originally, the term necroptosis was used to indicate a specific case of regulated necrosis [i.e. tumor necrosis factor receptor-1 (TNFR1)-stimulated that can be inhibited by necrostatin-1]. However, the 2012 Nomenclature Committee on Cell Death<sup>169</sup> recommends that the definition of necroptosis to describe RIPK1 and/or RIPK3-dependent regulated necrosis. More recent recommendations include findings on RIPK3-downstream mixed lineage kinase domain-like (MLKL).<sup>170-173</sup>

First characterized in L929 mouse fibrosarcoma cells, caspases were found to play a major role in the switch between apoptosis to necrosis,<sup>174</sup> and Hitomi and colleagues compared apoptosis and necroptosis in murine cells.<sup>175</sup> They described the signaling network behind necroptosis initiated by death receptor ligation or pan-caspase inhibition with Z-VAD-fmk and a genome-wide search of the genetic and epigenetic mechanisms involved with its regulation.<sup>175</sup> Necroptosis was originally found to be a novel mechanism for cells to undergo death when apoptotic proteins are inhibited, such as in the presence of virally encoded caspase inhibitors<sup>176-179</sup> and viral FLICE inhibitors.<sup>180</sup> Since then, necroptosis has been found to be the fate of many cell types and pathological conditions, including kidney tubular epithelial cells<sup>181-183</sup> and retinal ganglion cells<sup>184,185</sup> of the ischemia reperfusion injury model,<sup>183,186,187</sup> traumatic brain and spinal cord injury,<sup>188,189</sup> myocardial infarction,<sup>190</sup> pathogenic infections,<sup>176,179,191</sup> inflammation,<sup>192-195</sup> and atherosclerosis.<sup>196</sup>

### 1.3.3 Molecular Pathway after Death Receptor Signaling

Regulated necrosis can be triggered by various factors such as viral infections,<sup>197</sup> ligation of death receptors [TNFR1,<sup>198,199</sup> Fas (CD95),<sup>175,200</sup> TRAILR1 and TRAILR2<sup>200</sup>] with

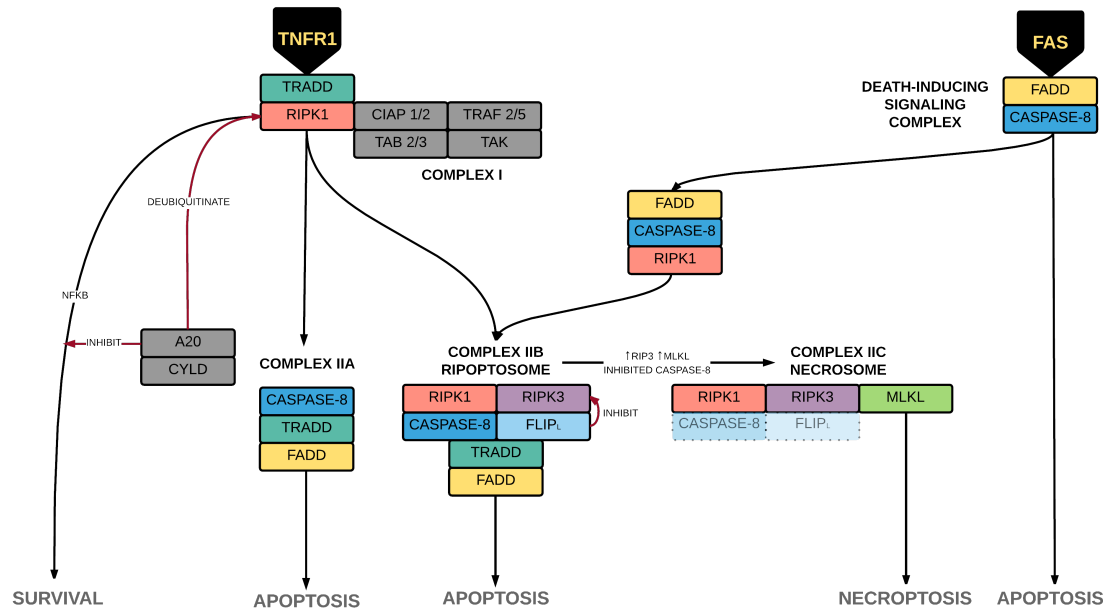
caspase inhibition.<sup>201</sup> The most extensively characterized inducer of necroptosis is TNF- $\alpha$  ligation to TNFR1.<sup>202,203</sup> TNF- $\alpha$  has the ability to induce apoptosis or necroptosis depending on the inhibition status of caspase 8.<sup>175</sup> This mechanism has been reviewed extensively,<sup>166,204-209</sup> but will be briefly summarized in the following paragraphs and in Figure 2.

Upon stimulation at TNFR1, TNFR1-associated death domain (TRADD) is recruited to the plasma membrane, attracting protein receptor interacting protein kinase-1 (RIPK1), cellular inhibitors of apoptosis protein (cIAP)1, cIAP2, TNF receptor-associated factor (TRAF)2 and TRAF5 to form receptor-bound complex I. RIPK1 is subsequently polyubiquitylated at Lys63, allowing the docking of transforming growth factor- $\beta$ -activated kinase 1 (TAK1), TAK1 binding protein (TAB)2 or TAB3, and inhibitor of NF- $\kappa$ B kinase (IKK) complex. This results in activation of the canonical NF- $\kappa$ B activation. This also results in the upregulation of A20, which acts as negative feedback and reduces NF- $\kappa$ B activity by removing Lys63-linked polyubiquitin chains from RIPK1. Dissociation of RIPK1 from TNFR1 allows it to interact with FAS-associated death domain (FADD), RIPK3, cellular FLICE (FADD-like IL-1 $\beta$ -converting enzyme)-inhibitory protein (FLIP) and pro-caspase 8, forming the TRADD-dependent death-inducing signaling complex (DISC) (Complex IIa). Pro-caspase 8 and the long isoform of FLIP (FLIP<sub>L</sub>) form a heterodimeric caspase that cleaves and inactivates RIPK1 and RIPK3 interactions to prevent necroptosis. Pro-caspase 8 can also form homodimers, undergo autoproteolysis and activation, causing its dissociation from TRADD-dependent complex IIa and subsequent activation of executioner caspase 3 and caspase 7, resulting in apoptosis.

When caspase 8 is inhibited (with chemical inhibitors or virally encoded proteins),<sup>210</sup> RIPK1 and RIPK3 are allowed to interact at their RIP homotypic interaction motif (RHIM) domains and will dimerize, auto- and transphosphorylate, and form the necrosome. Cyldromatosis (CYLD) also deubiquitylates RIPK1 in this complex to further promote kinase activation. Phosphorylation of RIPK3 recruits MLKL, which oligomerizes and is subsequently phosphorylated, translocates to the plasma membrane, where it mediates cell membrane damage and calcium influx and ultimately loss of cell

integrity. When second mitochondria-derived activator of caspase (SMAC or DIABLO) mimetic are used to inhibit cIAPs, thus, leaving RIPK1 deubiquitylated, canonical NF- $\kappa$ B signaling is reduced.<sup>211</sup> A TRADD-independent, RIPK1-dependent complex called the ripoptosome (complex IIb) is formed, which includes RIPK1, RIPK3, FADD and FLIP<sub>L</sub> that stimulates non-canonical NF- $\kappa$ B signaling. RIPK1 and RIPK3 are inactivated through cleavage mediated by caspase 8—FLIP<sub>L</sub> heterodimers, which can result in apoptosis. RIPK1/3 can mediate necroptosis depending on the presence of caspase-8. Morphologically similar to necrosis, the consequential loss of membrane integrity of necroptotic cells leads to the release of cDAMPs, further exacerbating injury and inflammation.

Signaling through another member of the TNF family, Fas (Apo-1 or CD95), a well-defined mechanism of prototypic extrinsic apoptosis induction, is also associated with necroptosis induction.<sup>212</sup> The binding of FasL stabilizes aggregated Fas trimers at the membrane and induces a conformational change that, independent of TRADD, recruits FADD and pro-caspase 8 to the death domain of the cytosolic tail of the receptor. The formation of the DISC and downstream activation of caspase 8, leads to activation of other caspases. The death domain of Fas allows it to associate with RIPK1 which has been shown to be important for programmed necrosis induction in caspase 8 deficient Jurkat cells.<sup>200,212,213</sup> It is possible that in activated primary T lymphocytes, Fas-mediated death is the dominant mode of death,<sup>200</sup> which might explain why caspase activity inhibition in mouse T lymphocytes *in vivo* does not induce autoimmune disease that is usually manifested in mice with inactivating mutations in Fas or Fas ligand.<sup>214</sup>



**Figure 2. Death receptor signaling lead to apoptosis and necroptosis.**

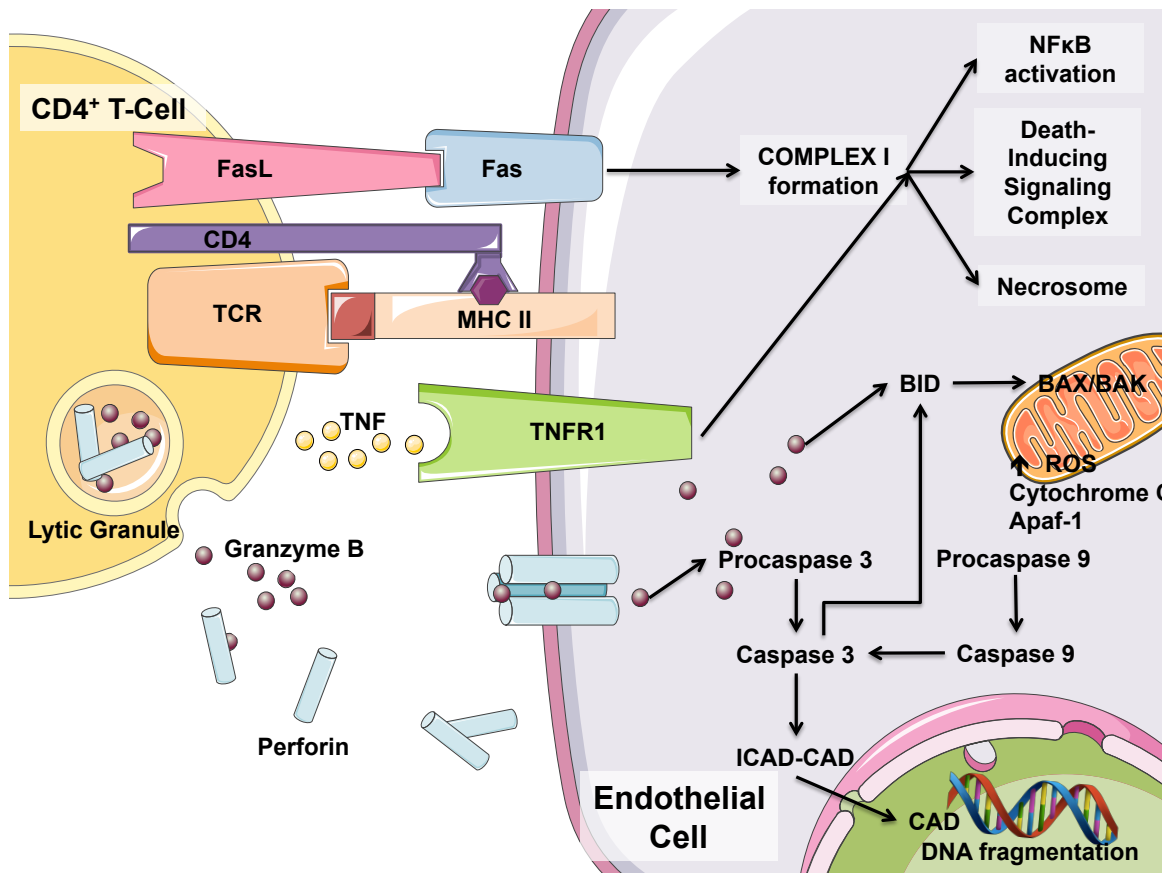
Upon TNFR1 stimulation, complex I is formed by TRADD binding to RIPK1, TRAF2 and TRAF5, cIAP1 and cIAP2, leading to NF-κB and MAPK pathway activation. Polyubiquitination of RIPK1 by cIAPs lead to its interaction with TAK1 and TAB2/3. TAK1 activates the IKK complex, leading to polyubiquitination and proteosomal degradation of IκB, resulting in NF-κB translocation to the nucleus and pro-survival gene transcription. In a negative feedback loop, this upregulates A20 and CYLD, which target RIPK1 for deubiquitination, releasing it to form secondary complexes. The formation of TRADD-dependent complex IIa, involves FADD-mediated recruitment and activation of caspase-8 and its active cleavage of RIPK1 and RIPK3 dimers result in apoptosis. In the presence of smac mimetic, which facilitate cIAP proteasomal degradation, formation of the TRADD-independent complex IIb can similarly activate caspase-8 and result in apoptosis in a RIPK1-dependent mechanism. In the absence of caspase-8 activity, RIPK1 and RIPK3 interacts in a complex called the necrosome, associating with FADD, caspase-8, and TRADD, where phosphorylation of RIPK1 and RIPK3 in the necrosome will lead to downstream MLKL activation and necroptosis. The dimerization of cFLIP and pro-caspase 8 in the DISC inhibits its activation and downstream apoptosis.

