The Endothelial Response to Injury: Defining the Role of Epidermal Growth Factor-Like Domain 7 and Endothelial Protective Strategies

by

Mitesh Vallabh Badiwala, B.Sc., M.D.

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Institute of Medical Science
University of Toronto

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The Endothelial Response to Injury:
Defining the Role of Epidermal Growth Factor-Like Domain 7 and Endothelial Protective Strategies

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Doctor of Philosophy
Institute of Medical Science
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2013

Abstract

Background: Currently, the optimal long-term therapy for end stage heart failure is heart transplantation. Cardiac allograft vasculopathy contributes to a significant number of deaths following transplantation. This vasculopathy is related to early endothelial injury sustained at the time of organ transplantation and to persistent endothelial injury as a result of cytotoxic immunosuppression, as well as chronic rejection.

Epidermal growth factor-like domain 7 (Egfl7), is expressed in endothelial cells upon arterial injury and may have a role in maintaining vascular endothelial integrity and regeneration following injury. Similarly, novel pharmacologic agents such as Bosentan, an endothelin-1 antagonist, and Cilostazol, a phosphodiesterase 3 inhibitor, have been demonstrated to attenuate calcineurin inhibition induced endothelial dysfunction and neointimal hyperplasia, respectively. We hypothesized that, 1) Egfl7 will attenuate endothelial activation, cell adhesion molecule expression and neutrophil adhesion following simulated ischemia-reperfusion injury or exposure to calcineurin inhibition and that, 2) Bosentan and Cilostazol will inhibit neointimal hyperplasia following endothelial injury in a mouse model of vascular injury.
Methods: Human coronary artery endothelial cells were subjected to hypoxia-reoxygenation injury or the calcineurin inhibitors Cyclosporine A and Tacrolimus to examine the effects of Egfl7 on these injury mechanisms. Cell adhesion molecule expression, neutrophil adhesion to endothelial cells, and NF-κB activation were measured. Cell adhesion molecule and Egfl7 expression were also examined in a mouse model of neointimal. This model was used to examine the effects of Bosentan and Cilostazol on neointimal hyperplasia.

Results: Egfl7 had potent anti-inflammatory properties including inhibition of NF-κB pathway activation, ICAM-1 expression and neutrophil adhesion to injured endothelium. Within vessels exhibiting neointimal hyperplasia, Egfl7 was expressed in regions lacking ICAM-1 expression. Both cilostazol and bosentan attenuated neointimal hyperplasia in isolation as well as during co-treatment with CNI therapies.

Conclusions: Egfl7 is an endothelial protective signaling protein with anti-inflammatory properties effective against simulated ischemia-reperfusion injury and calcineurin inhibition mediated injury. Cilostazol and Bosentan are pharmacologic strategies demonstrating efficacy against the development of neointimal hyperplasia. These observations provide a novel therapeutic target and strategies that may be relevant to endothelial protection and prevention of cardiac allograft vasculopathy following heart transplantation.
Acknowledgments

First and foremost, I would like to thank the many mentors, colleagues, and friends within the Division of Cardiac Surgery, Department of Surgery and the Heart Transplant Program at the University of Toronto who have nurtured and supported my aspirations, both clinical and academic. Without their efforts, none of this work would have been possible.

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<tbody>
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<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>AGE</td>
<td>Advance glycation end-product</td>
</tr>
<tr>
<td>ARD</td>
<td>Ankyrin repeat domains</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>BH₄</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAV</td>
<td>Cardiac allograft vasculopathy</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CNI</td>
<td>Calcineurin inhibition</td>
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<tr>
<td>CPT</td>
<td>Cell preparation tube</td>
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<td>Centromere Binding Factor 1/Recombining Binding Protein-Jx/Suppressor of Hairless/Longevity Assurance Gene-1</td>
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<td>CyA</td>
<td>Cyclosporine A</td>
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<td>Diacylglycerol</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<td>DCFDA</td>
<td>Dichlorofluorescein diacetate</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>ECE</td>
<td>Endothelin converting enzyme</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>Egfl7</td>
<td>Epidermal growth factor-like domain 7</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>EGM</td>
<td>Endothelial cell growth medium</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<tr>
<td>ESL</td>
<td>E-selectin ligand</td>
</tr>
<tr>
<td>ET</td>
<td>Endothelin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FKBP</td>
<td>FK506 binding protein</td>
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<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>H/R</td>
<td>Hypoxia/Reoxygenation</td>
</tr>
<tr>
<td>H$_2$DCFDA</td>
<td>Dichlorodihydrofluorescein diacetate</td>
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<tr>
<td>HBEC</td>
<td>Human brain endothelial cell</td>
</tr>
<tr>
<td>HCAEC</td>
<td>Human coronary artery endothelial cell</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>Hey1</td>
<td>Hairy/enhancer-of-split related with YRPW motif protein 1</td>
</tr>
<tr>
<td>HIMEC</td>
<td>Human intestinal microvascular cell</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>HMG-CoA</td>
<td>3-Hydroxy-3-methylglutaryl-coenzyme A</td>
</tr>
<tr>
<td>HMVEC</td>
<td>Human microvascular endothelial cell</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>I/R</td>
<td>Ischemia/Reperfusion</td>
</tr>
<tr>
<td>I:M</td>
<td>Intima to Media</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgSF</td>
<td>Immunoglobulin superfamily</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>IVUS</td>
<td>Intravascular ultrasound</td>
</tr>
<tr>
<td>Jag1</td>
<td>Jagged1</td>
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<tr>
<td>LAD</td>
<td>Left anterior descending</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LFA</td>
<td>Lymphocyte function-associated antigen</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Mac-1</td>
<td>Macrophage-1 antigen</td>
</tr>
<tr>
<td>MAdCAM</td>
<td>Mucosal vascular addressin cell adhesion molecule</td>
</tr>
<tr>
<td>MAIL</td>
<td>Molecule possessing ankyrin-repeat induced by lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>MMF</td>
<td>Mycophenolate mofetil</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export sequences</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κ-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization sequences</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet-activating factor</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase enzyme</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PEST</td>
<td>Proline, glutamate, serine and threonine</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PI3KR2</td>
<td>Phosphoinositide 3-kinase regulatory subunit 2</td>
</tr>
<tr>
<td>PNAd</td>
<td>Peripheral node addressins</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PNT</td>
<td>Peroxynitrate</td>
</tr>
<tr>
<td>PO$_2$</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PSGL</td>
<td>P-selectin glycoprotein ligand</td>
</tr>
<tr>
<td>PSI</td>
<td>Proliferation signal inhibitor</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp1-Cullin1-Roc1-F-box</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>Spred-1</td>
<td>Sprouty-related, EVH1 domain-containing protein 1</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>Tac</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>TACE</td>
<td>TNF-$\alpha$ converting enzyme</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VE</td>
<td>Vascular endothelial</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLA</td>
<td>Very late antigen</td>
</tr>
<tr>
<td>XTT</td>
<td>2,3-Bis(2-methoxy-4-nitro-5- sulfophenyl)-2H-tetrazolium-5-carboxanilide</td>
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Chapter 1
KNOWLEDGE TO DATE
1.1 Introduction
Heart failure remains the only cardiovascular diagnosis increasing in incidence.\textsuperscript{1, 2} Currently, the accepted optimal long-term therapy for end stage heart failure is heart transplantation in eligible recipients. However, the increasing number of patients eligible for transplant far outstrips the organs available for transplantation. Over the past decade, nearly 25\% of patients died while waiting for a heart transplant in Canada.\textsuperscript{3} Because of the scarcity of donor organs, heart transplant programs are forced to restrict available organs to the “best” recipients. This rationing has precluded many patients from consideration for heart transplantation, despite the fact that transplant remains their best (and sometimes only) option for therapy. Thus, optimizing the outcomes of transplanted hearts is of critical importance.

Those patients fortunate enough to receive a heart transplant still suffer from early and late complications that limit their survival and quality of life. The median survival following heart transplantation is currently 10 years, and is 13 years for patients surviving the first year after transplantation.\textsuperscript{4} Complications that occur early after transplantation are predominantly related to primary graft failure. Primary graft failure after heart transplantation is defined as the presence of severe cardiac mechanical dysfunction without obvious anatomic (surgical) or immunologic causes such as hyperacute rejection.\textsuperscript{5} Primary graft failure may result from the use of marginal donor allografts, prolonged ischemic times and/or inadequate myocardial preservation. Complications that occur late following transplant are predominantly attributed to infection, malignancy, and the development of diffuse intimal hyperplasia (thickening of the intimal layer of the arterial wall resulting in narrowing of arterial lumen), otherwise
known as cardiac allograft vasculopathy (CAV).\textsuperscript{4} Currently, CAV is responsible for between 11 and 14% of deaths beyond the first year following heart transplantation.\textsuperscript{4}

CAV may be related to early endothelial injury sustained at the time of organ transplantation, persistent antibody mediated rejection, and lastly, persistent endothelial injury as a result of cytotoxic immunosuppressive agents.\textsuperscript{6} Angiographic studies have documented CAV in up to 45% of transplant recipients as early as 3 years following transplantation.\textsuperscript{6, 7} The use of intravascular ultrasound imaging of the coronary arteries has demonstrated intimal thickening in 58% of patients at 2 years.\textsuperscript{8} Unfortunately, the pattern of CAV tends to be diffuse and involves the entire coronary vascular tree, which distinguishes itself from focally stenotic coronary artery disease that is amenable to angioplasty, stenting or bypass surgery.

Therefore, the only therapeutic option available to individuals with severe CAV is re-transplantation. However, survival following re-transplantation is poorer than after primary transplantation and this remains an option only in very highly selected patients.\textsuperscript{9} Thus, the major protective strategy against CAV is prevention. However, despite best efforts to date, the incidence of CAV following heart transplantation continues to be constant over time (Figure 1-1).\textsuperscript{4} The identification of novel targets to attenuate endothelial injury and subsequent intimal hyperplasia that underlies CAV will lead to new treatment strategies aimed at averting CAV after transplantation. In doing so, the durability and longevity of heart transplantation will improve in our patients.
Figure 1-1. Freedom from Cardiac Allograft Vasculopathy for Adult Heart Recipients. Reprinted from The Journal of Heart and Lung Transplantation, Vol. 29, Stehlik J, et al., The Registry of the International Society for Heart and Lung Transplantation: Twenty-seventh official adult heart transplant report—2010, Pages 1089-1103, Copyright (2010), with permission from Elsevier.
1.2 Endothelial Injury in Heart Transplantation

Endothelial cells are arranged along the lumen of blood vessels in a contiguous monolayer. The uninjured endothelium serves to maintain vascular homeostasis by regulating vascular tone, inhibiting both immune cell activation and vascular smooth muscle cell proliferation, and preventing thrombus formation. Central to normal endothelial function is the existence of preserved endothelial nitric oxide (NO) production/availability by endothelial nitric oxide synthase (eNOS) that catalyzes the production of NO from L-arginine. NO is a potent vasodilator that serves to maintain basal vascular smooth muscle tone while counteracting the effects of vasoconstrictors such as endothelin-1 (ET-1). Importantly, NO inhibits the adherence of circulating leukocytes and platelets to the endothelium, and suppresses the expression of cell adhesion molecules and inflammatory cytokines.

Following heart transplantation, evidence of endothelial dysfunction is demonstrated by impaired endothelium-dependent vasomotor function and dysregulation of the eNOS system. Early endothelial dysfunction has been demonstrated to predict the development of CAV as early as 1 year following transplantation. In the setting of heart transplantation, there are a number of factors that result in endothelial injury including the mechanism of brain death, ischemia/reperfusion injury around the time of donor organ procurement and transplantation, alloimmune responses underlying the process of rejection, and viral infections such as Cytomegalovirus (CMV). Following transplantation, a number of metabolic abnormalities that adversely affect endothelial function can occur as a result of immunosuppressive therapies. These conditions include hypertension, diabetes mellitus, hypercholesterolemia, hypertriglyceridemia, and hyperhomocysteinemia. In particular, immunosuppression using calcineurin-inhibitors (CNIs) is associated with the development
of some of these metabolic conditions, and moreover, can directly cause endothelial injury and dysfunction.\textsuperscript{19-24} Ultimately, this multifactorial basis for endothelial injury in the context of heart transplantation is thought to initiate and propagate the proliferative intimal hyperplastic response to injury that underlies CAV.\textsuperscript{10, 16, 18}

1.2.1 Ischemia/Reperfusion (I/R) Injury

I/R injury is defined as damage that occurs following the reperfusion of previously viable but ischemic tissues.\textsuperscript{25} Ischemia, defined as the absolute or relative shortage of blood supply (which includes oxygen and other blood-borne nutrients) to tissues or organs, results in well described organ dysfunction – in the case of myocardial ischemia, diminished myocardial function occurs. Though restoring adequate blood flow to an ischemic organ is vital to preventing irreversible dysfunction due to tissue death, the act of reperfusion itself is known to be capable of causing cellular damage which exceeds that of ischemia alone.\textsuperscript{26}

Injury of coronary endothelial cells following I/R can occur in a number of clinical settings including reperfusion following cardioplegic arrest during cardiac surgery.\textsuperscript{27} In particular, I/R induced endothelial injury that occurs with cardiac allograft arrest, storage and subsequent reperfusion during heart transplantation is believed to be a key event that incites development and progression of CAV.\textsuperscript{28-31} Therefore, therapies aimed at the preservation of endothelial function during the acute and early phases of heart transplantation have the potential to reduce the incidence of both primary graft dysfunction and CAV.