### 1.3.4 Granule-mediated cell death

The granule pathway is a primary mechanism used by T-cells and NK cells to directly lyse allogeneic cells. Upon recognition of foreign antigen, T-cells release granules that contain serine proteases called granzymes and the cytolytic protein, perforin, into the immune synapse. Perforin plays a key role in this cytolytic mechanism, as it forms pores on the target membrane surface and facilitates the delivery of granzymes into target cells to induce apoptosis. Secretory lysosomal granules polarize rapidly to the cell surface towards the immune synapse upon recognition of a target. Once in the cytoplasm, granzyme B can promote cell death through two main pathways: BH3 interacting-domain death agonist (BID)-dependent mitochondrial permeabilization; or direct caspase activation. In the former mechanism, granzyme B activates pro-apoptotic BID which induces oligomerization of BAX and/or BAK in outer mitochondrial membrane,<sup>215-217</sup> leading to cytochrome *c* release into the cytosol, assembly of apoptosome, and resultant caspase-9 activation. In the latter pathway, effector caspases 3 and 7 can be directly activated by granzyme B cleavage, resulting in cleavage of caspase 2, 6, 9<sup>218</sup> and ultimately cell death. Granzyme B can also cleave the inhibitor of caspase-activated DNase, (ICAD) which mediates DNA degradation.<sup>219</sup>

Although it is well known that CD8<sup>+</sup> T-cells are the main source of granzyme B, CD4<sup>+</sup> T-cells can also use granzyme B to kill target cells. Activated CD4<sup>+</sup> and CD8<sup>+</sup> T-cells secrete similar amounts of granzyme B,<sup>220</sup> however, the levels are maintained by CD4<sup>+</sup> T-cells for longer periods.<sup>221</sup>

There is substantial evidence that perforin and granzyme B are involved in endothelial injury in transplantation. T-cells that express perforin are found in the subendothelial space in vasculopathy,<sup>222</sup> and perforin and granzyme B proteins both localize to human vessel intima,<sup>223</sup> where granzyme B protein is observed around apoptotic cells.<sup>224</sup> Both perforin and granzyme B has been shown to play an important role in EC death induction, resulting in vasculopathy in cardiac transplantation.<sup>147,225</sup>



**Figure 3. Cytotoxic CD4<sup>+</sup> T-cell interactions with an endothelial cell.**

CD4<sup>+</sup> T-cells can interact with endothelial cell (EC) via direct recognition of foreign MHC Class II or indirect recognition of processed peptides presented on the MHC Class II. CD4<sup>+</sup> T-cells use cell contact forms of cell death such as FasL (Apo-1) and granzyme B to lyse target cells and the inflammatory cytokine TNF- $\alpha$  to induce cell death. Fas (CD95) stimulation can lead to NF- $\kappa$ B activation, formation of death-inducing signalling complex (apoptosis), or necrosome complex (necroptosis). Granzyme B can enter target cells via the pore-forming protein perforin and cause cell death via caspase dependent and independent mechanisms. Granzyme B is a pro-apoptotic protease that activates apoptosis via cleavage of caspase-3, Bid, causing oligomerization of Bax and Bak that augment mitochondria membrane potential. Release of cytochrome C into the cytosol work with caspase-9 and apoptosis-activating factor 1 (Apaf-1) to further process procaspase 3. Lastly, the cleavage of inhibitor of caspase-activated DNase (ICAD) can lead to DNA fragmentation and resultant apoptosis.

## 1.4 Rationale and Hypothesis

### 1.4.1 Rationale

Given the lack of interventions for CAV which remains to be one of the leading causes of death after cardiac transplantation, and the implication of EC injury in chronic rejection,<sup>111-113</sup> further studies into the mechanism of EC death in a long-term transplant model are pertinent. Previously, it was found that RIPK3 deficiency in donor hearts prevented necroptosis and acute rejection with immunosuppression.<sup>226</sup> Hence, we wanted to define the role of RIPK3 and necroptosis in CAV using a mouse model of CD4<sup>+</sup> T-cell-mediated chronic cardiac transplant injury.

### 1.4.2 Hypothesis

We hypothesize that RIPK3 deficiency protects endothelial cells against cytotoxic CD4<sup>+</sup> T-cell mediated cell death and chronic rejection in cardiac allograft transplantation.

### 1.4.3 Objectives

1. To determine if donor heart grafts deficient in RIPK3 prolong graft survival in a single MHC Class II mismatch transplantation;
2. To determine mechanisms of CD4<sup>+</sup> T-cell mediated death in wild type and RIPK3<sup>-/-</sup> MVEC *in vitro*.



## Chapter 2

### 2 Materials and Methods

#### 2.1 Animals

Male inbred C57BL/6 (H-2<sup>b</sup>; B6; 4-6 weeks old; Charles River Laboratories) and B6.129R1-RIPK3<sup>tm1Vmd</sup> (H-2<sup>b</sup>; RIPK3<sup>-/-</sup>; 4-6 weeks old; H-2b; Genentech, Inc.) and the single MHC class II mismatch B6.C-H-2<sup>bm12</sup> (H-2Ab1<sup>bm12</sup>; bm12; 9-10 weeks old; Jackson Laboratories) mice were maintained at the animal facility at University of Western Ontario. All experimental procedures were approved by The University of Western Ontario Animal Care Committee (Appendix A).

#### 2.2 Microvascular Endothelial Cell Culture

B6 and RIPK3<sup>-/-</sup> microvascular endothelial cells (B6 MVEC, RIPK3<sup>-/-</sup> MVEC) were isolated and purified as previously described<sup>226,227</sup> and immortalized by SV40 transfection. Cells were grown in complete Endothelial Growth Media-2 (EGM-2 medium) supplemented with fetal bovine serum and EGM-2 SingleQuots (Lonza) and were used between passages 3-6.

#### 2.3 T-Cell purification and MLR stimulation

CD4<sup>+</sup> T-Cells were purified from bm12 mouse spleens using anti-CD4 magnetic beads (MACs; Miltenyi Biotec) and were only used for assays if purity was at least >85%. Purified CD4<sup>+</sup> T-cells were co-cultured with 50µg/mL mitomycin C (Cayman Chemicals)-treated T-cell depleted B6 splenocytes in RPMI-1640 (Gibco) supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 lg/mL), glutamine (2 mM), sodium pyruvate (1 mM), HEPES (10 mM), and β-mercaptoethanol (0.5 mM). One hundred IU IL-2 was added immediately after co-culture and 20-50IU IL-2 was added every other day until day 4-7.

## 2.4 Antibodies

Phenotype of CD4<sup>+</sup> T-cells and MVECs were characterized using the following antibodies: anti-mouse CD4, CD107a (BD Pharmingen), CD262, H-2k<sup>b</sup>, IA/IE<sup>b</sup>, TNF- $\alpha$ , IFN- $\gamma$ , granzyme B, perforin, Fas, FasL, E-Cadherin, I-CAM1, CD31, isotype control IgG (eBioscience).

## 2.5 Heterotopic heart transplantation and post-operative monitoring

Heterotopic abdominal heart transplantation was conducted as previously described.<sup>226,228-231</sup> Briefly, B6 (n=25; H-2<sup>b</sup>) or RIPK3<sup>-/-</sup> (n=36; H-2<sup>b</sup>) heart grafts were heterotopically transplanted into the abdomen of bm12 (H-2<sup>bm12</sup>) mice by anastomoses of the graft ascending aorta to the donor abdominal aorta, and the graft pulmonary artery to the donor inferior vena cava. The pulmonary veins and vena cava of the graft were ligated.

Animals were treated with subcutaneous injections of ketoprofen after surgery. Palpation of the graft was monitored daily and hearts were considered rejected when transplanted heart pulse ceased.

## 2.6 Histology and Immunohistochemistry

Donor hearts were collected on day 24 and perfused with a 5 $\mu$ M Ethidium Homodimer-1 saline solution (Life Technologies) followed by 10mL PBS (Gibco) at 1mL/minute as previously described.<sup>226</sup> It was cut transversely and either frozen using Tissue-Tek® O.C.T. Compound (Sakura® Finetek) or fixed with 5% formalin for paraffin embedding. Paraffin sections were used for hematoxylin and eosin as well as elastic trichrome staining.

Formalin-fixed (5 $\mu$ m) or snap-frozen (5 $\mu$ m) sections were fixed in acetone and stained with anti-mouse CD4 biotin (clone: GK1.5; affymetrix eBioscience) followed by immunohistochemistry stain according to standard protocol.

All injury scores were evaluated by a pathologist in a blinded manner. Graft injury was evaluated based on change in endothelium as compared with naïve. Endothelial damage was scored on a scale of 0–4 (0: no change, 1: 0-24% change, 2: 25-49% change, 3: 50-74% change, 4: >75% change). Vasculopathy was defined by morphometric analysis measured with ImageJ software from a total of 33 vessels (n=5, B6) and 25 vessels (n=3, RIPK3<sup>-/-</sup>) that had a diameter  $\geq 80\mu\text{m}$ . The neointima index (NI) was calculated according to the formula  $\text{NI} = [\text{intima area}/(\text{intima area} - \text{luminal area})]$ . The percentage of occluded vessels was calculated according to: (number of occluded vessels/total number of vessels) x 100% per sample. CD4<sup>+</sup> T-cell infiltration was measured as percentage of positive area for anti-CD4 over total image area (ImageJ). An average of percentage of positivity was obtained from ten HPF of the most cell dense areas per sample.