Several changes occur in the endothelial cell with ischemia, and these include the repression of eNOS which results in decreased bioavailability of NO, the expression of proinflammatory gene products such as cell adhesion molecules and cytokines, and the production of endothelins.\textsuperscript{32-35} These changes induced by ischemia result in a proinflammatory state that
primes the endothelium for further injury upon reperfusion. Upon reperfusion, nitric oxide is rapidly consumed as it combines with reactive oxygen species (ROS) including superoxide anions to produce the free radical, peroxynitrate (PNT). PNT then goes on to hydroxylate and nitrate aromatic compounds, which leads to protein and DNA degradation.

Tetrahydrobiopterin (BH₄) has been demonstrated to be an essential co-factor for eNOS. In cell culture studies, Shiono et al. demonstrated that exposure of cardiomyocytes to L-arginine led to enhanced nitric oxide production and decreased cellular injury following ischemia and reperfusion. A similar effect was observed when cells were exposed to BH₄. Other investigators have demonstrated in both regional and global ischemic models that L-arginine supplemented cardioplegia reduces infarct size, decreases neutrophil accumulation and improves coronary endothelial function.

While activated neutrophils are considered the primary source of ROS following I/R injury, endothelial cells can produce ROS upon I/R injury via a number of pathways. These sources of ROS include production via uncoupling of eNOS (secondary to decreased bioavailability of BH₄ or L-arginine) or via NADPH oxidase activation, and/or by oxidation of hypoxanthine which accumulates during ischemia due to adenine nucleotide catabolism. With regard to the effect of ROS on endothelial function, Seccombe et al. demonstrated that direct exposure of coronary arteries to oxygen radicals in a canine model resulted in impaired endothelium-dependent vasomotor function.

Hypoxia/reoxygenation (H/R), which simulates I/R in a cell culture model, is known to injure endothelial cells through oxidant production, which leads to the expression of inflammatory cytokines and activates inflammatory cell signaling pathways. This endothelial inflammatory response includes the expression of cell surface adhesion molecules such as
ICAM-1, VCAM-1 and E-selectin. In addition, activated endothelial cells produce and release cytokines such as MCP-1, IL-1 and IL-8 which, in conjunction with the increased expression of cellular adhesion molecules, promote recruitment and adhesion of circulating leukocytes. Once bound to the activated endothelium, these leukocytes migrate through the endothelial layer into the media and produce oxygen derived free radicals and toxic inflammatory cytokines.

Early endothelial injury and activation following I/R injury is thought to predispose the vasculature to persistent immune mediated injury following heart transplantation leading to CAV. I/R injury has been demonstrated to stimulate major histocompatibility (MHC) class II expression and the shedding of donor antigens from allograft endothelial cells. I/R injury has also been shown to up-regulate the release of innate immune ligands such as heat shock proteins, which may then be detected by the toll-like receptor-4 (TLR-4) on mononuclear cells. These events likely promote both the allo- and innate immune responses including cellular and vascular rejection, and are thought to increase the risk of subsequent CAV. In a rodent model of aortic allograft transplantation, Gohra et al. demonstrated that I/R causes endothelial injury that results in allograft vasculopathy, akin to CAV, within 60 days of transplantation. Interestingly, I/R injury also caused similar, albeit less severe, vasculopathy in isografts transplanted in this model, suggesting that I/R injury may stimulate an endothelial response to injury that is unrelated to alloimmune mediated mechanisms of rejection. In another model of rodent heterotopic heart transplantation, Poston et al. demonstrated that inducing an inflammatory state in donor animals using lipopolysaccharide 24 hours prior to organ procurement resulted in more severe reperfusion injury and allograft vasculopathy following transplantation. These findings were also associated with increased
ICAM-1 expression, while recipient treatment with an anti-ICAM-1 monoclonal antibody was able to attenuate both reperfusion injury and allograft vasculopathy in this model.\textsuperscript{28}

In summary, I/R induced endothelial injury that occurs during heart transplantation seems to cause endothelial dysfunction as a result of deranged NO homeostasis and injury from ROS. I/R injury also stimulates the production of cytokines and cell adhesion molecules which renders the endothelium in a proinflammatory state that attracts circulating leukocytes. Furthermore, I/R injury has been implicated in the initiation of ongoing immune mediated rejection that is thought to play a role in the development of CAV.

1.2.1.1 NF-κB Pathway

1.2.1.1.1 NF-κB Proteins

Nuclear factor-κB (NF-κB) proteins refers to group of structurally related, inducible transcription factors that are ubiquitously expressed and phylogenetically conserved down to the Drosophila species.\textsuperscript{79} Activation of the NF-κB pathway plays a major role in the expression of a host of proinflammatory genes coding for a variety of cytokines and cell adhesion molecules including ICAM-1, VCAM-1, E-selectin, TNFα, IL-1, IL-6, IL-8, MCP-1, tissue factor, plasminogen activator inhibitor-1, and cyclo-oxygenase-2.\textsuperscript{79} Members of the NF-κB family include NF-κB1 (p50), RelA(p65), RelB, c-Rel, and NF-κB2 (p52).\textsuperscript{79} These proteins are characterized by a highly conserved 300-amino acid Rel homology domain (RHD) made of two immunoglobulin-like structures.\textsuperscript{80} The RHD allows for the dimerization and DNA binding functions of these proteins, as well as their interaction with their inhibitory proteins known as IκBs.\textsuperscript{80-82} Although NF-κB protein subunits can form either homo- or heterodimers, the most common active form is the p50/p65 heterodimer that binds to the
decameric consensus sequence GGGRNNYYCC (where R=G or A, Y=C or T and N=any nucleotide).  

### 1.2.1.1.2 NF-κB Inhibition by IκB Proteins

The activity of NF-κB is regulated by the IκB group of proteins, which include IκBα, IκBβ, IκBγ, IκBε, IκBζ, and MAIL (molecule possessing ankyrin-repeat domains induced by lipopolysaccharide). These proteins are characterized by six to eight copies of ankyrin repeat domains (ARD) that facilitate binding of IκB to the NF-κB dimers, while concealing nuclear localization sequences (NLS) on NF-κB. Aside from structural differences, IκB proteins differ from one another by their modes of activation and preference for binding to different NF-κB dimers. NF-κB dimers that contain RelA (p65) preferentially bind to IκBα and IκBβ. In its inactive state, NF-κB is retained in the cytoplasm through its association with IκB. In the most common scenario of IκBα binding to the p65/p50 complex, IκBα conceals the NLS on the p65 subunit but not on p50. This results in partial blockade of NF-κB nuclear translocation, however, IκBα contains nuclear export sequences (NES) that allows for active transport of the IκBα/NF-κB complex back to the cytoplasm. Since the effects of the NES dominate over that of the NLS, NF-κB is predominated located in the cytoplasm through its binding to IκBα.

### 1.2.1.1.3 Activation of NF-κB Pathway

Currently, there are in excess of 460 known activators of the NF-κB pathway. Those germane to endothelial biology include TNF-α, IL-1, LPS, advanced glycation end-products (AGEs), hyperglycemia, PAF, shear stress, oxidized lipids, oxidant stress and I/R injury. The upstream signaling pathways involved in transducing these stimuli into NF-κB activation are numerous and complex; however, they canonically converge on a complex of three
proteins known together as the IκB kinase (IKK) complex. The IKK complex consists of two catalytic subunits (IKKα and IKKβ) and one regulatory subunit (NEMO) that couples the catalytic subunits to upstream activators. Activation of the IKK complex by any of the upstream signaling pathways results in rapid phosphorylation of IκB proteins (Ser32 and Ser36 on IκBα, and Ser19 and Ser23 on IκBβ). Once phosphorylated, IκB is recognized by the E3RSIKB/β-TrCP receptor subunit of the Skp1-Cullin1-Roc1-F-box (SCF) ubiquitin ligase complex, is polyubiquitinated, and then degraded by the 26S proteasome. The liberation of IκB in this manner reveals the NLS on NF-κB which allows nuclear translocation of NF-κB to occur, where it binds to its consensus sequence on promoter/enhancer regions of genes, resulting in gene transcription (Figure 1-2).

While NF-κB pathway activation by most stimuli follows the canonical pathway described above, there is some evidence that stimuli such as exogenous oxidants, hypoxia and/or H/R can activate the NF-κB pathway via non-canonical pathways. For example, in murine T cells exposed to H₂O₂, IκBα is phosphorylated at Tyr42 and Thr/Ser residues within a proline, glutamate, serine and threonine (PEST) rich region of its C-terminal. Phosphorylation in this manner results in subsequent degradation by calpain proteases. Similarly, hypoxia has been demonstrated to induce NF-κB activation via IκBα phosphorylation at Tyr42. Furthermore, H/R has been demonstrated to result in c-Src-dependent tyrosine phosphorylation of IκBα independent of IKK activation. H/R has also been shown to activate p56/Lck tyrosine kinase, which similarly results in tyrosine phosphorylation of IκBα.
In the canonical NF-κB pathway, dimers of NF-κB such as p65/p50 are sequestered in the cytoplasm by interaction with IκB proteins such as IκBα. Upon signal mediated cell surface receptor activation, the IKK complex (containing IKKα and IKKβ catalytic subunits and the NEMO regulatory subunit) is assembled which then phosphorylates IκB at two serine residues. Phosphorylation of IκB leads to its ubiquitination and degradation by the proteasome. This degradation of IκB then allows the NF-κB dimer to translocate to the nucleus where it binds to its consensus sequence on promoter/enhancer regions of genes, resulting in gene transcription.
1.2.1.1.4  NF-κB Activation by I/R Injury Mechanisms

Oxidative stress has been demonstrated to induce NF-κB activation and a concomitant increase in monocyte adhesion to endothelial cells via induction of cell adhesion molecules in a rat model of endothelial injury.\textsuperscript{98} Conversely, inhibition of NF-κB activation using the proteasome inhibitor, MG132, or oligonucleotide decoys for NF-κB binding have been shown to prevent increased cell surface expression of E-selectin with a concomitant decrease in neutrophil adhesion to anoxia/reoxygenation injured endothelial cells in monolayer cultures.\textsuperscript{99, 100} With regard to heart transplantation, Ma et al. have demonstrated in an elegant series of experiments that NF-κB in cardiac allografts is activated upon I/R injury during heart transplantation in a mouse model using bioluminescence imaging.\textsuperscript{101} Both anti-oxidant therapy (using high dose riboflavin) and NF-κB transcription factor decoy treatment have been demonstrated to decrease the severity of CAV in rodent models of cardiac transplantation.\textsuperscript{102, 103} Thus, there is evidence in animal and cellular models that oxidative stress results in NF-κB activation that leads to cell adhesion molecule expression and neutrophil adhesion. Furthermore, NF-κB activation has been observed during I/R injury that occurs with cardiac transplantation, while treatment that has targeted NF-κB in animal models has successfully attenuated CAV.

1.2.1.2  Cell Adhesion Molecules

The adherence of cells to one another, to the endothelium and/or to their extracellular matrix is facilitated by membrane proteins collectively known as cell adhesion molecules (CAMs).\textsuperscript{104} The recruitment of circulating leukocytes to sites of injury occurs through a cascade of events orchestrated by CAMs on both activated endothelial cells and leukocytes. Different subset of CAMs including selectins, immunoglobulin adhesion molecules and integrins are involved at each step of this sequence of events.
1.2.1.2.1 NF-κB and CAM Expression

It is well established that NF-κB plays a key role in regulating the expression of endothelial CAMs such as ICAM-1, VCAM-1 and E-selectin. Nuclear translocation of NF-κB during endothelial cell activation by a variety of stimuli (e.g. TNFα, LPS, IL-1, ROS, I/R injury) has been implicated in the enhanced transcription-dependent expression of CAMs because the promoter regions of the genes for these molecules contain NF-κB binding sites that are essential for the expression of these proteins on endothelial cells. As mentioned previously, studies using chemical inhibitors or oligonucleotide decoys for NF-κB have been successful at inhibiting the expression of CAMs.