## 2.7 Cell death measurement

Cell death was induced by cytokine (human TNF- $\alpha$ ) or CTL (cytotoxic T lymphocytes). Twenty thousand MVECs were plated on 96-well flat-bottomed plates for 24 hours in complete EGM-2 media (Lonza). Cell death was assessed as either a measure of SYTOX<sup>®</sup> Green Nucleic Acid Stain (100nM in serum-free EBM-2 media; Lonza) at a concentration of 100nM, or by 7-AAD Viability Staining Solution (BioLegend) according to manufacturers' protocols. Cell death was captured using the IncuCyte ZOOM<sup>®</sup> System (Essen Bioscience) and the CytoFLEX flow cytometer (Beckman Coulter). Flow cytometry data was analyzed using FlowJo Single Cell Analysis Software v.10.08 (FlowJo Enterprise).

### 2.7.1 Cytokine-induced MVEC death

The cytokine cocktail used for cell death assay consisted of: recombinant human TNF- $\alpha$  (100ng/mL; PeproTech), GDC-0152 (smac mimetic; 100nM; Selleckchem), Z-VAD-fmk (50 $\mu\text{M}$ ; R&D Systems), with or without Necrostatin-1s (10 $\mu\text{M}$ ; EMD Millipore) in serum-free EBM-2.

### 2.7.2 CD4<sup>+</sup> T-cell-induced MVEC death

MVEC were pre-treated 24 hours prior with mouse-IFN- $\gamma$  (100ng/mL; PeproTech) and labeled with CellTrace<sup>TM</sup> CFSE (ThermoFisher Scientific). Mixed lymphocyte reaction-stimulated CD4<sup>+</sup> T-cells were added at an effector to target ratio of 5:1 and the 96-well U-bottom plate was centrifuged at 31xg (Beckman Coulter) for 2 minutes prior to incubation at 37.5°C and 5% CO<sub>2</sub> for 7 hours. MVEC death was quantified 7-AAD staining of CFSE gated cell population.

## 2.8 Immunoblot Analyses

Supernatants were collected from treated MVEC (seeded in 6-well plates at 3 x 10<sup>5</sup> cells and grown to a confluent monolayer in EGM-2 over 24 hours). Cells were trypsinized, centrifuged at 1,500 x g for 5 minutes and 50 $\mu$ L nuclear lysis buffer (20mM HEPES, 0.4mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM PMSF) was added to each sample followed by a 30 minute incubation at 37.5°C. The nuclear fraction was collected by centrifugation at 10,000 x g for 15 minutes at 4°C.

Protein in supernatants were concentrated by centrifugation for 15 minutes using Amicon<sup>®</sup> Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 membrane (EMD Millipore). An equal volume of loading buffer (2-ME, glycerol, bromophenol blue, Tris-HCl) was added to the protein and was separated by gel electrophoresis with 4% stacking gel and 12% running gel. Protein was transferred to a nitrocellulose membrane using the iBlot<sup>®</sup> 7-minute blotting system (Invitrogen). 5% skim milk (Carnation) in Tris buffered saline and Tween 20 (TBS-T) was used for blocking.

HMGB1 protein was detected using rabbit polyclonal anti-mouse HMGB1 antibody–ChIP Grade (Abcam) after overnight incubation in 2.5% milk TBS-T. Protein was visualized using secondary anti-rabbit IgG horseradish peroxidase-linked antibody (Cell Signaling Technology) and chemiluminescent HRP substrate (EMD Millipore) and detected by enhanced chemiluminescence. Protein was semi-quantitated by densitometry (Alphaview<sup>®</sup>) and normalized using  $\beta$ -actin that is detected by anti- $\beta$ -actin antibody (Sigma-Aldrich).

## 2.9 Statistical Analyses

Data was compared using the Student's one tailed t-test for unpaired values. The Mantel-Cox log-rank test was used to determine graft survival differences. Differences were considered significant when  $p\text{-value} \leq 0.05$ .

## Chapter 3

### 3 Results

#### 3.1 RIPK3 deficiency protect donor heart graft from T-cell-mediated rejection *in vivo*

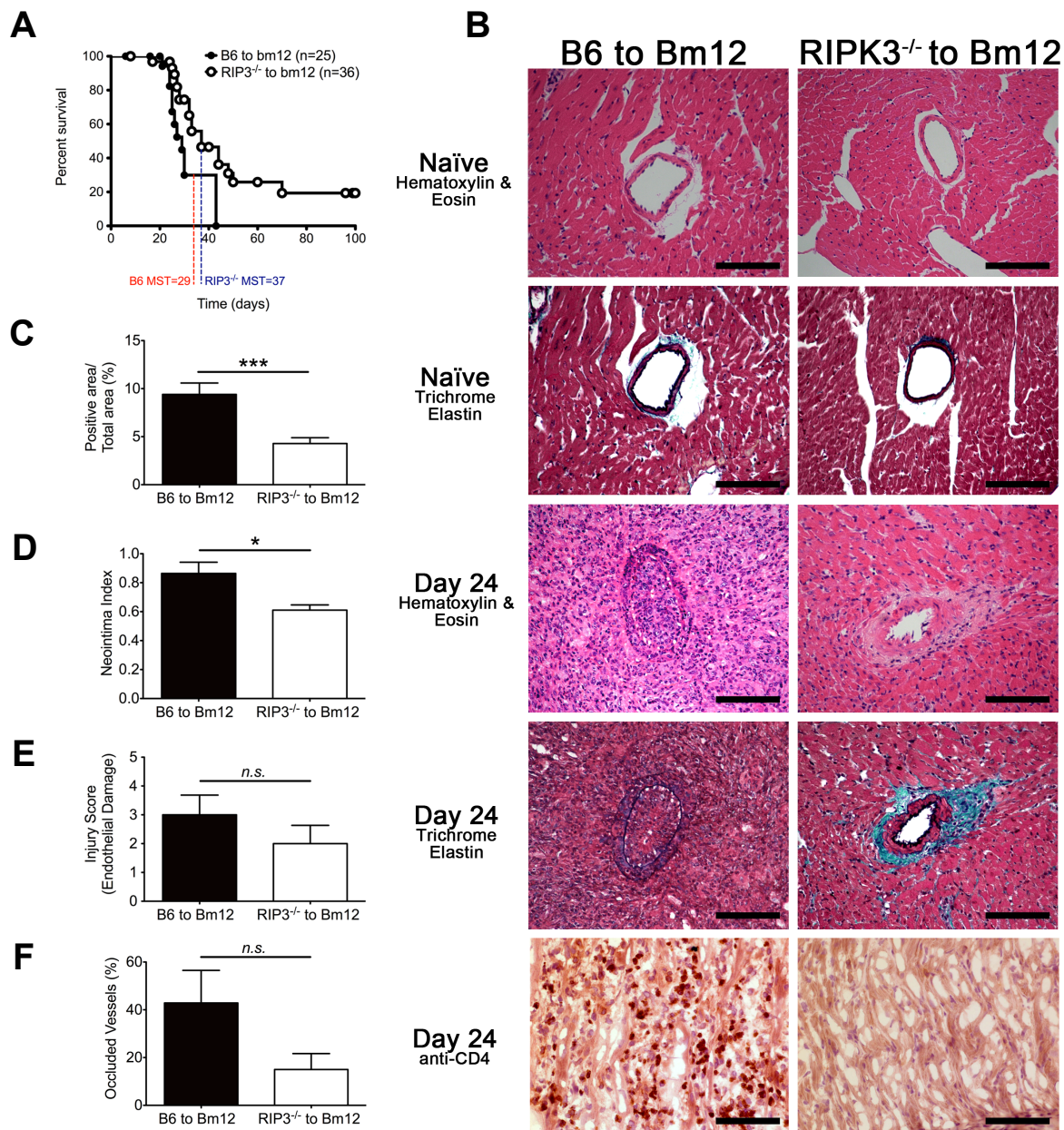
##### 3.1.1 Graft Survival

To determine if RIPK3 deficient donor hearts resulted in improved graft survival when compared to wild type in a single MHC Class II mismatch model, male wild type C57BL/6 (B6; n=25; H-2<sup>b</sup>) mice or B6-RIPK3<sup>-/-</sup> (RIPK3<sup>-/-</sup>; n=36; H-2<sup>b</sup>) heart grafts were heterotopically transplanted into abdomens of bm12 (H-2Ab1<sup>bm12</sup>) mice. We found that RIPK3<sup>-/-</sup> donor hearts survived significantly longer than B6 hearts (Log Rank test; p=0.0033, Figure 4a). The median survival time (MST) was 29 days for B6 and 37 days for RIPK3<sup>-/-</sup> grafts. Hence, CD4<sup>+</sup> T-cell-mediated heart graft rejection was reduced in RIPK3<sup>-/-</sup> donor graft.

Next we further characterized the mechanism rejection by examining CD4<sup>+</sup> T-cell infiltration, endothelium damage, and vasculopathy in heart grafts on day 24.

##### 3.1.2 Measures of Graft Rejection

Hematoxylin and eosin staining showed extensive infiltration in both RIPK3<sup>-/-</sup> and B6 heart grafts at day 24. (Figure 4b) There was a significantly lower amount of CD4<sup>+</sup> T-cell infiltration in RIPK3<sup>-/-</sup> heart grafts than in wild type (4.287±0.6016% vs. 9.397±1.187%; n=3-5 per group, p=0.0002, Figure 4c). Lumen stenosis was observed in both groups; however, vasculopathy was more pronounced in wild type donor hearts, as a significantly smaller neointima index in RIPK3<sup>-/-</sup> heart grafts was observed (0.6107±0.0361 vs. 0.8641±0.0771; n=3-5 per group, p=0.0271, Figure 4d). No significant differences were found in endothelial damage (n=3-5 per group, p=0.159, Figure 4e) or percentage of occluded vessels (n=3-5 per group, p=0.069, Figure 4f).



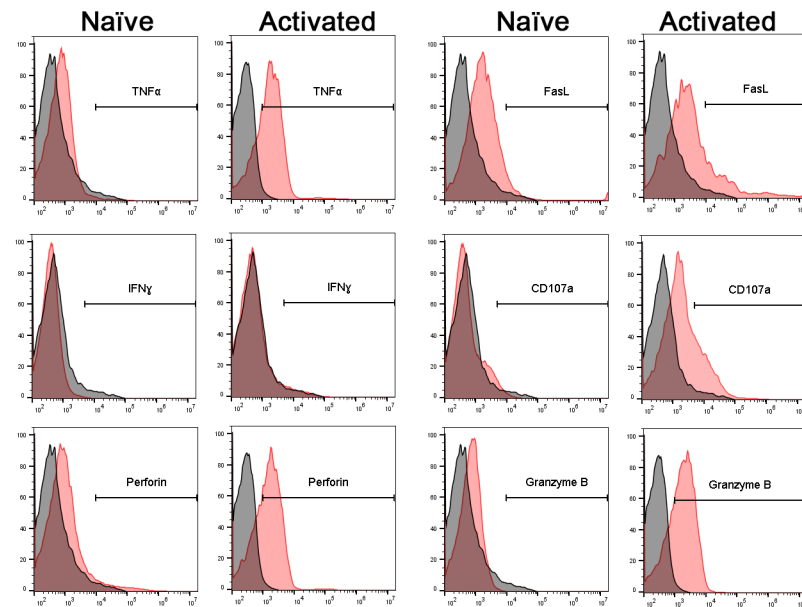
**Figure 4. Heart grafts deficient in RIPK3 attenuates graft rejection.**

Male wild type C57BL/6 (●B6; H-2<sup>b</sup>) or B6-RIPK3<sup>-/-</sup> (○RIPK3; H-2<sup>b</sup>) heart grafts were heterotopically transplanted into abdomens of B6-Bm12 (Bm12; H-2A<sup>b</sup>1<sup>Bm12</sup>) mice as described in the methods. Hearts were palpated and scored daily. Grafts were considered rejected upon cessation of the heartbeat and confirmed by histological examination. (A) Kaplan Meier Survival Curve analysis showed that RIPK3<sup>-/-</sup> (n=36) heart grafts survived longer than B6 (n=25). (B) Extensive vasculopathy and CD4<sup>+</sup> T-cell infiltration in B6 donor grafts was evidenced in histological sections. Images representative of n=5-7/group. (C) CD4<sup>+</sup> T-cell infiltration was measured as percentage of positive area positive for anti-CD4<sup>+</sup> over total image area. (D) Neointima index as a measure of vasculopathy was measured from all vessels  $\geq 80\mu\text{m}$  in diameter in samples (n=3-5) and was calculated according to:  $\text{NI} = [\text{intima area}/(\text{intima area} - \text{luminal area})]$ . (E) Endothelial damage was scored on a scale of 0–4 (0: no change, 1: 0-24% change, 2: 25-49% change, 3: 50-74% change, 4: >75% change). (F) Percentage of occluded vessels was calculated according to: (number of occluded vessels/total number of vessels) x 100%. Scale bar, 100  $\mu\text{m}$ . \*  $p \leq 0.05$  \*\*  $p \leq 0.01$  \*\*\*  $p \leq 0.001$



### 3.2 Allo-reactive CD4<sup>+</sup> T cells express TNF- $\alpha$ , IFN- $\gamma$ , FasL, granzyme B, and perforin

We first characterized the expression of cytotoxic molecules in naïve and Day 6-MLR B6 alloactivated bm12 CD4<sup>+</sup> T-cells. As illustrated in Figure 5, flow cytometric analyses showed that activated CD4<sup>+</sup> T-cells had increased surface expression of FasL and CD107a. An increase in intracellular expression of perforin, granzyme B, and TNF- $\alpha$  was also detected.



**Figure 5. Allo-activated CD4<sup>+</sup> T-cells have cytokine producing and cytotoxic phenotype.**

Bm12 CD4<sup>+</sup> T-cells were purified by MACs system and cultured in MLR for six days with mitomycin-C treated B6 splenocytes. Intracellular flow cytometry analysis revealed increase in TNF- $\alpha$ , perforin, and granzyme B and surface expression of CD107a and FasL in MLR activated cultures when compared with naïve. Histograms are representative of three separate experiments. (n=3).

### 3.3 RIPK3 deficiency in endothelial cells is protective against *in vitro* TNF- $\alpha$ -mediated death and release of cDAMPs

As RIPK3<sup>-/-</sup> donor heart grafts survived longer than wild type donor grafts in our chronic rejection model, we aimed to determine the mechanisms of cell injury by exploring the effects of inflammatory cytokines as a mediator of cell death. TNF- $\alpha$ , smac-mimetic, and Z-VAD-fmk with or without Necrostatin-1s was added to B6 MVECs and  $\Delta$ SYTOX® fluorescence (cell death) was measured over 12 hours (Figure 6a).

At hour 12,  $\Delta$ SYTOX® fluorescence significantly increased in B6 MVEC treated with TNF- $\alpha$ , smac-mimetic and Z-VAD-fmk when compared to untreated B6 MVEC (4478.0 $\pm$ 999.9 vs. 60.81 $\pm$ 8.207; purple vs. blue; n=5 per group; p<0.0001). When compared to cytokine-treated B6 MVEC, there was a significant reduction in  $\Delta$ SYTOX® fluorescence when Necrostatin-1s, an inhibitor of RIPK1 (4478.0 $\pm$ 999.9 vs. 83.02 $\pm$ 16.35; purple vs. brown; n=5 per group; p<0.0001), was added (Figure 6a). This suggests that TNF- $\alpha$  induced necroptosis in B6 MVECs.

In RIPK3<sup>-/-</sup> MVECs treated with human TNF- $\alpha$  and smac-mimetic,  $\Delta$ SYTOX® of fluorescence was significantly reduced with the apoptosis inhibitor, Z-VAD-fmk (865.0 $\pm$ 183.8 vs. 103.9 $\pm$ 26.50; green vs. orange; n=5 per group; p<0.0001, Figure 6a). Taken together, this data showed that at 12 hours, TNF- $\alpha$ -induced necroptosis in B6 MVECs can be recovered by pan-caspase inhibition, RIPK1 inhibition, or genetic deletion of RIPK3.

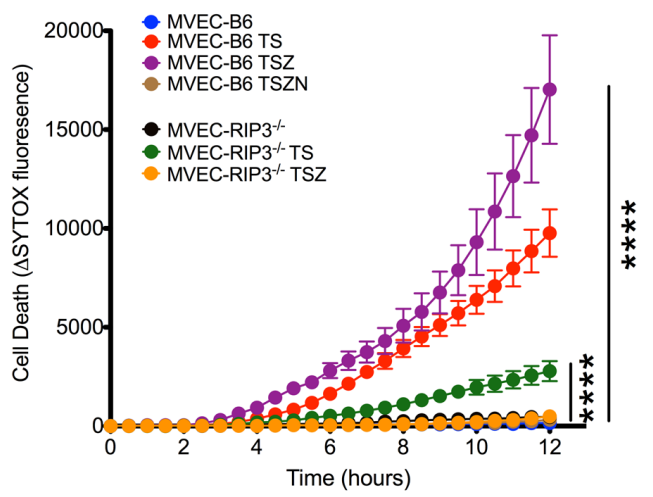
As illustrated in Figure 6b, flow cytometry analysis confirmed these findings. There was a significant increase in 7-AAD staining from B6 MVEC treated with TNF- $\alpha$  and smac mimetic to B6 MVEC treated with the addition of Z-VAD-fmk (21.80 $\pm$ 7.78% vs. 32.56 $\pm$ 3.78%; n=3 per group; p=0.0048; red vs. purple, Figure 6c). Addition of Necrostatin-1 significantly decreased 7-AAD positive cells when compared with B6 MVEC treated TNF- $\alpha$  (10.42 $\pm$ 5.32% vs. 32.56 $\pm$ 3.78%; n=3 per group; p=0.002; brown vs. purple, Figure 6c). Further, addition of Z-VAD-fmk with TNF- $\alpha$  significantly decreased 7-AAD staining of TNF- $\alpha$  treated RIPK3<sup>-/-</sup> MVEC (6.60 $\pm$ 2.45% vs.

13.46±1.75%; n=3 per group, p=0.008; yellow vs. green, Figure 6c). Finally, a reduction in 7-AAD was seen in RIPK3<sup>-/-</sup> MVEC when compared with B6 after cytokine treatment with Z-VAD-fmk (6.60±2.45% vs. 32.56±3.79%; n=3 per group, p=0.0002; yellow vs. purple, Figure 6c). This data confirmed earlier findings that TNF- $\alpha$ -mediated necroptosis is abrogated by RIPK1 inhibition or RIPK3 deletion.

cDAMPs such as HSP70 and HMGB1 are released upon cell lysis<sup>161</sup> and in acute rejection<sup>226</sup>. HMGB1, a nuclear DNA-binding protein, is a potent modulator of inflammation in organ rejection when passively released into the extracellular space, such as when cells are damaged or are necrotic.<sup>162,232</sup> To confirm necrotic death in our system, released HMGB1 were measured in the supernatants of MVEC treated with TNF- $\alpha$  with/without Necrostatin-1s for 24 hours (Figure 7a).

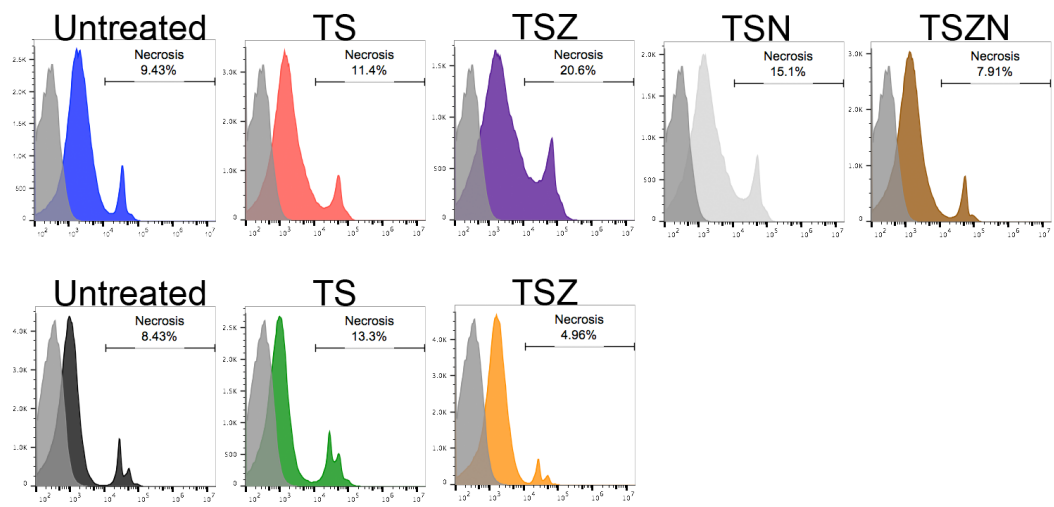
Although no significant increase in HMGB1 was detected in TNF- $\alpha$  and Z-VAD-fmk-treated B6 MVEC compared with TNF- $\alpha$  treatment alone, released HMGB1 was significantly decreased with RIPK1 inhibition by Necrostatin-1s (intensity index: 1.09±1.49 vs. 17.35±5.48; n=3 per group, p=0.0038). No HMGB1 was detected in the supernatant of any RIPK3<sup>-/-</sup> MVEC (Figure 7a).