1.2.1.2.2 Selectins

Selectins are a family of glycoprotein adhesion molecules that contain an N-terminal C-type lectin domain, followed by an epidermal growth factor (EGF) domain and two to nine short consensus repeats similar to domains found in complement binding proteins. Members of this family include P-selectin (CD62P), E-selectin (CD62E) and L-selectin (CD62L). P-selectin is expressed on activated endothelial cells and platelets, while E-selectin is only expressed on endothelial cells. L-selectin is only expressed on leukocytes. The lectin and EGF domains are responsible for selectin binding to sialylated and fucosylated oligosaccharides, such as sialyl-Lewis X tetrasaccaride.

P-selectin is the predominate selectin involved in neutrophil recruitment to activated endothelial cells following I/R injury. It is stored in specific granules known as Weibel-Palade bodies, present in both platelets and endothelial cells. Within minutes of reperfusion, P-selectin is translocated from these Weibel-Palade bodies and is expressed on the cell surface. Cell surface expression of P-selectin is also stimulated by a variety of
inflammatory agents including thrombin, histamine from mast cells, and ROS. Cytokines such as IL-4 can also stimulate transcriptional upregulation of P-selectin resulting in sustained endothelial P-selectin expression. Once expressed at the cell surface, P-selectin interacts with its binding partner, P-selectin glycoprotein ligand-1 (PSGL-1), a disulfide link-containing homodimer located on the microvilli of leukocytes.

E-selectin is expressed on endothelial cells in response to stimulation by cytokines such as TNFα and IL-1. In contrast to P-selectin, the expression of E-selectin does not rapidly occur after reperfusion injury, as its de novo synthesis and cell surface expression peaks at 4 to 6 hours following cytokine stimulation. As such, E-selectin may not play a significant role in leukocyte adhesion to endothelium early after I/R injury; however, E-selectin is believed to mediate neutrophil, monocyte and memory T-cell adhesion to endothelium following later cytokine stimulation. Once expressed on the cell surface, E-selectin is believed to bind E-selectin ligand-1 (ESL-1) and potentially PSGL-1; however, this remains unclear. The role of E-selectin may in fact be somewhat redundant, as mice deficient for E-selectin do not exhibit impaired leukocyte recruitment to inflamed peritoneum unless P-selectin is also inhibited.

L-selectin is constitutively expressed on the microvilli of circulating leukocytes and is believed to mediate leukocyte binding to activated endothelium at inflammatory sites, as well as lymphocyte homing to secondary lymphoid organs such as peripheral lymph nodes and Peyer’s Patches. The ligands for L-selectin on the high endothelial venules of peripheral lymph nodes are peripheral node addressins (PNAd/CD34) and in Peyer’s Patches are mucosal addressin cell-adhesion molecule-1 (MAdCAM-1). In late phases of
inflammation, PSGL-1 is shed from circulating leukocytes onto endothelial cells where they can also act as ligands for L-selectin.\textsuperscript{117, 122} There is also some evidence that L-selectin can act as a ligand for both P- and E-selectin.\textsuperscript{104, 123-125}

1.2.1.2.3 Immunoglobulin Supergene Family Adhesion Molecules

The immunoglobulin supergene family (IgSF) is a family of cell adhesion molecules that include ICAM-1(CD54), VCAM-1(CD106) and platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31).\textsuperscript{110} All IgSF members have a variable number of repetitive extracellular Ig-like domains, which are followed by a transmembrane domain and a cytoplasmic sequence.\textsuperscript{110} It is the Ig-like domains that recognize and bind to heterodimeric glycoproteins, otherwise known as integrins.\textsuperscript{110}

ICAM-1 contains 5 Ig-like domains and is constitutively expressed at low levels on the endothelium.\textsuperscript{126} While its expression exhibits marked heterogeneity amongst different vascular beds, it is reliably expressed in the coronary vascular endothelium.\textsuperscript{104, 127} As mentioned previously, following exposure to proinflammatory stimuli such as ROS, IL-1 or TNFα, endothelial expression of ICAM-1 is significantly up-regulated by 4 hours following stimulation and can remain upregulated beyond 48 hours with continued stimulation.\textsuperscript{111, 128} This is accomplished through NF-κB pathway activation and subsequent upregulation of ICAM-1 mRNA synthesis.\textsuperscript{79} In a feline model of myocardial I/R injury, Weyrich et al. demonstrated that ICAM-1 levels remained relatively low during the first 120 minutes of reperfusion, however by 150 and 270 minutes, there was a significant increase in expression.\textsuperscript{112} In cellular studies, both Howard et al. and Kokura et al. have demonstrated increased ICAM-1 expression in HBECs and HUVECs respectively, beginning 4 hours and
persisting beyond 10 hours following reoxygenation in I/R models of endothelial injury.\textsuperscript{100, 129}

VCAM-1 contains 7 Ig-like domains, and in contrast to ICAM-1, is not constitutively expressed in endothelial cells.\textsuperscript{130} However, it is dramatically upregulated by inflammatory cytokines such as TNF\textsubscript{a}, IL-1, IL-4, and INF\textsubscript{y}.\textsuperscript{104} Kokura et al. have also demonstrated that HUVECs subjected to anoxia/reoxygenation injury express VCAM-1.\textsuperscript{100} VCAM-1 has been implicated in the development of atherosclerotic plaques. Cybulsky et al. have shown that cholesterol induces the endothelial expression of VCAM-1 on aortic endothelium at sites that develop into atherosclerotic plaques.\textsuperscript{131} It seems that the expression of VCAM-1 occurs at an early stage of atherosclerotic plaque formation, prior to the recruitment of monocytes, and thus may be involved in recruitment of monocytes that go on to regulate plaque development.\textsuperscript{132, 133} VCAM-1 may also play a role in allograft rejection as its expression has been spatially and temporally linked to T-cell infiltration and a rejection response following cardiac transplantation.\textsuperscript{134}

PECAM-1 contains 6 Ig-like domains, and is constitutively expressed on neutrophils, monocytes, platelets, T-cells and significantly on endothelial cells at their intercellular junctions.\textsuperscript{135} Because of its high basal expression on endothelial cells, inflammatory cytokines are generally not able to stimulate PECAM-1 expression further; however, in vitro evidence suggests that prolonged incubation with IL-1, TNF\textsubscript{a}, lymphotoxin or LPS for >48 hours does slightly increase PECAM-1 expression.\textsuperscript{110} PECAM-1 is thought to primarily bind to itself in a homophilic interaction, which accounts for its localization to intercellular junctions.\textsuperscript{135} Current evidence suggests that PECAM-1 plays a role in leukocyte transmigration through the endothelium.\textsuperscript{135, 136}
1.2.1.2.4 Integrins

Integrins are a family of cell adhesion molecules that are expressed constitutively on leukocytes amongst many other cell types. Integrins are characterized by their transmembrane glycoprotein structure incorporating one α-chain subunit and one β-chain subunit that form a heterodimer in a noncovalent fashion. Currently, there are at least 18 α and 8 β subunits known to occur in humans, together forming 24 different integrins. The major integrins expressed on leukocytes include the β₂ integrins, α₄β₂ (LFA-1, CD11a/CD18) and α₅β₂ (Mac-1, CD11b/CD18), as well as the α₄β₁ (VLA-4, CD49d/CD29) and α₄β₇ integrins. A unique feature of integrins is their variable affinity for their ligands. This variability is based upon conformational changes in the extracellular regions induced by either G-protein coupled receptor mediated phosphorylation of the integrin β cytoplasmic tail, or by binding of the cytoskeletal adaptor protein, talin, to the β tail. This binding results in dissociation of the α and β cytoplasmic tails, which confers a conformational change in the extracellular region that increases affinity for the integrin’s ligand in a process known as “inside-out signaling”.

LFA-1 and Mac-1 are considered to be the integrin binding partners for ICAM-1. LFA-1 is expressed on all leukocytes subtypes, whereas Mac-1 is only expressed on monocytes, macrophages and neutrophils. In addition to ICAM-1, LFA-1 binds the other members of the ICAM family (ICAM-2 and ICAM-3), while Mac-1 is able to bind to the complement cleavage fragment iC3b, factor X, fibrinogen and heparin. In contrast to LFA-1, Mac-1 is stored in intracellular granules which can rapidly release Mac-1 for translocation to the cell membrane upon leukocyte stimulation by proinflammatory cytokines/mediators. Stimulation by proinflammatory cytokines has also been
demonstrated to increase the binding affinity state of both LFA-1 and Mac-1 via inside-out signaling described previously.\textsuperscript{137, 147}

VLA-4 is expressed by a variety of leukocytes including monocytes, eosinophils, lymphocytes, mast cells and hematopoietic progenitor cells, but is notably absent from neutrophils.\textsuperscript{117, 148} In contrast, $\alpha_4\beta_7$ integrin is restricted primarily to lymphocytes, with some expression in NK cells and stimulated monocytes, macrophages and eosinophils.\textsuperscript{149, 150} Both of these integrins serve as the primary binding partners for VCAM-1. The $\alpha_4\beta_7$ integrin, on the other hand, seems to play a unique role in binding to MAdCAM-1 in Peyer’s Patches.\textsuperscript{151}

1.2.1.3 Neutrophil-Endothelial Cell Interactions

Neutrophils are a subtype of leukocytes that play a central role in the host defense mechanism that seeks out and removes invading pathogens. They respond to intercellular signals that identify the foreign pathogens, and subsequently initiate a complex sequence of events that leads to extermination of the pathogens.\textsuperscript{111, 128} However, similar signals of inflammation are caused by endothelial cells following I/R injury.\textsuperscript{26} As mentioned previously, neutrophils that are recruited to sites of activated endothelial cells following I/R injury are believed to be the primary source of ROS and other inflammatory products that subsequently contribute to endothelial injury and dysfunction.\textsuperscript{26, 111, 128} Evidence from animal models of myocardial I/R injury have demonstrated that ischemia alone, without reperfusion, elicits a slow recruitment of neutrophils into the area of ischemic myocardium over a period of between 12 to 24 hours.\textsuperscript{111, 152} However, following reperfusion there is a rapid accumulation of neutrophils in the area at risk with neutrophil adhesion to coronary endothelium within minutes.\textsuperscript{111, 153, 154} In these animal models, this adhesion has been
observed to parallel a progressive decline in coronary endothelial function. The rapid recruitment of neutrophils to sites of activated endothelium is mediated by a highly coordinated series of neutrophil-endothelial cell interactions that occur in three stages: tethering and rolling, arrest/firm adhesion, and finally, transendothelial migration (Figure 1-3).