**A**

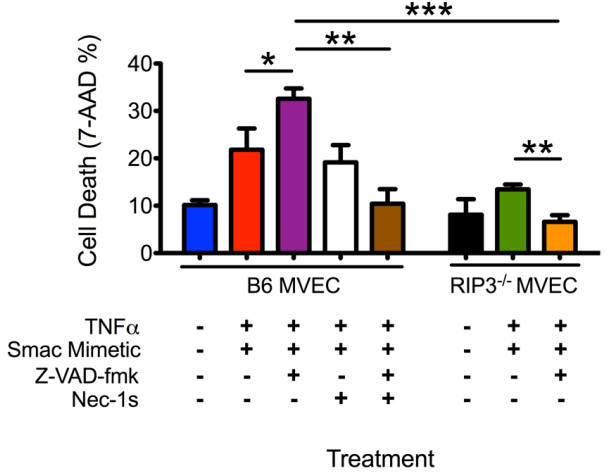


**Legend:**  
 T = TNF- $\alpha$   
 S = smac mimetic  
 Z = Z-VAD-fmk  
 N = Necrostatin-1s

**B**

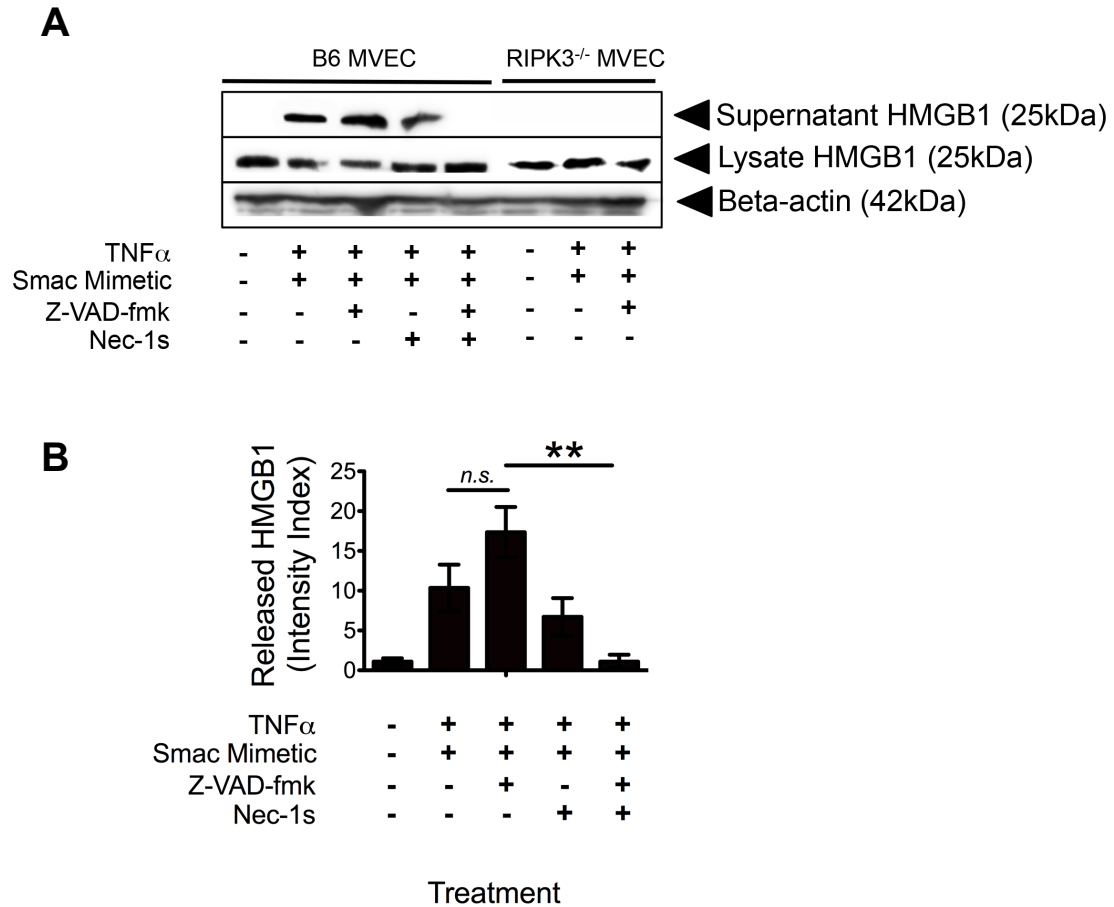


**C**



**Figure 6. RIPK3 deficient MVEC are protected from cytokine-induced cell death.**

B6 and RIPK3<sup>-/-</sup> MVEC were plated in equal numbers at a density of  $3 \times 10^5$  cells and treated with 100ng/mL TNF $\alpha$ , 100nM smac mimetic, with or without 50 $\mu$ M Z-VAD-fmk and 10 $\mu$ M Necrostatin-1s for 12-24 hours in serum-free media. Cell death was detected by (A) IncuCyte ZOOM live imaging system and (B-C) 7-AAD staining by flow cytometry. Graphs are representative of three separate experiments. (C) Average of three independent experiments (n=3). Data shown as mean $\pm$ SEM. \*\*\*\* $p \leq 0.0001$  \*\*\* $p \leq 0.001$  \*\* $p \leq 0.01$  \* $p \leq 0.05$ .



**Figure 7. RIPK3 deficient MVEC are resistant to cytokine-induced release of cDAMPs.**

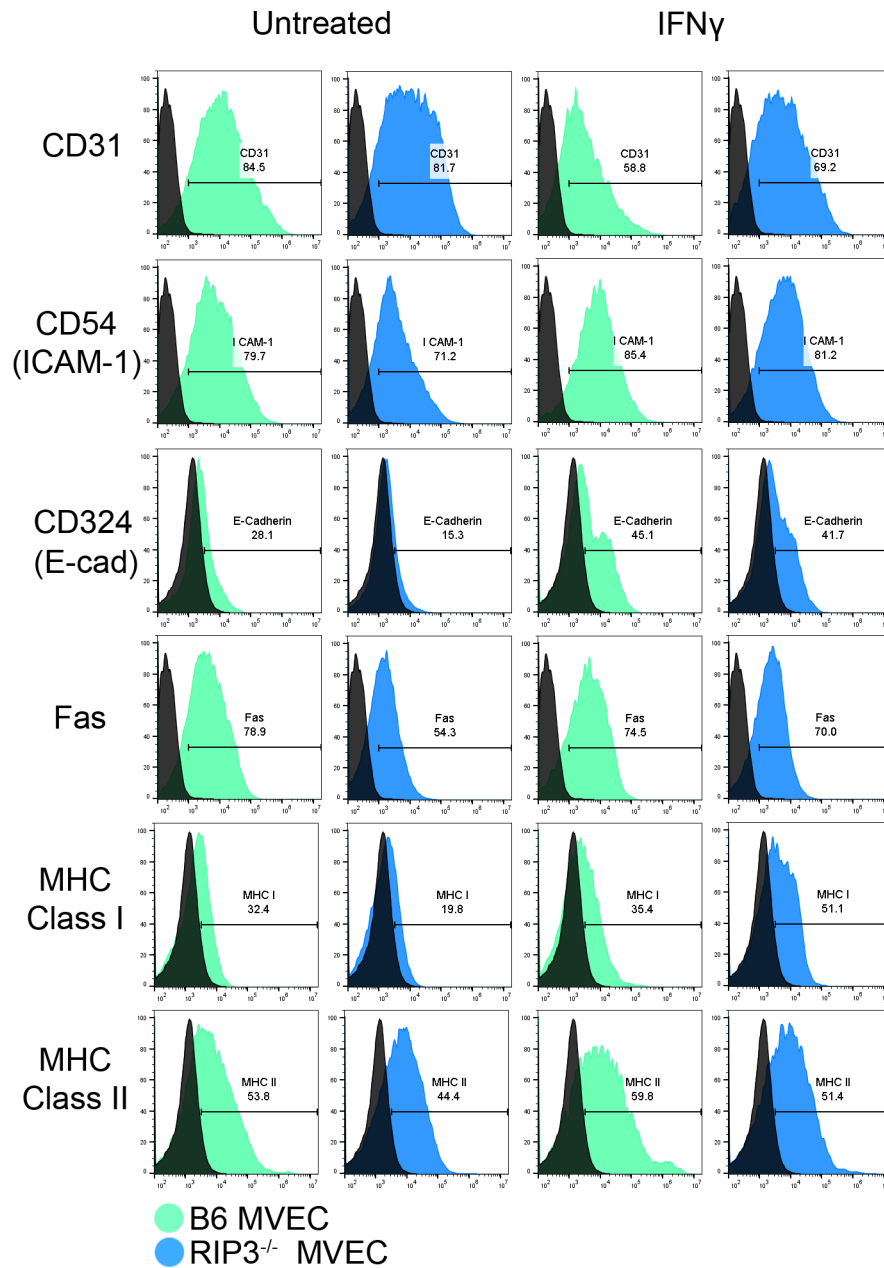
B6 and RIPK3<sup>-/-</sup> MVEC were plated in equal numbers at a density of  $3 \times 10^5$  treated with 100ng/mL TNF- $\alpha$ , 100nM smac mimetic, with or without 50 $\mu$ M Z-VAD-fmk and 10 $\mu$ M Necrostatin-1s for 48 hours in serum-free media. (A) Equal amounts of supernatants were loaded. Released HMGB1 protein was quantified by Western Blot. Remaining B6 and RIPK3<sup>-/-</sup> MVEC lysates were used as loading controls. (B) Data shown as mean $\pm$ SEM and representative of three independent experiments (n=3). \*\* $p \leq 0.01$ .

### 3.5 CD4<sup>+</sup> T-cell induce apoptosis and necroptosis in target MVEC

Next, we sought to determine the contribution of RIPK3 in a CD4<sup>+</sup> T-cell specific single minor MHC mismatch chronic rejection model. Since mouse endothelium does not constitutively express MHC Class II but can be induced with mouse IFN- $\gamma$ ,<sup>233</sup> MVECs were characterized after overnight treatment with mouse IFN- $\gamma$ . In both B6 and RIPK3<sup>-/-</sup> MVECs, there was an increase in surface expression of CD54 (ICAM-1), CD324 (E-cadherin), MHC Class I, and MHC Class II compared with untreated (Figure 8). Interestingly, there was an increase of surface expression of Fas on RIPK3<sup>-/-</sup> MVECs but a decrease on B6 MVECs. Surprisingly, there was a decrease in surface CD31 in both B6 and RIPK3<sup>-/-</sup> MVECs after IFN- $\gamma$  treatment.