Figure 1-3. Neutrophil–Endothelial Cell Adherence and Transmigration after Ischemia–Reperfusion. Activated leukocytes interact with the vascular endothelium via a series of distinct steps. The initial “rolling” step is initiated by ischemia–reperfusion–induced increases in endothelial P-selectin expression, which interacts with its leukocyte counter-receptor, P-selectin glycoprotein 1 (PGSL-1) (1). Interaction of leukocyte β2 integrins, CD11a/CD18 and CD11b/CD18, with endothelial intercellular adhesion molecule 1 (ICAM-1) results in firm leukocyte adherence and aggregation (2). Leukocyte transmigration into the interstitial compartment is facilitated by platelet-endothelial cell adhesion molecule 1 (PECAM-1) within the endothelial cell junctions (3). Reproduced with permission from: Collard CD and Gelman S. Pathophysiology, Clinical Manifestations, and Prevention of Ischemia-Reperfusion Injury. Anesthesiology 2001;94:1133-1138.
1.2.1.3.1 Tethering and Rolling

The first stage in neutrophil-endothelial cell interaction involves neutrophil tethering and rolling along the endothelial surface that is dependent on the expression of appropriate cell adhesion molecules. This initial tethering and rolling is largely dependent on endothelial selectin expression (as previously described). Initially, endothelial cell surface P-selectins interact with neutrophil PSGL-1, and results in loose tethering and rolling.\(^{26, 155}\)

Within the circulation, neutrophils interacting with the endothelium are identified as rolling when the velocity of the neutrophils is below that of the free flowing cells in circulation.\(^{156}\)

The rolling velocity of the neutrophils has been demonstrated to be dependent on the relative level of selectin expression, and can be described as fast or slow rolling (<5 \(\mu\)m/s).\(^{157}\) Since E-selectin is not expressed by endothelial cells until hours after initial injury, P-selectin is primarily responsible and sufficient for early neutrophil tethering and rolling.\(^{128, 158}\)

However, P-selectin has been found to mediate fast rolling, whereas E-selectin has been shown to mediate slow rolling, which suggests that the interaction between E-selectin and its binding partners is stronger than P-selectin. Indeed, models of mice deficient in E-selectin have demonstrated reduced number of adherent leukocytes in cytokine-stimulated models of endothelial injury.\(^{158, 159}\)

Tethering can also occur between leukocytes and activated endothelial cells independent of selectins via low affinity binding of VLA-4 and \(\alpha_4\beta_7\) integrins to VCAM-1; however, given that neutrophils generally do not express VLA-4 or \(\alpha_4\beta_7\), the contribution of endothelial VCAM-1 in neutrophil-endothelial cell interactions is likely negligible. Importantly, the binding of the inactive LFA-1 and Mac-1 integrins on neutrophils with ICAM-1 is thought to facilitate tethering and rolling via low affinity interactions.\(^{137}\)
As neutrophils roll along activated endothelium, they are exposed to cytokines released by activated endothelial cells. Binding of these cytokines to neutrophil cell surface receptors initiates intracellular signaling cascades within the neutrophil that results in inside-out signaling and activation of integrins into a high-affinity ligand-binding state. Thus, rolling allows time for integrin activation to occur, which is a necessary step for subsequent arrest and firm adhesion on activated endothelium.

1.2.1.3.2 Arrest/Firm Adhesion

Once neutrophils engage in tethering and rolling, integrins are activated as described previously. In the absence of IgSF binding partners for these integrins on endothelial cells, the rolling neutrophils will continue to roll and eventually be released back into the flow of the bloodstream. However, within a few hours of activation, cell surface expression of ICAM-1 on endothelial cells markedly increases. Thus, activated LFA-1 and Mac-1 integrins on neutrophils will bind to this ICAM-1 with high affinity and facilitate cessation of neutrophil rolling and allow firm adhesion.

In addition to the inside-outside signaling that activates integrins and increases their affinity for their binding partners, cytokine signaling also stimulates the rapid release of integrins from their cytoskeletal attachments. This release then allows the integrins to form clusters by redistribution in the cell membrane, at sites of integrin bond formation with IgSF CAMs. In this manner, the number of integrin-IgSF bonds increases, which strengthens the binding interaction between cells in a process known as valency regulation.

1.2.1.3.3 Transendothelial Migration

Following the rolling and subsequent firm adhesion of neutrophils to activated endothelium, neutrophils pass through the endothelium into the underlying tissues in a process known as
transendothelial migration (diapedesis). The initiation of this process is dependent on the previous stage of firm adhesion mediated by ICAM-1 and LFA-1/Mac-1 binding. Smith et al. have demonstrated that antibody mediated inhibition of CD18 (β2 integrin) or ICAM-1 function results in >85% inhibition of neutrophil transendothelial migration through IL-1 or LPS activated endothelium.162

Transendothelial migration predominately occurs at the junction between endothelial cells and is facilitated by the Ig-SF CAM, PECAM-1, which has been described previously.135,136 PECAM-1 is expressed on both neutrophils and endothelial cells (particularly highly expressed at intercellular junctions) and bind to one another via homophilic interactions.135 Muller et al. demonstrated that pretreating neutrophils with monoclonal antibodies specific for PECAM-1 is sufficient to block transendothelial migration.136 It is generally thought that neutrophils migrate between endothelial cells in the direction of higher density PECAM-1 expression in process known as haptotaxis.136

1.2.1.3.4 ICAM-1 in Neutrophil-Endothelial Adhesion

Smith et al. were the first group to describe the key role that ICAM-1 plays in neutrophil adhesion to endothelial cells.162 Using anti-ICAM-1 monoclonal antibodies, they demonstrated that neutrophil adhesion was significantly blocked to HUVECs exposed to either IL-1 or LPS.162 Using an anti-CD18 monoclonal antibody, they were able to reproduce this inhibition of neutrophil adhesion under identical conditions. Furthermore, when they used both anti-ICAM-1 and anti-CD18 antibodies, there was no additive inhibitory effect on neutrophil adhesion, suggesting that neutrophil adhesion occurs in an ICAM-1 dependent fashion through its interaction with β2 integrins.162 Bochner et al. confirmed these findings in cellular studies examining the adhesion of various leukocytes (basophils, eosinophils and
neutrophils) to IL-1 stimulated HUVECs. Interestingly, they also demonstrated that VCAM-1 does not contribute to neutrophil adhesion and found that neutrophils do not express the VCAM-1 binding integrin, VLA-4, as previously mentioned.

ROS induced endothelial injury upregulates the expression of cell adhesion molecules such as ICAM-1. Lo et al. were the first to demonstrate that this upregulated ICAM-1 expression is responsible for ROS induced promotion of neutrophil adhesion to endothelial cells. Again, using both anti-ICAM-1 and anti-CD18 monoclonal antibodies, they demonstrated >90% blockade of the adhesive interaction between neutrophils and HUVECs injured with \( \text{H}_2\text{O}_2 \). Briaud et al. examined the effect of neutrophil adhesion to coronary endothelium after myocardial I/R injury in ICAM-1/P-selectin knockout mice. They observed a significant reduction in the adhesion of neutrophils to coronary endothelium at 24 hours following reperfusion in knockout mice; however, no difference at 3 hours when compared to wild-type mice. This suggest that early neutrophil adhesion can occur in a non P-selectin dependent manner, while later adhesion relies significantly on ICAM-1 expression, which typically occurs beyond the 3 hour time-point examined in this study. Finally, Ma et al. studied the effect of ICAM-1 inhibition using a monoclonal antibody, on myocardial injury, endothelial dysfunction and neutrophil adhesion in a feline model of myocardial I/R injury. Their results revealed that administration of an anti-ICAM-1 antibody 10 minutes prior to reperfusion resulted in a significant decrease in myocardial necrosis, enhanced endothelium-dependent vasorelaxation of isolated LAD coronary arteries, and a significant inhibition of neutrophil adhesion to the I/R injured coronary artery endothelium compared to controls.
The results of these and other preclinical studies raised enthusiasm toward developing therapeutic strategies employing monoclonal antibodies toward integrins and ICAM-1 to prevent injury following MI, stroke and other inflammatory diseases.\textsuperscript{117} Unfortunately, aside from some success using anti-\(\alpha_4\) monoclonal antibodies in the settings of multiple sclerosis and irritable bowel disease, these efforts have generally failed in clinical trials.\textsuperscript{117, 166, 167} Clinical trials using anti-CD18 monoclonal antibodies (Rovelizumab and Erlizumab) were unsuccessful at demonstrating any benefit in reducing either myocardial or cerebral ischemic injury following MI or stroke.\textsuperscript{117, 168-170} Similarly, a trial using an anti-ICAM-1 monoclonal antibody (Enlimomab) in stroke patients did not reveal any benefit, and moreover, suggested that they may be a worsening of outcomes with use of the antibody.\textsuperscript{171} Thus, although animal data seemingly suggest that a simple anti-CD18 and/or anti-ICAM-1 antibody should attenuate I/R injury, these strategies have yet to be demonstrated to be beneficial in human trials. Novel methods or mechanisms to attenuate neutrophil adhesion to injured endothelium may eventually yield these benefits.

1.2.2 Calcineurin Inhibition (CNI) induced Injury

The widespread clinical application of CNI for immunosuppression has facilitated solid organ transplantation and has indisputably improved the survival of heart transplant recipients. Cyclosporine A (CyA) was the first calcineurin inhibitor used in clinical heart transplantation; however, more recently tacrolimus (also known as FK506) has become the predominate calcineurin inhibitor used in these patients.\textsuperscript{4}

CyA is a cyclic peptide of 11 amino acids derived from the soil fungus, \textit{Tolypocladium inflatum gams}, originally discovered in Norway in 1969.\textsuperscript{172} Tacrolimus is a macrolide lactone derived from the bacteria, \textit{Streptomyces tsukubaensis}, originally discovered in
Japanese soil in 1984. Both drugs enter cells by diffusion, but bind to different intracellular immunophilins. Cyclosporine binds to cyclophilin, whereas tacrolimus binds to FK506 binding protein-12 (FKBP-12). Both of these bound complexes are able to then bind to and inhibit calcineurin. Active calcineurin is a phosphatase that can dephosphorylate the nuclear factor of activated T cells (NFAT). Dephosphorylated NFAT, in turn, can then translocate to the cell nucleus where it acts as a transcription factor by binding to the promoter regions of cytokine genes, including IL-2. IL-2 is a potent stimulator of lymphocyte activation and proliferation. Thus, by inhibiting calcineurin, both CyA and tacrolimus ultimately block the production of IL-2, which subsequently suppresses activation of lymphocytes. Despite sharing similar mechanisms of action, these two drugs differ in their side effect profiles. Tacrolimus causes less gingival hyperplasia and hirsutism than CyA, and is also less nephrotoxic of the two. While tacrolimus is also believed to cause less hypertension than CyA, it does seem to trigger more post-transplant diabetes mellitus. On balance, the shift towards more recent clinical use of tacrolimus is likely due to its more favourable side effect profile.

1.2.2.1 CNI Induced Endothelial Dysfunction

Both clinical and animal data have allowed a number of investigators to demonstrate that CNI induces endothelial dysfunction. Clinical studies assessing endothelial function using forearm blood flow, primarily in renal transplant patients, have demonstrated impaired endothelial function in patients treated with CyA. In an observational clinical study, Weis et al. examined the effect of tacrolimus compared to CyA on endothelial function within 6 months following heart transplantation. They observed significant epicardial endothelial dysfunction (defined by a vasoconstrictive response to acetylcholine), microvascular smooth muscle cell dysfunction (defined by flow velocity increases in
response to adenosine and nifedipine), and decreased eNOS expression with increased expression of IL-6 in myocardial biopsies from patients treated with tacrolimus, azathioprine and prednisone versus cyclosporine, azathioprine and prednisone.

Animal studies have generally demonstrated that CyA causes endothelial dysfunction, however, there is debate in the literature as to whether tacrolimus causes similar dysfunction. In a rat model, Ramzy et al. demonstrated that 2 weeks of systemic CyA treatment impaired the endothelial dependent vasorelaxation of thoracic aortic segments. They also demonstrated that CyA increased the sensitivity of these segments to ET-1 with a concomitant 34% increase in ET\textsubscript{A} receptor expression. Additionally, they observed that eNOS expression in the thoracic aortic segments was reduced by 30% following treatment with CyA, and that oxidative injury, as measure by 8-isoprostane levels, were also increased in this group. In a subsequent study, this group was able to attenuate the CyA induced impaired vasomotor function and ET-1 sensitivity using concomitant systemic treatment with the ET-1 antagonist, bosentan. Similarly, Jeanmart et al. examined the effect of CyA and tacrolimus on coronary endothelial function of porcine epicardial coronary arteries in vitro. They observed that exposure to cyclosporine impaired endothelial dependent vasorelaxation in response to serotonin but not bradykinin, whereas tacrolimus caused a more severe impairment to vasorelaxation in response to both stimuli. However, when Tepperman et al. examined the effect of a 2 week systemic course of tacrolimus treatment on the vasomotor function of rat thoracic aortic segments, they observed that tacrolimus did not impair endothelial dependent vasorelaxation. Sodium nitroprusside mediated endothelial independent vasorelaxation was impaired with tacrolimus; however, diltiazem mediated vasorelaxation was not affected. They also observed a decrease in sensitivity to ET-1 and levels of ET-1; however, noted an increased in ET\textsubscript{B} receptor expression.
In summary, there is both clinical and animal data to suggest that CyA clearly causes endothelial dysfunction manifest as impaired vasomotor function. However, whether tacrolimus causes similar endothelial dysfunction is less clear given the conflicting data in the literature.

1.2.2.2 CNI induced Endothelial CAM expression and Cell Adhesion

Whether CNI treatment leads to the expression of CAMs and/or modulates cell adhesion to endothelial cells is also unclear, since there is conflicting data in the literature. The relative effect of CyA and tacrolimus in this regard is also unclear, given the mixed results that have been published.

Data from some animal models suggest that CyA increases leukocyte adhesion. In a rat cremaster flap model, Unsal et al. demonstrated that after 6 weeks of CyA administration, a significant increase in the number of adherent leukocytes occurred in the flap microvasculature and this was associated with increased microvascular permeability, interstitial inflammation and venous vasculitis.\(^{189}\) Similarly, in a rat model of autologous arterial grafting that generates neointimal hyperplasia, Jurado et al. demonstrated that CyA promotes the adhesion and infiltration of circulating neutrophils to the injured arterial graft wall.\(^{190}\)

Animal data has also suggested that tacrolimus is associated with increased CAM expression that may mediate the development of CAV. In a heterotopic murine model of heart transplantation, Suzuki et al. demonstrated that early (5 days) treatment of recipient mice with anti-ICAM-1 and anti-LFA-1 monoclonal antibodies successfully inhibits the occurrence of graft arteriopathy (coronary intimal hyperplasia) compared to control recipients that received tacrolimus immunosuppression at 60 days post-transplant.\(^{191}\)
Interestingly, ICAM-1, VCAM-1 and PDGF-B mRNA expression were observed to be significantly increased in the allografts of mice receiving tacrolimus treatment.

Data from cellular studies also suggest that CNI increases CAM expression and leukocyte adhesion. Gallego et al. examined the effect of CyA on leukocyte adhesion to HUVEC under flow conditions. They observed that CyA increased the number of leukocytes that adhered to treated HUVECs; however, they did not observe any difference in the number of leukocytes that engaged in rolling. They also characterized the mechanism of this adhesion using anti-ICAM-1, VCAM-1 and E-selectin monoclonal antibodies and found that blocking any of these CAMs significantly inhibited CyA induced leukocyte adhesion. Furthermore, using confocal microscopy, they demonstrated that ICAM-1, VCAM-1 and E-selectin were all upregulated on the endothelial surface following CyA treatment. Similarly, Schlichting et al. have demonstrated that dendritic cell (DC) adhesion and transmigration on allogeneic human microvascular endothelial cells is increased by endothelial incubation with either CyA or tacrolimus. Using monoclonal blocking antibodies, they showed that LFA-1 was a participant in this increased adhesion and transmigration. Furthermore, they made the observation that ICAM-1 and PECAM-1 mediated the increased DC adhesion and transmigration stimulated by tacrolimus, whereas VCAM-1 was partially responsible for the increased adhesion yielded by CyA. These data suggest that CyA, and tacrolimus both increase cell adhesion to treated endothelial cells, via the upregulation of CAMs. However, others have presented somewhat mixed results in this regard.

Rafiee et al. examined the effect of CyA on human intestinal microvascular cells (HIMECs). They observed that CyA increased the binding of leukocytes to treated HIMECs; however, paradoxically decreased the expression of E-selectin, ICAM-1 and
VCAM-1. They also observed the CyA inhibited p38 MAPK phosphorylation and iNOS expression, but did not inhibit NF-κB activation. Similarly, Sasakawa et al. demonstrated that tacrolimus down-regulated expression of E-selectin, ICAM-1 and VCAM-1 on HMVECs stimulated by the supernatant of PBMCs that had been treated with anti-CD3 and anti-CD2 antibodies. They also demonstrated that tacrolimus inhibited the secretion of TNFα and IL-1β from the HMVECs and that this inhibition of cytokine secretion was the mechanism by which tacrolimus decreased CAM expression. Others have observed that CyA not only decreases CAM expression, but inhibits cell adhesion. Charreau et al. demonstrated that CyA inhibits the expression of E-selectin on cultured porcine endothelial cells in response to LPS stimulation. They also observed decreased adhesion of U937 cells to endothelial cells treated in this manner. Markovic et al. also demonstrated that CyA inhibited the in vitro expression of E-selectin and VCAM-1 on HUVECs stimulated by TNFα, IL-1β or a cytokine mixture from the sera patients during allograft rejection (IL-1β, IL-2, IL-4, IL-6, IL-10, TNFα and INFγ). Data from animal models have also supported these observations. In a rat model of systemic inflammation induced by i.v. TNFα administration, CyA was observed to decrease the expression of E-selectin on coronary vascular endothelial cells. Molossi et al. demonstrated that CyA suppressed vascular expression of ICAM-1 and VCAM-1 in a rabbit model of heterotopic cardiac transplantation at 9-10 days following transplant.

Some have observed that CyA potentiates I/R injury of the endothelium. Squadrito et al. demonstrated that i.v. CyA administration 5 minutes prior to reperfusion in a rat model of myocardial I/R injury increases ICAM-1 expression and leukocyte accumulation in the reperfused myocardium. Azizian et al. have demonstrated that CyA augments I/R injury by decreasing endothelial cell viability in vitro. In contrast, Zhang et al. demonstrated that
CyA inhibits the stimulated expression of ICAM-1 in human cerebromicrovascular endothelial cells subjected to 4 hours of hypoxia followed by 4 to 24 hours of reoxygenation.\textsuperscript{201}

Thus, there are entirely conflicting data on whether CyA or tacrolimus increase or decrease the expression of CAMs on endothelial cells, and whether they promote or inhibit the adhesion of leukocytes.

1.3 Cardiac Allograft Vasculopathy (CAV)

Despite the advances that have been made to improve survival and reduce the incidence of acute rejection after heart transplantation, CAV remains what some have described as the “Achilles heel” of cardiac transplantation.\textsuperscript{202} Currently, CAV is responsible for between 11 and 14% of deaths beyond the first year following heart transplantation.\textsuperscript{4} CAV may be related to persistent antibody-mediated rejection, persistent endothelial injury as a result of immunosuppressive agents and lastly, early endothelial injury sustained at the time of organ transplantation.\textsuperscript{6} Angiographic studies have documented CAV in up to 45% of transplant recipients as early as 3 years following transplantation.\textsuperscript{6, 7} The use of intravascular ultrasound imaging of the coronary arteries has demonstrated intimal thickening in up to 58% of patients at 2 years.\textsuperscript{8} Unfortunately, patient survival following a diagnosis of CAV is reduced significantly.\textsuperscript{4}

1.3.1 Histopathology of CAV

CAV is characterized by a diffuse, concentric fibrous intimal hyperplasia that primarily affects the arteries, arterioles, and capillaries of the donor heart.\textsuperscript{16, 203} It affects both the epicardial and intramyocardial coronary arteries and includes some features of both atherosclerosis and arteritis.\textsuperscript{16, 203, 204} The atherosclerotic features include incorporation of
lipids and the development of plaques in later stages. The features of arteritis include infiltration by mononuclear inflammatory cells and progression in later stages to destruction of the internal elastic lamina.\textsuperscript{16,205} CAV distinguishes itself from CAD by its diffuse and concentric involvement of the entire coronary tree, whereas CAD typically manifests as focal, eccentric plaques involving the epicardial coronary arteries with calcification in later stages.\textsuperscript{203}

1.3.2 Pathophysiology of CAV

Although a precise mechanism causing CAV has not been found, it is currently thought that the pathophysiology of CAV involves multiple mechanisms that include both immunologic and non-immunologic factors. These factors seem to contribute to endothelial dysfunction that underlies the development of CAV (Figure 1-4).\textsuperscript{18}
1.3.2.1 Immunologic factors

Several immunologic factors have been demonstrated to lead to CAV and these include human leukocyte antigen (HLA) mismatching, activation of host T-cells, and cytokine production.\textsuperscript{204}

While donor and recipient HLA typing is performed prior to transplantation, formal HLA matching is not done and hence mismatches are common. In particular, HLA-DR mismatching has been found to have the strongest correlation with the development of
CAV.204, 206, 207 This correlation may be due, in part, to an increase in cellular rejection episodes that be been observed with HLA-DR mismatches.204, 206, 208

Cell mediated rejection following T-cell activation may occur through direct or indirect alloreognition pathways.209 The direct pathway occurs when foreign major histocompatibility complex (MHC) molecules on the surface of donor cells are recognized directly by recipient CD8⁺ or CD4⁺ T-cells, which then stimulates a cytotoxic CD8⁺ T-cell response.209 The indirect pathway occurs when donor antigens are taken up and processed by recipient antigen presenting cells (dendritic cells), which are then presented to and recognized by helper CD4⁺ T-cells that then stimulate a cytotoxic CD8⁺ T-cell response.209 Szeto et al. determined that CAV occurs in CD8⁺ T-cell-depleted rat heart transplant recipient animals, whereas it did not occur in CD4⁺ T cell-depleted recipient rats, suggesting that CD4⁺ T cells, through an indirect alloreognition pathway, are important in the pathophysiology of CAV.210 Along these lines, it has been observed that dendritic cells frequently adhere to allograft endothelial cells and subsequently activate a T cell response that includes the production of cytokines such as INFγ and TNFα that in turn lead to endothelial cell activation and thus to endothelial dysfunction.18, 211, 212

1.3.2.2 Non-Immunologic Factors

Several non-immunologic factors have been associated with the development of CAV and these include donor brain death (including mechanism of brain death), I/R injury that occurs at the time of transplantation, various metabolic abnormalities, and infection.10, 16, 203

1.3.2.2.1 Brain Death

The occurrence of donor brain death triggers a catecholamine surge that is associated with peripheral vasoconstriction and the release of hormones and inflammatory cytokines.18, 203, 213
These events that occur with brain death have been demonstrated to result in endothelial activation as part of an inflammatory response.\textsuperscript{18, 203, 213} This response includes the expression of CAMs as well as the induced expression of MHC molecules that can activate allore cognition pathways in the recipient as described previously.\textsuperscript{18, 209} Recently, Mehra et al. evaluated the mode of brain death and its association with CAV.\textsuperscript{214} They determined that explosive donor brain death (accompanied by a sudden and rapid increase in intracranial pressure) was associated with greater coronary intimal thickening as assessed by IVUS in heart transplant recipients.\textsuperscript{214} This suggests that injury to the endothelium after brain death occurs via circulating factors such as catecholamines, hormones and cytokines, and moreover, results in increased CAV.\textsuperscript{10, 203}

1.3.2.2.2 I/R Injury

I/R injury contributes to endothelial dysfunction and injury as previously discussed earlier in this chapter. Gohra at al. have demonstrated that I/R injury leads to the development of allograft vasculopathy in a rodent model of aortic allograft transplantation.\textsuperscript{31} Others have observed that I/R injury up-regulates MHC expression and the shedding of donor antigens, which in turn, augments the alloimmune response following transplantation.\textsuperscript{10, 77, 203} The release of innate immune ligands, such as heat shock proteins, can be detected by toll-like receptor-4 (TLR-4) on monocytes.\textsuperscript{78} Interestingly, the increased gene expression of TLR-4 in monocytes has been correlated with increased allograft coronary artery endothelial dysfunction.\textsuperscript{18, 215} Thus, strategies aimed at attenuating I/R injury may confer benefit beyond preventing endothelial dysfunction, and may attenuate factors that influence the alloimmune response that also contributes to CAV.
1.3.2.2.3 Metabolic Abnormalities

Metabolic abnormalities such as hyperlipidemia, hyperhomocysteinemia, hypertension, and hyperglycemia are common in heart transplant recipients owing to the routine use of immunosuppression in this population.\textsuperscript{10, 216, 217} While these abnormalities are associated with the development of atherosclerosis amongst the general population, they have also been demonstrated to be associated with the development of CAV.\textsuperscript{10}

In a rabbit heterotopic heart transplant model, hypercholesterolemia has been demonstrated to be associated with the development of CAV.\textsuperscript{218} Histologic comparison of the native and allograft coronary arteries revealed that hypercholesterolemia elicited more profound pathologic changes in the transplanted arteries. Furthermore, the investigators observed that hypercholesterolemia promoted more fibrofatty proliferative changes in the intimal hyperplasia seen in this model.\textsuperscript{218}

Hyperhomocysteinemia has been demonstrated to cause endothelial dysfunction by inhibiting endothelial NO production and results in a blunted vasomotor response to vasodilator challenges in health human subjects.\textsuperscript{219} It also occurs in relatively high frequency amongst transplant recipients,\textsuperscript{220} and has been associated with the development of CAV.\textsuperscript{221} Unfortunately, strategies aimed at lowering homocysteine levels, such as with folate therapy, have not demonstrated any benefit with regard to decreasing the onset of CAV.\textsuperscript{222}

1.3.2.2.4 Infection

Infection, particularly with the cytomegalovirus (CMV), has been associated with the development of CAV.\textsuperscript{16, 18, 203, 204, 223} Grattan et al. were the first to observe that 28\% of heart transplant recipient who had CMV developed severe CAV, as compared to only 10\% of those without CMV.\textsuperscript{223} Interestingly, CMV is able to infect human endothelial cells and
upregulates the constitutive expression of ICAM-1.\textsuperscript{224, 225} In addition, Weis et al. have recently demonstrated that CMV impairs endothelial eNOS function in vitro.\textsuperscript{226} These data suggest that CMV infection may contribute to CAV by directly causing endothelial dysfunction.