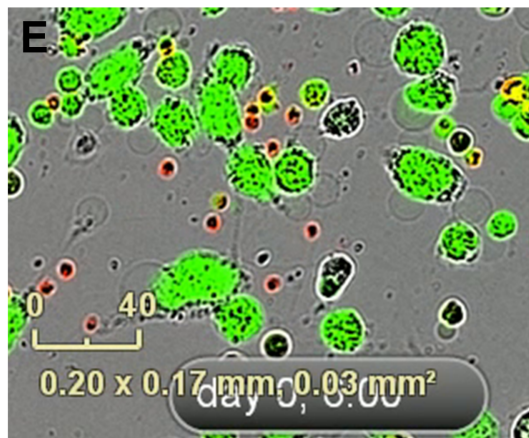
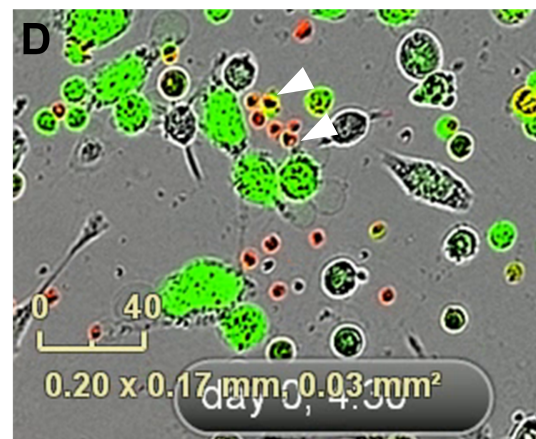
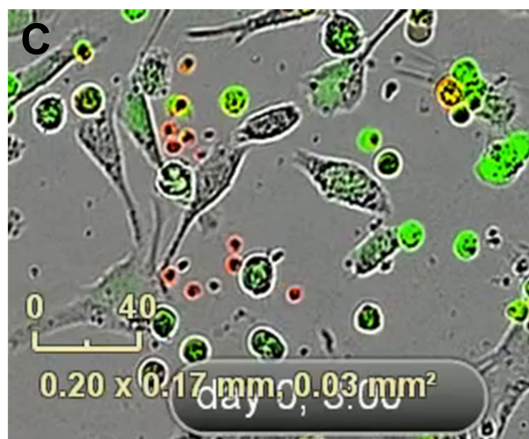
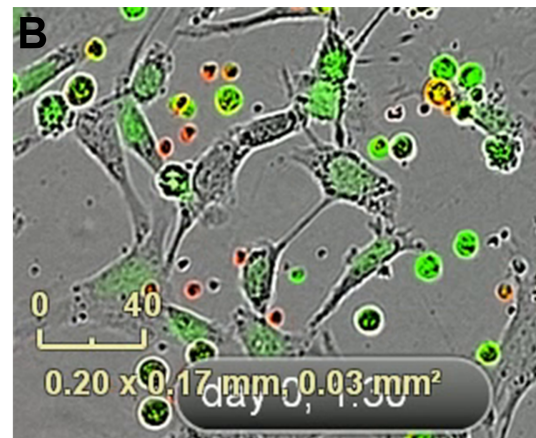
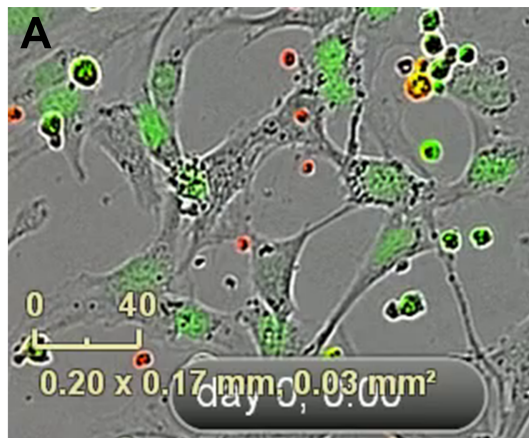
B6 and RIPK3<sup>-/-</sup> MVEC treated with 100ng/mL mouse IFN- $\gamma$  after 24 hours were challenged with MLR activated bm12 CD4<sup>+</sup> T-cells and cell death was measured after seven hours using 7-AAD staining. Interactions between CD4<sup>+</sup> T-cell and B6 MVECs were captured using the IncuCyte ZOOM real time imaging system (Figure 9). Flow cytometry analysis indicated that bm12 CD4<sup>+</sup> T-cells induced cell death in both CFSE-gated B6 and RIPK3<sup>-/-</sup> MVEC (Figure 10). There was significantly higher 7-AAD positive cells in B6 MVECs when compared with RIPK3<sup>-/-</sup> MVECs (22.89 $\pm$ 6.67% vs. 16.68 $\pm$ 4.61%; n=6 per group, p=0.045). A similar result was obtained with GSK'872, a RIPK3 inhibitor, as cell death was significantly reduced in RIPK3 inhibited B6 MVEC when compared with untreated (15.44 $\pm$ 2.04% vs. 22.89 $\pm$ 6.67%; n=6 per group, p=0.013). When compared with untreated B6 MVECs (22.89 $\pm$ 6.67%; n=6), no significant differences were observed in Necrostatin-1 treated B6 MVECs (24.56 $\pm$ 6.71%; n=6, p=0.34), or Z-VAD-fmk and Necrostatin-1 treated B6 MVEC (21.10 $\pm$ 4.81%, n=6, p=0.30, Figure 10).





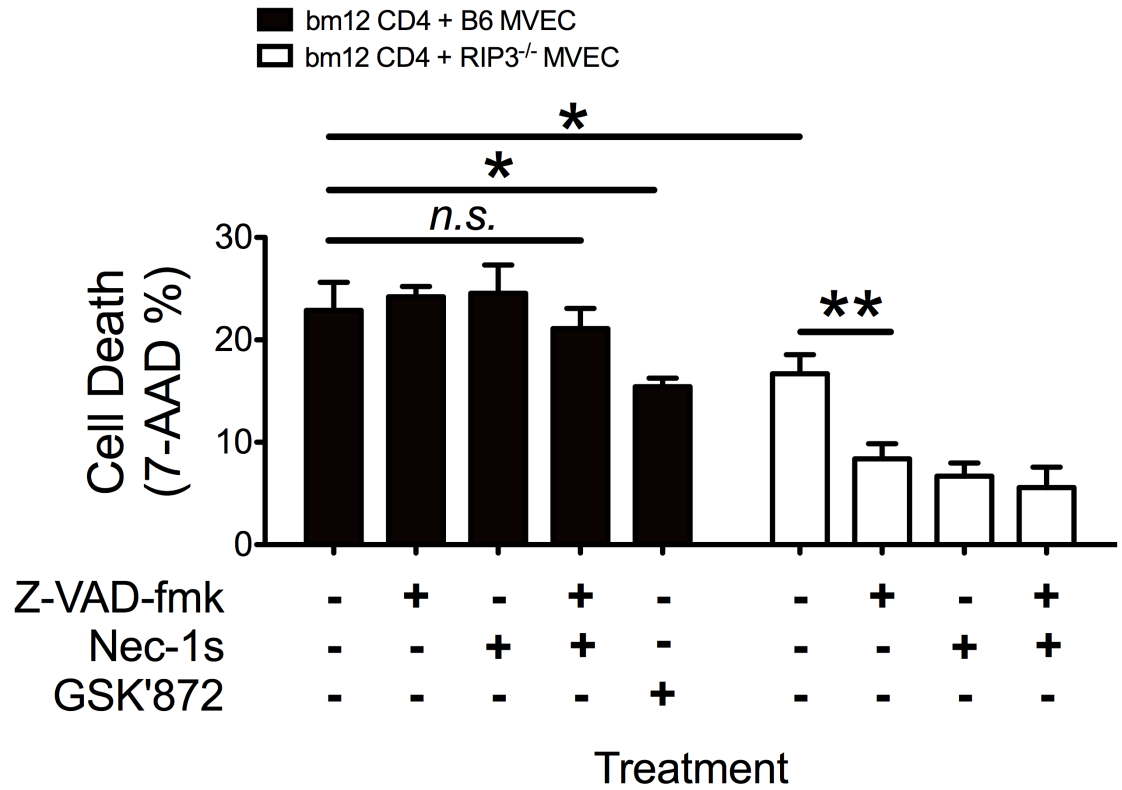
**Figure 8. Characterizing surface proteins of mouse interferon-gamma treated MVEC.**

B6 and RIPK3<sup>-/-</sup> MVECs were characterized before and after 24 hours of 100ng/mL mouse interferon-gamma (IFN- $\gamma$ ) treatment and the surface proteins CD31, CD54 (ICAM-1), CD324 (E-cadherin), Fas, MHC Class I, and MHC Class II were detected by flow cytometry. Histograms are representative of three separate experiments.



**Figure 9. CD4<sup>+</sup> T-cells lyse B6 MVEC *in vitro*.**

Bm12 CD4<sup>+</sup> T-cells were stained with Far Red and B6 MVEC with membrane impermeable nucleic acid stain SYTOX® Green. Cells appear bright green and round when they lose membrane integrity and undergo necrosis. (A-B) At hour 0, red CD4<sup>+</sup> T-cells are added to adherent, healthy MVECs. (C) T-cells interact with MVEC. (D) At hour 3, red CD4<sup>+</sup> T-cells (arrows) approach and come in close proximity of MVECs. (E) At hour 4.5, MVECs appear rounded and fluoresce bright green, indicative of SYTOX® Green binding with free nucleic acids released by MVECs undergoing necrosis. (F) By hour 6, most of MVECs have undergone necrosis.



**Figure 10. B6 MVEC are more sensitive than RIPK3<sup>-/-</sup> MVEC to alloactivated CD4<sup>+</sup> T-cell-induced death *in vitro*.**

CFSE-stained B6 and RIPK3<sup>-/-</sup> MVEC were pre-treated with pan-caspase inhibitor Z-VAD-fmk, RIPK1 inhibitor Necrostatin-1s (Nec-1s), or RIPK3 inhibitor GSK'872 for 30 minutes and co-cultured with day 6 MLR activated bm12 CD4<sup>+</sup> T-cells. Cell death was measured by flow cytometric detection of 7-AAD. Data shown as mean±SEM and are representative of three independent experiments (n=3). \*\* $p \leq 0.01$  \* $p \leq 0.05$ .

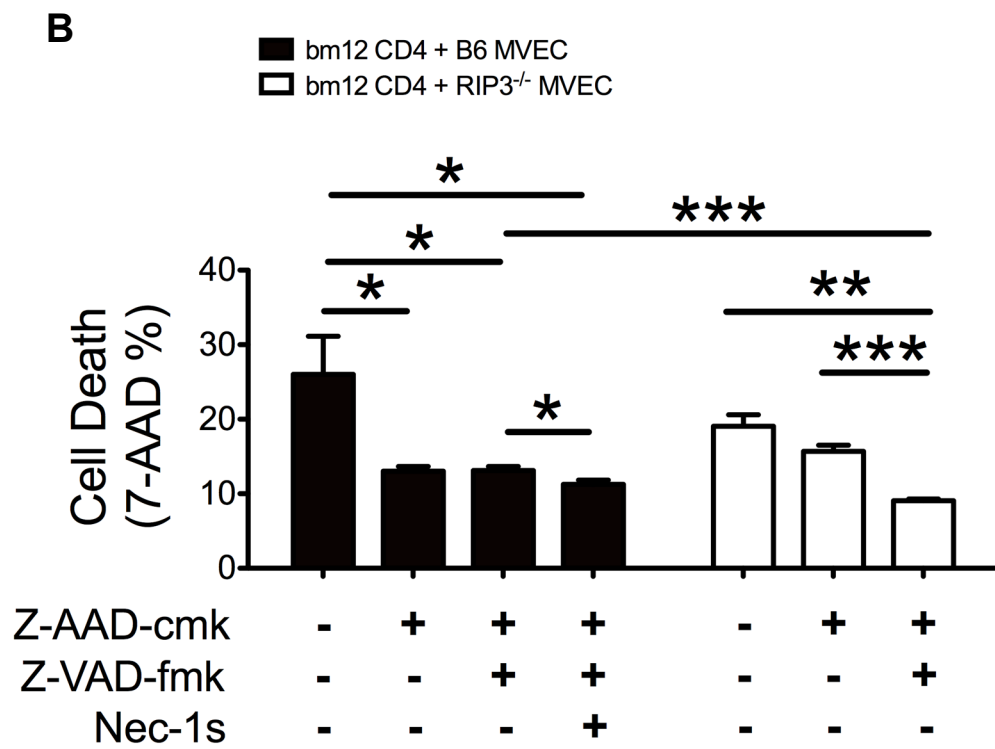
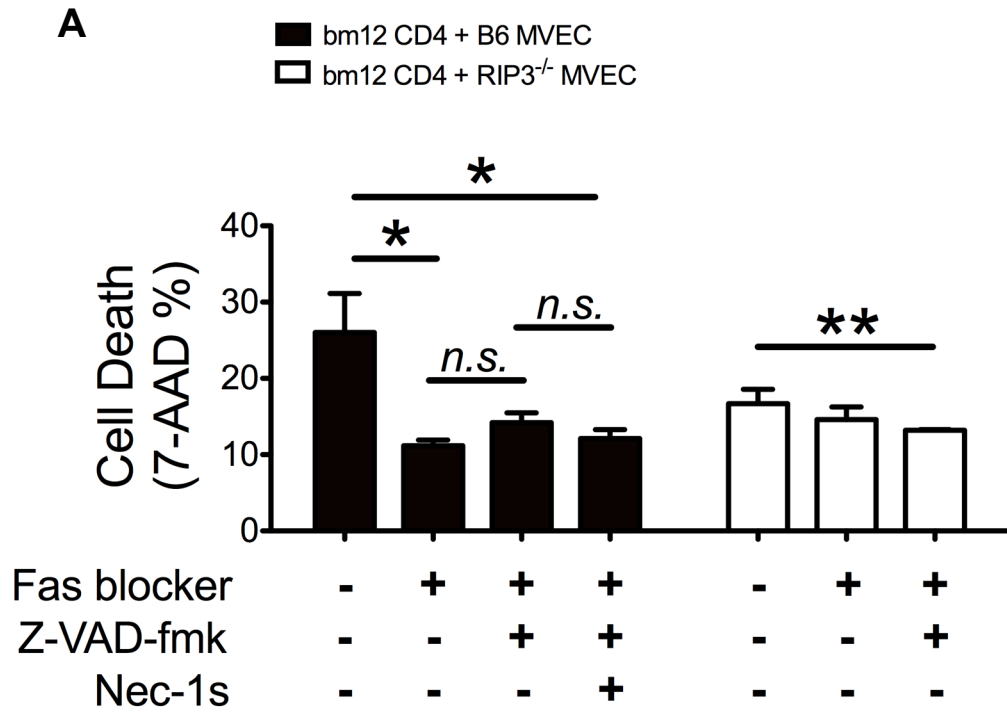
### 3.6 CD4<sup>+</sup> T-cell induced death are mediated by both FasL-Fas and granzyme B/perforin interactions