1.3.3 Current Clinical Strategies to Prevent CAV

Treatment of established CAV is difficult owing to its diffuse involvement of the coronary tree. Therapies such as angioplasty, stenting and/or coronary artery bypass grafting that are used to treat focal CAD are usually not suitable or effective in dealing with CAV. Thus, current clinical strategies have focused on the prevention of CAV.

1.3.3.1 Statins

HMG-CoA reductase inhibitors, also known as statins, reduce cholesterol levels by interrupting the cholesterol synthesis pathway at the level of HMG-CoA reductase. Kobashigawa et al. were the first to demonstrate the efficacy of early initiation (< 2 weeks following transplant) of pravastatin therapy on decreasing the incidence of CAV at 1 year following heart transplantation in a randomized clinical trial.\textsuperscript{227} A subsequent study using simvastatin demonstrated reduced progression of CAV and improved 8-year survival in heart transplant recipients.\textsuperscript{228} Thus, statin therapy has become a routine preventive therapy in heart transplantation.

1.3.3.2 Vasodilators

Vasodilator therapy, using calcium channel blockers and/or angiotensin converting enzyme (ACE) inhibitors, is typically used in the treatment of hypertension. Preliminary studies have demonstrated that calcium channel blockade using diltiazem early after heart transplantation reduces the severity, progression and mortality related to angiographic CAV.\textsuperscript{229} Similarly
ACE inhibitor use has been associated with plaque regression in CAV. Recently, a synergistic effect with calcium channel blockade on reducing the development of CAV has been identified. These early results, though encouraging, have yet to be evaluated in larger prospective randomized trials.

1.3.3.3 **Mycophenolate Mofetil (MMF)**

MMF is used as an immunosuppressant, commonly along with a calcineurin inhibitor, and is the prodrug of mycophenolic acid, which inhibits purine biosynthesis. The use of MMF was compared to azathioprine, another purine biosynthesis inhibitor, in a randomized controlled trial of heart transplant recipients who were also receiving CyA and corticosteroids. Eisen et al. demonstrated a 35% reduction in 3-year mortality with the use of MMF in this trial. Importantly, Kobashigawa et al. demonstrated a reduced incidence of CAV (defined by a maximal intimal thickness of $\geq 0.3$ mm) within the first year following transplant using MMF. Thus, MMF treatment has rapidly replaced the use of azathioprine in heart transplantation.

1.3.3.4 **Proliferation Signal Inhibitors (PSIs)**

Rapamycin, a macrocyclic immunosuppressant and product of *Streptomyces hygroscopicus*, inhibits cellular proliferation stimulated by growth factor–driven signal transduction in response to alloantigens. It binds to the same immunophilin as tacrolimus, FKBP-12; however, the rapamycin-FKBP-12 complex goes on to inhibit the mammalian target of rapamycin (mTOR) kinase instead of calcineurin. mTOR is responsible for phosphorylation of proteins involved in cell-cycle regulation and proliferation. Thus, by inhibiting mTOR, rapamycin interrupts the cell cycle and causes arrest at the G1 and S phases. The mTOR pathway is central to IL-2 induced lymphocyte activation and
proliferation. Thus, rapamycin is used as an immunosuppressant to inhibit the response to IL-2, whereas CyA and tacrolimus block IL-2 production.\textsuperscript{17,175}

Both rapamycin and everolimus, a derivative of rapamycin, have been demonstrated to reduce the progression of IVUS proven CAV out to two years following transplantation in randomized clinical trials comparing them to azathioprine.\textsuperscript{235,236} In animal models, rapamycin has been demonstrated to significantly attenuate the development of transplant vasculopathy, although the mechanism of action remains unclear.\textsuperscript{237} Fukuda et al demonstrated that rapamycin had a direct inhibitory effect on circulating vascular progenitor cells.\textsuperscript{238} In this study, the authors examined the hyperplastic response to wire-induced arterial injury and demonstrated that rapamycin attenuated neointimal hyperplasia by directly inhibiting the number of bone marrow derived cells present in the hyperplastic lesion. With regard to endothelial function, as compared to CyA, Ramzy et al demonstrated that rapamycin does not impair endothelial dependent vasorelaxation nor does it alter sensitivity to endothelin-1 induced vasospasm.\textsuperscript{239} Although rapamycin portends endothelial functional benefits over CyA, which may be an impetus for its use early after transplantation, it has the adverse side effect of impairing wound healing that severely limits its use immediately post-transplantation and often results in cessation of its use.\textsuperscript{240,241} The effect of delayed initiation of rapamycin therapy on preventing endothelial dysfunction and subsequent development of intimal hyperplasia post-transplantation is unclear. The discontinuation of rapamycin therapy on the development of intimal hyperplasia is also currently unclear.
1.4 Epidermal Growth Factor-Like Domain 7 (Egfl7)

1.4.1 Egfl7 Expression in During Development

Egfl7 is a novel protein that is secreted and expressed in a restricted manner in endothelial and early endothelial progenitor cells. Fitch et al characterized the expression of Egfl7 by RNA in-situ hybridization studies as being localized to highly vascularized adult tissues including lung, heart, uterus and ovary.\textsuperscript{242} They also described its expression in embryos at sites of vascular system development.

1.4.2 Known Functional and Structural Properties of Egfl7

Egfl7 was originally described and named by Soncin et al in 2003 as vascular endothelial (VE)-statin, since it was characterized as an endothelial-specific secreted ligand that inhibited platelet-derived growth factor-BB induced smooth muscle cell migration.\textsuperscript{243} Subsequently in 2004, two developmental biology research groups almost simultaneously published the “discovery” and embryologic function of Egfl7. Parker et al described the loss of vascular tubulogenesis in zebrafish embryos that underwent Egfl7 gene knockdown.\textsuperscript{244} They further showed that human umbilical vein endothelial cells adhered to Egfl7 coated culture plates, but in a weaker fashion than other classic cell-adhesion substrates, such as fibronectin and collagen I.

Subsequent studies have elucidated the structure of Egfl7 to be a ~30 kDa secreted protein that contains an Emilin-like domain and 2 epidermal growth factor domains.\textsuperscript{245} The presence of the Emilin-like domain has led to the hypothesis that Egfl7 may self-assemble like extracellular matrix (ECM) proteins since this domain is known to be a multimerization motif.\textsuperscript{245} Interestingly, the Egfl7 mRNA contains a micro-RNA sequence in its intron 7, named miR-126.\textsuperscript{246} miR-126 has been demonstrated to positively regulate vascular
endothelial growth factor (VEGF) dependent signaling by decreasing the expression of Spred-1 and PI3KR2, which are known repressors of VEGF signaling.\textsuperscript{246} Homozygous knockout mouse models of Egfl7 have demonstrated \textasciitilde50% in utero mortality and associated delayed vascular development in multiple organ systems, with impaired sprouting angiogenesis.\textsuperscript{247} However, it is unclear whether the phenotypes observed in these models are due to a lack of Egfl7 or a reduction in miR-126 expression.\textsuperscript{248} Nichol et al. have recently developed transgenic mice that overexpress Egfl7.\textsuperscript{248} They observed that overexpression of Egfl7 in their transgenic mice was not associated with altered miR-126 levels, and resulted in a hyperangiogenic response with excess arterial branching and tortuosity observed within the retinas of these mice.\textsuperscript{248} This suggests that Egfl7 itself, independent of the effect of miR-126, can elicit changes to the vascular phenotype.

1.4.3 Egfl7 Expression upon Arterial Injury

In 2005, Campagnolo et al published their results examining the expression of Egfl7 in the setting of arterial injury.\textsuperscript{249} Both a balloon aortic injury model in rats and a ferric chloride carotid artery injury model in mice revealed Egfl7 expression restricted only to the regenerating endothelium, but not in the neointima that formed following injury. They also demonstrated that Egfl7 acts as a chemoattractant for embryonic endothelial cells, and to a lesser degree, fibroblasts in a cell migration assay. These results suggest that Egfl7 may have a role in the maintenance of vascular endothelial integrity and regeneration following injury. Supporting this hypothesis are the results of Egfl7 gene expression inhibition studies with siRNA targeting Egfl7. Jiang et al reported that Egfl7 inhibition in this manner resulted in decreased HUVEC survival/viability and reduced LDH and ATP release.\textsuperscript{250} These results have also been confirmed by Nichol et al. who knocked down Egfl7 expression in HUVECs using siRNA and found strong suppression of endothelial cell proliferation and migration.\textsuperscript{248}
1.4.4  Egfl7 and Hyperoxia-Induced Endothelial Injury

More recently, Xu et al have published data that suggests that Egfl7 overexpression can protect endothelial cells from hyperoxia-induced cell death.\(^{251}\) They demonstrated that in both in-vitro (cultured human umbilical vein cells) and in-vivo (neonatal rat lungs) models of hyperoxic exposure, Egfl7 gene expression was significantly reduced. Interestingly, Egfl7 overexpression in endothelial cells inhibited hyperoxia-induced cytochrome-c release and caspase-3 activation, and reduced the expression of the pro-apoptotic protein Bax while increasing the expression of the anti-apoptotic protein Bcl-xL. These findings suggest that Egfl7 may have a protective role in endothelial cell biology.

1.4.5  Egfl7 Expression and Hypoxia

Recently, Gustavsson et al examined the effect of hypoxic preconditioning on vascular gene expression in the brains of neonatal rats subjected to global hypoxic preconditioning.\(^ {252}\) Interestingly, they observed that after 3 hours of exposure to hypoxia (8.0% O\(_2\) in nitrogen), Egfl7 gene expression was upregulated by 1.4-fold. This upregulation persisted at 2 hours post hypoxia (1.3-fold upregulation), but returned to baseline by 8 hours. These results suggest that Egfl7 expression may be influenced by oxygen tension. Whether hypoxic injury directly influences Egfl7 expression in endothelial cells has not been previously reported. Furthermore, the role of Egfl7 in the context of endothelial injury and activation has not been previously examined.

1.4.6  Egfl7 and the Notch Signaling Pathway

The Notch signaling cascade is an evolutionarily conserved pathway that regulates fundamental processes of cell/tissue development, cell-fate determination and cell differentiation.\(^ {253}\) Activation of the Notch pathway begins by stimulation of Notch receptors,
which are transmembrane proteins (Notch1 through Notch4), that have extracellular domains containing multiple EGF-like repeats (Figure 1-5). Upon stimulation by canonical ligands such as Delta-like1, Delta-like4, Jagged1 and/or Jagged2, the Notch receptors undergo a series of proteolytic cleavage events that releases the Notch intracellular domain (NICD). The NICD then translocates to the nucleus where it forms a complex with DNA-binding proteins of the CSL family leading to the formation of active transcription factors that promote the expression of various Notch target genes. Interestingly, cross-talk between the Notch and NF-κB signaling pathways has been recently identified, with the observation that the NICD augments nuclear retention and activity of NF-κB.
Figure 1-5. Notch Signaling Pathway. Once the Notch receptor interacts with a ligand such as Delta-like or Jagged ligands, an ADAM-family metalloprotease called TACE (Tumor Necrosis Factor Alpha Converting Enzyme) cleaves the Notch receptor just outside the membrane. After this first cleavage, an enzyme called γ-secretase cleaves the remaining part of the Notch receptor just inside the cell membrane. This releases the Notch intracellular domain (NICD), which then translocates to the nucleus, where it can regulate gene expression by activating the transcription factor CSL (CBF1/RBP-Jκ/Suppressor of Hairless/LAG-1).
With regard to vascular biology, Notch signaling is known to play a key role in angiogenesis, both during development and in tumor angiogenesis.\textsuperscript{258} Inhibition of Notch signaling in endothelial cells using a Notch1 decoy has been demonstrated to attenuate tumor growth and neoangiogenesis.\textsuperscript{259} In addition to its role in angiogenesis, Notch1 has been demonstrated to mediate neointima formation after vascular injury in a mouse model of carotid injury.\textsuperscript{260} Furthermore, Notch signaling has been implicated in the pathophysiology of atherosclerosis, as blockade of Notch signaling using a gamma-secretase inhibitor has been shown to reduce its development in diet-induced mouse models of atherosclerosis.\textsuperscript{261} Of note, blockade of Notch signaling has been shown to decrease macrophage expression of ICAM-1,\textsuperscript{261} while Notch1 upregulation has been shown to increase stimulated macrophage expression of ICAM-1.\textsuperscript{262}

Recently, Egfl7 has been identified as the first naturally occurring, non-canonical inhibitor of Notch signaling.\textsuperscript{255,263} In an elegant series of studies, Schmidt et al. were the first to identify that the extracellular domains of Notch1-4 act as Egfl7 interacting molecules in yeast two-hybrid screens using human Egfl7 as bait. They then identified Egfl7 as a competitive inhibitor of Jagged1 and Jagged2 binding to Notch1 expressed in transfected HEK293 cells.\textsuperscript{263} Functionally, Egfl7 was found to reduce proliferation and self-renewal of neural stem cells in vitro.\textsuperscript{263} These results were confirmed more recently by Nichol et al. who performed similar studies and found that Egfl7 is a competitive inhibitor of Delta-like4 binding to Notch4.\textsuperscript{248} They also found that the expression of the Notch target gene, Hey1, was significantly down-regulated in transgenic mice that overexpress Egfl7. These studies suggest that Egfl7 may exert some of its effects through a Notch signaling pathway.
1.5 Endothelin-1 Receptor Antagonism

1.5.1 Endothelin-1 and Endothelial Dysfunction

Endothelin-1 was discovered by Yanagisawa et al. in 1988 and is one of the most potent naturally occurring vasoconstrictor known to man.\(^{264}\) It is produced primarily by endothelial cells, but can also be produced by vascular smooth muscle cells.\(^{264-266}\) Production of ET-1 begins as a preproET peptide that is cleaved by endopeptidases to form BigET-1. BigET-1 is then secreted and subsequently converted to ET-1 by endothelin converting enzymes (ECEs) that are located on endothelial cells.\(^{267-270}\) ET-1 is then able to bind to one of two ET-1 receptors (ET\(_A\) and ET\(_B\)) located on a variety of cell types including endothelial cells, smooth muscle cells and myocardial cells.\(^{271-277}\) These transmembrane receptors are coupled to intracellular G proteins upon binding to ET-1.\(^{278,279}\) Depending on the cell type, downstream effector kinases (such as guanylyl and adenylyl cyclases, phospholipases C and A2, and PI3K), are inhibited or activated by G protein mediated ET-1 signaling.\(^{280-283}\) This results in modulated production of a variety of downstream second messengers. In the case of vasoconstriction, ET-1 leads to the activation of phospholipase C, that in turn results in production of DAG and IP\(_3\) that stimulate intracellular Ca\(^{2+}\) influx and resultant smooth muscle contraction.\(^{284-289}\)

A number of stimulators of endothelial ET-1 production are known and include IL-1, TNF\(\alpha\) and TGF\(\beta\).\(^{290-292}\) NO also regulates ET-1 production. Ohkita et al. have demonstrated that the NO donor, FK409, is able to attenuate basal and TNF\(\alpha\) stimulated ET-1 production in porcine aortic endothelial cells.\(^{293,294}\) They were also able to demonstrate that this inhibition was accomplished partially through NF-\(\kappa\)B pathway inhibition. Augmentation of NO levels by treatment with L-arginine has also been shown to reduce ET-1 levels in a rat model.\(^{295}\) As
mentioned previously, I/R injury to the endothelium also stimulates ET-1 production through the production of inflammatory cytokines and ROS.\textsuperscript{296, 297} Calcineurin inhibitors have also been shown to stimulate ET-1 production and sensitivity to ET-1. Haug et al. have demonstrated that both CyA and tacrolimus stimulate the expression (both at the peptide and mRNA levels) of ET-1 in HUVEC in culture.\textsuperscript{298} Petrakopoulou et al. have observed that heart transplant patients receiving CyA had elevated levels of ET-1.\textsuperscript{299} Cauduro et al. have demonstrated that renal transplant patients taking CyA also had elevated ET-1 levels.\textsuperscript{300, 301}

As previously discussed, Ramzy et al. have demonstrated that CyA treatment increases vascular sensitivity to ET-1 induced vasospasm with a concomitant increase in ET\textsubscript{A} receptor expression.\textsuperscript{20}

ET-1, in turn, has been demonstrated to modulate the expression of eNOS and decreases NO production.\textsuperscript{302, 303} It has also been found to increase ROS production in endothelial cells.\textsuperscript{303} Finally, Zouki et al. have demonstrated that ET-1 activates endothelial cells and increases neutrophil adhesion to human coronary artery endothelial cells in culture.\textsuperscript{304} These results suggest that ET-1 may exerts its detrimental effects on endothelial function through multiple pathways.

ET-1 has been implicated in the pathogenesis of CAV, primarily through its negative effect on endothelial function. Ravalli et al. have shown that that ET-1 is directly present in the coronary arteries of patients with CAV through immunoreactivity studies.\textsuperscript{305} Ferri et al. have demonstrated that myocardial interstitial ET-1 expression is associated with the presence of CAV.\textsuperscript{306} Wexberg et al. have determined that elevated plasma BigET-1 levels correlated with reduced coronary flow reserve and the development of increased coronary intimal hyperplasia 4 to 5 years following heart transplantation.\textsuperscript{307} Interestingly, Yamaguchi et al.
have demonstrated inhibition of CAV in a heterotopic heart transplant model by ET-1 blockade using antisense oligodeoxynucleotides of ET-1.\textsuperscript{308} Furthermore, Anggrahini et al. have demonstrated that ET-1 knockout mice have significantly reduced neointimal formation in a carotid artery ligation model.\textsuperscript{309} This reduction in neointimal formation was accompanied by reduced leukocyte and macrophage recruitment, reduced expression of ICAM-1, VCAM-1 and PECAM-1, as well as decreased smooth muscle cell proliferation. Thus, there seems to be an association of ET-1 and the development of CAV, with some evidence to suggest that blockade of ET-1 may inhibit neointimal hyperplasia.

1.5.2 Endothelin-1 Receptor Antagonism with Bosentan

ET-1 antagonism has revealed a number of beneficial effects following various injury mechanisms including I/R and CNI related injuries. Bosentan is a potent competitive ET\textsubscript{A} and ET\textsubscript{B} receptor antagonist that is currently used clinically in the treatment of pulmonary hypertension.\textsuperscript{310}

Cellular studies have demonstrated that bosentan exposure reduces cardiomyocyte ET-1 production leading to improved tolerance to ischemia.\textsuperscript{311, 312} In a rat heterotopic heart transplant model, Szabo et al. demonstrated that ET\textsubscript{A} and ET\textsubscript{B} receptor antagonism significantly improved myocardial and endothelial function following transplantation.\textsuperscript{313} A more recent study, employing a porcine model of orthotopic heart transplantation, suggest that supplementing a blood perfusate with 100 µM of bosentan improves load independent indices of myocardial function following 6 hours of ischemic allograft storage.\textsuperscript{314} Furthermore, bosentan conferred significant endothelial protection as evidenced by preservation of coronary vasomotor function following transplantation. Verma et al. have also demonstrated that bosentan augments endothelial function in a number of animal models
of diabetes, and well as in human internal mammary arteries.\textsuperscript{315-318} As mentioned previously, Ramzy et al. have been able to attenuate the CyA induced impaired vasomotor function and associated ET-1 sensitivity using concomitant systemic treatment with bosentan.\textsuperscript{186} These data suggest that ET-1 antagonism with bosentan has the potential to attenuate the detrimental effects of CNI therapy and may provide some benefit against the development of CAV in the face of CNI injury.

1.6 Phosphodiesterase 3 (PDE3) Inhibition

1.6.1 PDE3 Inhibition with Cilostazol

PDE3 is a primarily membrane-associated phosphodiesterase that catalyzes the hydrolysis of cAMP in various cell types, including vascular smooth muscle cells. Cilostazol is a specific inhibitor of PDE3 that inhibits platelet aggregation, and smooth muscle cell constriction and proliferation.\textsuperscript{319} Clinically, cilostazol is used for the treatment of peripheral arterial occlusive disease to alleviate the symptom of intermittent claudication.\textsuperscript{319-325}

1.6.1.1 Effect on Neointimal Hyperplasia

In 1995, Kubota et al. were the first group to report that cilostazol could prevent intimal hyperplasia after iliac artery stenting in dogs.\textsuperscript{326} Almost 10 year later, Fujinaga et al. reported that local application of cilostazol to a rat anastomotic stricture model of free artery graft stenosis resulted in significant inhibition of neointimal hyperplasia.\textsuperscript{327} Mechanistically, they identified decreased cell proliferation and tenascin-C (an extracellular matrix protein) expression in the neointima as potential mediators of cilostazol’s beneficial effects. Yamamoto et al have corroborated these results in a rat anastomotic stricture model of vein graft stenosis.\textsuperscript{328} Tsuchikane et al. tested cilostazol-eluting stents in a porcine model and reported significant suppression of in-stent intimal hyperplasia.\textsuperscript{329}
A number of studies have identified potential mechanisms that may explain the results observed in these animal models. Inoue et al. demonstrated that cilostazol treatment suppressed P-selectin-mediated platelet activation and Mac-1 mediated leukocyte activation in patients receiving bare metal coronary stents.\(^{330}\) Otsuki et al. demonstrated that cilostazol repressed VCAM-1 expression (protein and mRNA) via inhibition of NF-κB activation in cultured vascular endothelial cells.\(^{331}\) Gao et al. reported similar repression of VCAM-1 expression and NF-κB expression in the aortae of diabetic rats treated with cilostazol.\(^{332}\) Park et al. have reported that cilostazol inhibits monocytes adhesion to HUVEC activated by remnant lipoprotein particle via suppression of VCAM-1, E-selectin and MCP-1 release, as well as NF-κB inhibition.\(^{333}\) Others have also reported similar findings of cilostazol induced NF-κB inhibition and cytokine suppression, as well as decreased endothelial CAM expression and neutrophil adhesion.\(^{334-338}\) Interestingly, Omi et al. have demonstrated that cilostazol decreased neutrophil adhesion to activated endothelial cells by decreasing ICAM-1 and P-selectin through a mechanism that involved increasing NO production.\(^{339}\)

### 1.6.1.2 Human Studies

Clinically, a number of trials have demonstrated benefit of systemic use of cilostazol after coronary stent (drug eluting and bare-metal) implantation in reducing the incidence of in-stent restenosis.\(^{340-347}\) Most recently, the results from a randomized, double-blind, multicenter trial (the DECLARE-LONG II trial) involving 499 patients randomized to either cilostazol or placebo, in addition to aspirin and clopidogrel, for 8 months after zotarolimus-eluting stent implantation have been reported.\(^{348}\) The results from this trial demonstrated that patients receiving cilostazol had significantly less in-stent intimal hyperplasia volume (as assessed by IVUS), and this translated into a reduced requirement for target lesion re-intervention and revascularization at 12 months (7.2% vs. 12%, \(p=0.07\)).\(^{348}\)
Thus, there are data from both animal and clinical studies that suggest an inhibitory effect of cilostazol on neointimal hyperplasia. The effect of cilostazol on neointimal hyperplasia after endothelial injury in the context of immunosuppression, as in the post-transplant setting, is currently unknown. We believe that in the context of CNI induced endothelial dysfunction, cilostazol may portend benefit that abrogates the neointimal hyperplasia that occurs with CAV.

1.7 Summary of Proposed Investigations

1.7.1 Rationale

Whether hypoxic injury directly influences Egfl7 expression in endothelial cells has not been previously reported. Furthermore, the role of Egfl7 in the context of endothelial activation in response to injury has not been previously studied. We hypothesized that exposure to hypoxia would upregulate Egfl7 expression in cultured human coronary artery endothelial cells (HCAEC). We also hypothesized that exogenously administered Egfl7 would have a protective effect against H/R induced ICAM-1 expression, via inhibiting activation of the NF-κB signaling pathway, and in doing so, would inhibit neutrophil adhesion to injured endothelial cells.