To further determine the mechanisms of CD4<sup>+</sup> T-cell-induced MVEC death, cells were treated with Fas blocker and granzyme B inhibitor. Treatment with the Fas blocker, FasFc, in B6 MVEC significantly reduced cell death when compared with untreated ( $11.17 \pm 1.29$  vs.  $26.02 \pm 8.85\%$ ;  $n=3$ ,  $p=0.023$ , Figure 11a). Though no significant difference was detected in 7-AAD staining between Z-VAD-fmk and FasFc treated and B6 MVEC treated with FasFc only ( $14.20 \pm 2.24\%$  vs.  $11.17 \pm 1.29\%$ ;  $n=3$ ,  $p=0.056$ , Figure 11a), an increase in cell death was observed. Furthermore, no significant differences were detected between cells treated with Fas blocker, Z-VAD-fmk, and Necrostatin-1s when compared with B6 MVECs treated with Fas blocker and Z-VAD-fmk ( $12.13 \pm 2.01\%$  vs.  $14.20 \pm 2.24\%$ ;  $n=3$ ,  $p=0.149$ ), but a significant decrease in cell death was detected when compared with untreated B6 MVECs ( $12.13 \pm 2.01\%$  vs.  $26.02 \pm 8.85\%$ ;  $n=3$ ,  $p=0.0284$ ).

No significant difference in 7-AAD positivity was detected between untreated RIPK3<sup>-/-</sup> MVECs and cells treated with FasFc ( $19.07 \pm 2.66\%$  vs.  $14.61 \pm 2.85\%$ ;  $n=3$ ,  $p=0.059$ ). There was a significant reduction in cell death in Z-VAD-fmk and FasFc treated RIPK3<sup>-/-</sup> MVECs when compared with untreated ( $13.21 \pm 0.13\%$  vs.  $19.07 \pm 2.66\%$ ;  $n=3$ ,  $p=0.009$ ). This data indicates that CD4<sup>+</sup> T-cells use Fas ligand to induce B6 MVEC cell death that cannot be further inhibited by caspase inhibition.

To determine whether cytotoxic CD4<sup>+</sup> T-cells use granzyme B as a mechanism to induce MVEC death, the granzyme B inhibitor, Z-AAD-cmk was added. There was a significant decrease in cell death in Z-AAD-cmk treated B6 MVECs ( $13.03 \pm 1.11\%$  vs.  $26.02 \pm 8.85\%$ ;  $n=3$ ,  $p=0.032$ , Figure 11b). A significant decrease in 7-AAD was also detected in Z-AAD-cmk and Z-VAD-fmk treated B6 MVECs compared with untreated ( $13.11 \pm 0.96\%$  vs.  $26.02 \pm 8.85\%$ ;  $n=3$ ,  $p=0.032$ ). A significant decrease in cell death was detected in B6 MVECs treated with Z-AAD-cmk, Z-VAD-fmk and Necrostatin-1s when compared with cells treated with Z-AAD-cmk and Z-VAD-fmk only ( $11.27 \pm 1.00\%$  vs.  $13.11 \pm 0.96\%$ ;  $n=3$ ,  $p=0.041$ ) and when compared with untreated B6 MVECs ( $11.27 \pm 1.00$  vs.  $26.02 \pm 8.85\%$ ;  $n=3$ ,  $p=0.0227$ ).

Though no significant difference in 7-AAD positivity between untreated and Z-AAD-cmk treated RIPK3<sup>-/-</sup> MVECs was detected (19.07±2.67% vs. 15.69±1.43%; n=3, p=0.062, Figure 11b), co-treatment with Z-AAD-cmk and Z-VAD-fmk together significantly reduced cell death (9.07±0.4% vs. 19.07±2.67%; n=3, p=0.0015) and when compared with just Z-AAD-cmk alone (9.07±0.4% vs. 15.69±1.43%; n=3, p≤0.0001). A significant reduction in cell death was found in RIPK3<sup>-/-</sup> MVEC when compared with B6 MVEC treated with both Z-AAD-cmk and Z-VAD-fmk (9.07±0.40% vs. 13.11±0.96; n=3, p=0.0012). Taken together, this data suggests that CD4<sup>+</sup> T-cells use a combination of Fas ligand and granzyme B to induce MVEC death.



**Figure 11. Allo-activated CD4<sup>+</sup> T-cells use Fas-FasL and granzyme B as cytolytic mechanisms against MVEC.**

CFSE-stained B6 and RIPK3<sup>-/-</sup> MVEC were pre-treated with (A) Fas blocker, pan-caspase inhibitor Z-VAD-fmk, or RIPK1 inhibitor Necrostatin-1s (Nec-1s) or (B) granzyme B inhibitor Z-AAD-cmk, Z-VAD-fmk, or Nec-1s for 30 minutes and co-cultured with MLR stimulated allo-activated bm12 CD4<sup>+</sup> T-cells. Cell death was measured with 7-AAD nucleic acid stain. Data shown as mean±SEM and are representative of three independent experiments. \*\*\* $p \leq 0.001$  \*\* $p \leq 0.01$  \* $p \leq 0.05$ .



### 3.7 Summary of Results

In this study, we have shown that genetic deletion of RIPK3 in donor cardiac grafts significantly improved graft survival in a single MHC Class II mismatch chronic rejection model and that RIPK3 deficiency protected MVECs from TNF- $\alpha$  and allo-activated CD4<sup>+</sup> T-cell-induced necroptosis *in vitro*. Furthermore, in addition of TNF- $\alpha$ , allo-stimulated CD4<sup>+</sup> T-cells *in vitro* use both mechanisms of granzyme B and FasL to induce MVEC death.

## Chapter 4

### 4 Discussion

#### 4.1 Heart Transplantation Model

Despite advances in immunosuppression and improvement of acute rejection outcomes of heart grafts, chronic rejection still remains an obstacle in long-term graft survival. It is well established that T-cells, B-cells, macrophages, NK-cells, neutrophils and the complement pathway contribute to chronic graft rejection.<sup>35,119,126,234-238</sup> In this study, we chose a well-defined single MHC Class II mismatched model characteristic of its CAV development and chronic heart rejection.

The B6.C-H-2<sup>bm12</sup> (bm12) has a spontaneous mutation of the I-A<sup>b</sup> molecule resulting in a three amino acid substitution in the MHC class II antigen.<sup>239</sup> These mice develop severe vasculopathy characteristic of chronic rejection, which allows for investigation into immunological rejection mechanisms specific to the involvement of CD4<sup>+</sup> T-cells. Although genetic deletion of RIPK3 in donor graft attenuated rejection and significantly improved graft survival, a large sample number was needed to achieve significance. This was unexpected as the bm12 heart transplant model without immunosuppression is well reviewed in literature.

The general consensus of heart graft survival in the B6 to bm12 heart transplant model is beyond day 50,<sup>62,73,237,240-250</sup> and even day 80,<sup>251-254</sup> while severe CAV is observed by Day 24.<sup>36,73,255</sup> In our study, we unexpectedly found that chronic rejection events occurred earlier when compared to other studies using the bm12 model (earliest rejection day=17). It has been noted previously that this cardiac allograft transplantation model is variable, and one other group also obtained similar results of early rejection events with their bm12 recipient.<sup>256</sup>

It is possible that the more pronounced rejection events observed in our study could be attributed to a lower or altered ratio of Treg to Teff cells as regulatory T-cells play a crucial inhibitory role to control the size of Teff cell pool following heart

transplants.<sup>247,257</sup> Effector T-cell pool is influenced by the ability of responding precursor cells to optimally expand during antigen priming, and clonal expansion is influenced by the number of APCs, presence of costimulatory molecules and amplifying cytokines. A threshold number of effector T cells are required for rejection of allograft. Treg cells that develop later on after transplantation cannot limit the expansion of allo-reactive T-cells. Acute rejection of bm12 allografts in this model can be inhibited by CD25<sup>+</sup> Treg that restrict clonal expansion of allo-reactive T-cells.<sup>257</sup> This model seems to be Treg dependent, as B7:CD28 costimulatory signaling blockade with CTLA-4-Ig actually worsened chronic transplant rejection due to a higher effector T-cell/regulatory T-cell ratio as CTLA-4-Ig blocking impairs Treg development.<sup>258</sup> Others have reported that using a specific antibody that inhibits activation of naïve T-cells does not induce significant cytokine release *in vivo*, hence, selective CD28 blockade can attenuate chronic cardiac allograft rejection when combined with CD154 blockade or calcineurin inhibition.<sup>259</sup> Further assessment of Treg/Teff ratio in *in vivo* models at the time of heart collection could confirm these speculations.

Another limitation of this model is that the resultant injury perceived *in vivo* is not limited to CD4<sup>+</sup> T-cells. Carotid transplantation between various genetic models of mice found that active involvement of macrophages, B-cells and CD4<sup>+</sup> T-cells formed the immunologic basis of transplant-associated vasculopathy.<sup>35</sup> However, another group later found that CAV is reduced in a single MHC Class I mismatch miniature swine model after anti-CD8 monoclonal antibody therapy. It was suggested that CD8<sup>+</sup> T-cells may play a more important role early in the initiation of CAV.<sup>71</sup> Interestingly, direct recognition of mismatched MHC Class II on endothelial cells is not sufficient to initiate rejection.<sup>260</sup> It follows that CD8<sup>+</sup> T-cells could contribute to vasculopathy once activated by cross priming (exogenous antigens presenting on MHC class I as indirect presentation).<sup>126,42,261</sup> While CAV development is contingent on CD4<sup>+</sup> T-cells,<sup>62</sup> it has been suggested that cross-primed CD8<sup>+</sup> lymphocytes play a significant and additive role in vasculopathy by cytotoxic activity and IFN- $\gamma$  secretion.

Fischbein et al. suggested that CD8<sup>+</sup> T-cell activation is dependent on CD154-CD40 interaction by CD4<sup>+</sup> T-cells, which then act to augment chronic rejection in this

model.<sup>42,261</sup> Furthermore, it was demonstrated that vasculopathy is less severe in an allo-specific CD4<sup>+</sup> TCR transgenic mouse that is reactive to the bm12 mutation of the MHC Class II when compared with WT recipients, providing further proof that indirect allo-recognition play a major role in this process.<sup>254</sup> Since intimal thickening can occur in a model where host cells have a markedly reduced capacity in peptide presentation/indirect allo-reactivity,<sup>126</sup> other cells that can participate in effector functions are Th17 T-cells,<sup>62</sup> eosinophils,<sup>262</sup> or  $\gamma\delta$  T-cells.<sup>241</sup>

Perhaps a more sophisticated mouse model that is tailored to examining the specific cytotoxic effects of CD4<sup>+</sup> T-cells could be developed to eliminate other effector cells (macrophages, CD8, B-cells) by monoclonal antibody depletion in the bm12 recipient after wild type or RIPK3<sup>-/-</sup> heart transplantation.

In addition, as mice are maintained in a non-sterile environment, infections are likely after the surgery that could result in accelerated graft survival.<sup>263</sup> Nonetheless, our data showed that RIPK3 heart graft survives longer than wild type, and our study suggests that RIPK3 would be a good target for long-term graft survival induction.

## 4.2 Mechanisms of CD4<sup>+</sup> T-cell-mediated EC death

Our improved understanding of cell death pathways in recent years has led to characterization of various forms of necrotic death, such as necroptosis, parthanatos, oxytosis, ferroptosis, ETosis, NETosis, pyronecrosis and pyroptosis. It is currently unknown whether donor EC undergo these forms of cell death after heart transplantation. This study is the first to show that RIPK3 deficiency is protective of CD4<sup>+</sup> T-cell induced death.

Although cytotoxic activity has been regarded as a property of CD8<sup>+</sup> T-cells, there is unambiguous evidence that CD4<sup>+</sup> T-cells express IFN- $\gamma$  and perforin<sup>84</sup> and exert MHC Class II restricted cytotoxic activity<sup>76,83,84,92</sup> in clearing infectious diseases and cancer pathogenesis.<sup>109</sup> CD4<sup>+</sup> T-cells alone are sufficient to mount both acute<sup>260</sup> and chronic rejection responses. There is considerable debate on whether cytotoxic CD4<sup>+</sup> T-cells use FasL or perforin as its predominant cytotoxic mechanism. In an acute cardiac rejection

model, FasL<sup>113</sup> is the predominant cytotoxic mechanism used by CD4<sup>+</sup> T-cells *in vitro* while both FasL and perforin equally contribute to acute cardiac rejection *in vivo*.<sup>264</sup> Indeed, a combination of granzyme B and perforin induces endothelial cell apoptosis which contributes to luminal narrowing of transplant vascular disease<sup>111</sup> However, in a minor histocompatibility mismatched vasculopathy model, it was perforin that played a primary role in early endothelial damage and resultant onset of vascular disease, which was abrogated in animals with genetic deletion of perforin.<sup>111</sup> In this study, we have found that in addition to granzyme B, TNF- $\alpha$  and FasL also contributes to EC death. However, it is still unknown why CD4<sup>+</sup> T cells induce necroptosis in EC without caspase inhibition. It is possible that granzyme B or FasL induce RIPK3 activation without inhibition of caspase-8. This is supported by other studies on TLR signaling that IFN- $\gamma$  mediate necroptosis without participation of caspase-8 or RIPK1.<sup>265</sup>

Flow cytometry following cell permeabilization showed that there was an increase of granzyme B and perforin of activated CD4<sup>+</sup> T-cells compared to naïve. It is possible that the MLR activated CD4<sup>+</sup> T-cells used for characterizing experiments did not robustly express granzyme B and perforin since they were stimulated *in vitro*. Others have reported that CD4<sup>+</sup> T-cells generated *in vivo* express a higher amount of granzyme B than FasL<sup>98</sup> and granzyme B production is heavily dependent on the amount of IL-2 stimulation.<sup>266</sup>

It would be interesting to compare granzyme B and perforin expression of *in vitro* and *in vivo* generated CD4<sup>+</sup> T-cells in recipient blood on the day of graft procurement. Despite the low amount of granzyme B and perforin detected, there was a moderate increase in surface CD107a, a protein normally located in granules and are a marker of degranulation. Furthermore, its functional capacity is evidenced in our *in vitro* assays.

While RIPK3 deficiency enhanced survival but did not completely block rejection, it is possible that RIPK3 deficiency may only protect MVECs from TNF- $\alpha$  and FasL-mediated necrotic death. Though it is not known whether RIPK3 plays a role downstream of perforin and granzyme B mediated apoptosis (effector caspase 3 and 9).

Histological examination showed that there is less CD4<sup>+</sup> T-cell infiltration in the RIPK3<sup>-/-</sup> hearts compared to wild type. This supports our hypothesis that RIPK3 deficiency has a protective effect on EC as the lack of necroptosis reduces cDAMPs and inflammation, which attracts CD4<sup>+</sup> T-cells by chemotaxis.

### 4.3 Conclusions

This study explored the mechanisms of alloantigen specific CD4<sup>+</sup> T-cell mediated cytotoxicity on MVEC. We found that abrogation of RIPK3-mediated necroptosis in a single MHC Class II mismatch mouse model attenuated graft survival but did not completely eliminate rejection. We report that CD4<sup>+</sup> T-cells use FasL and granzyme B *in vivo* as cytotoxic mechanisms to induce endothelial cell apoptotic and necroptotic death. This study suggests that RIPK1/3-mediated necroptosis contributes to chronic rejection in cardiac allografts and that RIPK1 and RIPK3 are important therapeutic targets in addition to apoptotic molecules to induce long-term graft survival.

### 4.4 Future Directions

An interesting follow up experiment would be to transplant RIPK3 deficient hearts into bm12 mice deficient in FasL or perforin to further explore mechanisms of cytotoxic CD4<sup>+</sup> T-cells. The addition of immunosuppression to B6 and RIPK3 mice would elucidate all the mechanisms behind CD4<sup>+</sup> T-cell mediated EC death. As it has been reported that perforin expression is differential between *in vivo* and *in vitro* stimulated CD4<sup>+</sup> T-cells, it would be interesting to compare CD4<sup>+</sup> T-cell cytotoxic mechanisms depending on its method of stimulation. This could be correlated to *in vivo* experiments using FACs to quantify granzyme B and perforin levels in blood serum of animals on day of heart transplant harvest.

Currently, there are no pharmacological inhibitors of cell death that are clinically approved. The possibility that cell death inhibitors can be added to immunosuppression regimens is exciting. Currently, Necrostatin-1 has considerable potential in the preclinical stage<sup>267</sup> and exciting Granzyme B inhibitors are in the process of development for treatment of aging and deteriorating skin.<sup>268</sup> The testing of granzyme B inhibitors to

prolong graft survival in an appropriate model is important, as differences between human and mouse granzyme B activity has been reported.<sup>269</sup> For instance, murine granzyme B is not as effective as human granzyme B at cleaving BID.<sup>270</sup>

It is possible that granzyme-mediated killing is used to regulate the T-cell response by fratricide.<sup>271</sup> CTL can acquire MHC Class I molecules from targets and present on its own cell surface with the peptide, rendering them susceptible to lysis by neighbouring CTLs. Hence, the consideration of using granzyme B inhibitors as a drug regimen will need to take into account of the (accidental expansion of) recipient Teff population.

There is evidence that in human vascular disease, cytotoxic CD4<sup>+</sup> T-cells interact with target cells via stress-induced hsp60<sup>272</sup> and there is increased extracellular hsp60 in COPD patient bronchials which correlate to increased neutrophil counts.<sup>273</sup> Exploring various cDAMPs that are released by CD4<sup>+</sup> T-cell induced necroptotic ECs and their effects on neighbouring cells could lead to further insights on inflammatory and graft survival consequences of CD4<sup>+</sup> T-cell mediated chronic heart rejection.

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# Appendices

## Appendix A: Animal Protocol (2007-096-10) Approval



**AUP Number:** 2007-096-10

**PI Name:** Zhang, Zhuxu

**AUP Title:** 1. Therapeutic Potential and Mechanism of Double-Negative Regulatory T (DN-Treg) Cell-Mediated Tolerance in Heart Transplantation 2. Regulation of pre-transplant ischemic injury and cardiac allograft vasculopathy

**Approval Date:** 01/13/2012

**Official Notice of Animal Use Subcommittee (AUS) Approval:** Your new Animal Use Protocol (AUP) entitled "1. Therapeutic Potential and Mechanism of Double-Negative Regulatory T (DN-Treg) Cell-Mediated Tolerance in Heart Transplantation 2. Regulation of pre-transplant ischemic injury and cardiac allograft vasculopathy" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2007-096-10::5

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

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

Submitted by: Copeman, Laura  
on behalf of the Animal Use Subcommittee  
University Council on Animal Care

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### Platform Presentations:

1. **Kwok C**, Pavlosky A, Huang XY, Haig A, Jevnikar A, Zhang Z. CD4<sup>+</sup> T-cell mediated microvascular endothelial cell death in a chronic cardiac allograft rejection model is independent of RIPK3. Canadian Society of Transplantation 2015 Annual Scientific Meeting, Oct 9, 2015, Vancouver, British Columbia.
2. Pavlosky A, **Kwok C**, Jevnikar A, Zhang Z. Caspase-8 has paradoxical function in ischemia-reperfusion injury versus transplantation due to differences in

intracellular pH. Canadian Society of Transplantation 2015 Annual Scientific Meeting, Oct 9, 2015, Vancouver, British Columbia.

3. **Kwok C**, Pavlosky A, Huang XY, Haig A, Jevnikar A, Zhang Z. RIPK3 deficiency does not protect microvascular endothelial cells from CD4<sup>+</sup> T cell-mediated chronic rejection in cardiac allograft transplantation. Western University Annual Department of Medicine Research Day, May 7, 2015, London, Ontario.

### **Poster Presentations**

1. **Kwok C**, Pavlosky A, Huang XY, Haig A, Jevnikar A, Zhang Z. RIPK3 deficiency does not protect microvascular endothelial cells from CD4<sup>+</sup> T cell-mediated chronic rejection in cardiac allograft transplantation. London Health Research Day, April 1, 2015, London, Ontario.
2. **Kwok C**, Pavlosky A, Huang XY, Haig A, Jevnikar A, Zhang Z. Microvascular endothelial cells undergo RIPK3 independent, CD4<sup>+</sup> T-cell mediated death in chronic cardiac allograft rejection. Western University Department of Pathology and Laboratory Medicine Research Day, March 30, 2015, London, Ontario, Canada.
3. **Kwok C**, Lau, A, Pavlosky A, Jevnikar A, & Zhang Z (2014). Characterizing the impact of membrane vesicles produced by apoptotic and necrotic tubular epithelial cells on ischemia reperfusion injury and transplant rejection. Western University Department of Medicine Research Day, May 15, 2014, London, Ontario.
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