Whether CNI directly influences neutrophil adhesion specifically to coronary artery endothelial cells has not been previously reported. Furthermore, the role of Egfl7 in the context of endothelial activation in response to CNI injury has not been previously studied. We hypothesized that exposure to CNI would upregulate neutrophils adhesion to cultured human coronary artery endothelial cells (HCAEC). We also hypothesized that Egfl7 would have a protective effect against CNI induced ICAM-1 expression, via inhibiting activation of the NF-κB signaling pathway. In addition, given the recent evidence that Egfl7 may act by
inhibition of the Notch pathway, we hypothesized that the Notch receptor agonist, Jagged1, would inhibit the effect of Egfl7 on neutrophil adhesion, ICAM-1 production and NF-kB activation.

Although early initiation of rapamycin therapy following heart transplantation has been demonstrated to inhibit CAV, it is frequently discontinued owing to intolerable side effects or is initiated late after transplantation. The effects of rapamycin withdrawal or delayed initiation on neointimal hyperplasia have not been previously studied. We hypothesized that rapamycin withdrawal would lead to progressive neointimal hyperplasia after vascular injury, and that conversely, delayed initiation would result in suboptimal attenuation of neointimal hyperplasia.

Although ET-1 antagonism with bosentan and PDE3 inhibition with cilostazol have been demonstrated to have benefits with regard to improved endothelial function and inhibition of intimal hyperplasia, respectively, the effect of these strategies on inhibiting neointimal hyperplasia in the context of CNI induced vascular injury has not been studied. We hypothesize that the use of these drugs would attenuate neointimal hyperplasia after vascular injury.

1.7.2 Summary of Hypotheses

Effect of Hypoxic Injury on Endothelial Egfl7 expression

1. Exposure to hypoxia will up-regulate Egfl7 expression in cultured human coronary artery endothelial cells.
**Effects of Egfl7 on Endothelial Injury and Activation**

1. Egfl7 will decrease endothelial cell activation in response to hypoxia/reoxygenation and CNI injury by decreasing intercellular adhesion molecule (ICAM)-1 expression;
2. Egfl7 will block proinflammatory activation pathways by inhibiting NF-κB pathway activation;
3. By inhibiting endothelial cell activation, Egfl7 will inhibit neutrophil adhesion to injured endothelial cells;
4. The effect of Egfl7 will be reversed by co-treatment with Jagged1.

**Effects of Immunosuppressants on Neointimal Hyperplasia**

1. Immunosuppression with the calcineurin inhibitors CyA or tacrolimus will exacerbate the development of neointimal hyperplasia compared to Rapamycin;
2. Early initiation of rapamycin therapy will confer benefit against the development of neointimal hyperplasia compared to control, and will be superior to delayed initiation of rapamycin after vascular injury;
3. Discontinuation of rapamycin therapy after early initiation following vascular injury will result in the progression of neointimal hyperplasia and may result in a rebound effect with an accelerated progression of disease.

**Effects of Vascular Protective Strategies on Neointimal Hyperplasia**

1. Given their previously demonstrated beneficial effects on endothelial function and/or inhibiting neointimal formation, treatment with cilostazol or Bosentan will attenuate the development of neointimal hyperplasia in mice treated with CyA.
1.7.3 Objectives

These studies will hopefully reveal new protective strategies to prevent neointimal hyperplasia after heart transplantation. We hope to demonstrate that current therapy for post-transplant immunosuppression results in a significant exacerbation of endothelial injury and activation which accelerates neointimal hyperplasia leading to cardiac allograft vasculopathy. We will evaluate novel pharmacologic agents specifically targeted towards the endothelium, hoping to achieve enhanced preservation of endothelial function that will translate into attenuated neointimal hyperplasia. Since this study does not involve a transplanted organ, we will be able to differentiate the direct endothelial effects of our interventions from the injury caused by immune-mediated mechanisms. The overall outcome of these studies will be the identification of a pharmacologic strategy involving immunosuppressive and adjunctive pharmacologic agents that will minimize neointimal hyperplasia. This strategy can then be tested in a transplant model and then clinically in patients.

Furthermore, these studies will characterize the role of Egfl7 in endothelial injury and activation that occurs with hypoxia/reperfusion injury at the time of organ transplantation and with CNI induced injury following transplantation. Characterization of this potential therapeutic target will delineate whether it can abrogate endothelial injury that is thought to incite the subsequent development of vasculopathy. This knowledge may stimulate the use of this target as a means of conferring endothelial protection in the setting of endothelial injury.

The results of these experiments are transferable to the clinical realm since our pharmacologic agents of interest – cilostazol and bosentan - have been used previously in human patients. We hope to specifically identify which strategies mitigate the potent
endothelial damage caused by immunosuppression to improve the results of cardiac transplantation. Therefore, the proposed study has the potential to create a clinical impact in our heart transplant recipients. Due to the fact that congestive heart failure is emerging as the next cardiovascular epidemic, strategies to improve the results of current therapies, such as heart transplantation, will be vital to the health of patients suffering from end-stage heart failure.
Chapter 2
EGFL7 ATTENUATES THE ENDOTHELIAL RESPONSE TO HYPOXIA/REOXYGENATION INJURY
2.1 Introduction

Hypoxia/reoxygenation (H/R), which simulates ischemia/reperfusion (I/R) in a cell culture model, is known to injure endothelial cells through oxidant production, which leads to the production of inflammatory cytokines and activates inflammatory cell signaling pathways. This endothelial inflammatory response includes the expression of cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), that allow circulating neutrophils to adhere to sites of endothelial injury. Activation of coronary endothelial cells following I/R injury can occur in a number of clinical settings such as reperfusion following cardioplegic arrest during cardiac surgery. In particular, I/R induced endothelial injury that occurs with cardiac allograft storage and subsequent reperfusion during heart transplantation is believed to be a key event that incites subsequent development of allograft vasculopathy.

Epidermal growth factor-like domain 7 (Egfl7) is a novel protein that has been found to be expressed exclusively by endothelial and early endothelial progenitor cells. Studies in animal models of arterial injury have demonstrated Egfl7 to be upregulated in the regenerating endothelium following injury and have demonstrated that Egfl7 acts as a chemoattractant for embryonic endothelial cells. Upregulation of Egfl7 gene expression has also been demonstrated in the brains of neonatal rats subjected to global hypoxic preconditioning. Furthermore, overexpression of Egfl7 in pulmonary artery endothelial cells has been demonstrated to be protective against hyperoxic injury in both animal and cell culture models. Conversely, whether hypoxic injury directly influences Egfl7 expression in endothelial cells has not been previously reported. Furthermore, the role of Egfl7 in the context of endothelial activation in response to injury has not been previously studied. We hypothesized that exposure to hypoxia would upregulate Egfl7 expression in cultured human
coronary artery endothelial cell (HCAEC). We also hypothesized that Egfl7 would have a protective effect against H/R induced ICAM-1 expression, via inhibiting activation of the NF-κB signaling pathway, which would result in attenuated adhesion of neutrophils.

2.2 Methods

2.2.1 Methodology Outline

We first examined the effect of hypoxia on Egfl7 expression in HCAECs. Cells were subjected to 0, 3 or 6 hours of hypoxia and Egfl7 expression was detected by western immunoblot analysis. We then examined the effect of recombinant human Egfl7 (0.1 ng/mL to 100 ng/mL for 6 or 48 hours, Abnova, Taiwan) on HCAEC total cellular ICAM-1 expression determined by western immunoblot analysis. We then confirmed the results of these western immunoblot experiments by examining cell surface ICAM-1 expression determined by flow cytometry. In these and subsequent experiments, we chose to treat cells with 100 ng/mL of Egfl7 to ensure that any biologic effect was not missed while using lower doses. The effect of Egfl7 (100 ng/mL) on H/R induced ICAM-1 expression was determined by incubating cells with Egfl7 during the reoxygenation phase of the injury and determining ICAM-1 expression by both western immunoblotting and flow cytometry. Additionally, the effect of Egfl7 on ROS production and cell viability were assessed using DCFDA and XTT assays respectively. To examine the effect of Egfl7 on activation of the NF-κB signaling pathway during H/R injury, nuclear and cytoplasmic proteins were isolated from cells that had been subjected to H/R injury +/- Egfl7 during the reoxygenation phase. NF-κB translocation to the nucleus was determined by the cytoplasmic to nuclear NF-κB protein ratio as detected by western immunoblot analysis. Cytoplasmic levels of the NF-κB inhibitor, IkBα, were also determined from these samples by western immunoblot analysis.
Finally, a non-static neutrophil adhesion assay was then carried out as detailed below. Recombinant human tumor necrosis factor-α (1 ng/mL, TNFα, R&D Systems, Minneapolis, MN) was used as a positive control in these studies and phosphate-buffered saline (PBS) was used as the vehicle control for Egfl7.

2.2.2 Endothelial Cell Cultures

HCAECs (Lonza, Walkersville, MD) were cultured in endothelial growth medium (EGM-2 MV, Lonza, Walkersville, MD) containing endothelial growth factors, 5% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in humidified air (5% CO₂). Cells passaged 3 to 5 times and at 80-90% confluence were used for this study. For hypoxia studies, confluent HCAECs were exposed to hypoxia (0.1% O₂) by incubation in a ProOxC hypoxia chamber system (BioSpherix, Lacona, NY) at 37°C that subjected the cells to a controlled anoxic gas mixture consisting of N₂ and 5% CO₂. For reoxygenation and normoxic controls, cells were returned to/incubated in the standard CO₂ incubator under normoxic conditions (21% O₂, 5% CO₂, 74% N₂).

2.2.3 Western Immunoblotting

Western immunoblotting was performed using chemiluminescence detection. For Egfl7 and ICAM-1 protein detection, whole cell lysates were used. For NF-κB and IκBα protein detection, separate nuclear and cytoplasmic protein extracts were obtained from cell pellets using an NE-PER extraction kit (Thermo Scientific, Rockford, IL) as per the manufacturer’s directions. Protein samples were separated using 4% stacking and 10% running tris-glycine sodium dodecyl sulfate-polyacrylamide electrophoresis gels. Proteins were then transferred to polyvinylidene fluoride membranes. Blocking was performed in 5% milk solution for 1 hour at room temperature. The monoclonal primary antibodies were used at the following
dilutions in 1% milk: Egfl7 1:2000 (Abnova, Taiwan), ICAM-1 1:5000 (R&D Systems, Minneapolis, MN), NF-κB p65 and IκBα 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary horseradish peroxidase conjugated antibodies were used at a concentration between 1:5,000 and 1:15,000 (Santa Cruz Biotechnology, Santa Cruz, CA) with ECL Plus used as a substrate (GE Healthcare, UK). β-actin was detected as a loading control for all blots. X-ray films were analyzed using a Bio-Rad GS-800 calibrated densitometer and Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, CA).

2.2.4 Flow Cytometry

Flow cytometry was performed on cells grown on 60 mm plates detached and stained as follows. Cells were washed twice with PBS then incubated with 1 mL of Accutase cell detachment medium (eBioscience, San Diego, CA) at 37°C for 2 to 10 minutes to detach the cells. Once detached, 2 mL of staining buffer (1% bovine serum albumin and 20mM glucose in PBS) was added to the cell suspension which was then divided into samples for ICAM-1 and isotype IgG staining. The samples were centrifuged at 400x g for 5 minutes at 4°C and resultant cell pellets were then resuspended in 100 µL of staining buffer containing a 1:100 dilution of either ICAM-1 monoclonal antibody or IgG1 isotype control antibody (R&D Systems, Minneapolis, MN). The samples were incubated on ice for 40 minutes, washed with 1mL of staining buffer, and centrifuged at 400x g for 5 minutes at 4°C. The cell pellets were resuspended in 100 µL of staining buffer containing a 1:500 dilution of secondary antibody (Alexa Fluor 488, Invitrogen, Carlsbad, CA). The samples were incubated on ice in the dark for 25 minutes (to avoid the effect of photobleaching), washed with 1mL of staining buffer, and centrifuged at 400x g for 5 minutes at 4°C. The samples were then fixed in 2% paraformaldehyde and resuspended in 200 µL of staining buffer. Cell surface ICAM-1 expression on ≥15,000 cells was analyzed using a flow cytometer (Coulter EPICS XL,
Beckman Coulter, Brea, CA) and data analysis was performed using FlowJo software (version 7.2.5, Tree Star Inc., Ashland, OR).

2.2.5 **NF-κB DNA Binding Activity Assay**

Nuclear extracts were obtained from cell pellets using an NE-PER extraction kit (Thermo Scientific, Rockford, IL) as per the manufacturer’s directions. NFκB DNA binding activity within the nuclear fraction was determined using an NFκB (p65) transcription factor activity assay kit (Cayman Chemical Company, Ann Arbor, MI) as per the manufacturer’s directions.

2.2.6 **ROS Production Assay**

The effect of Egfl7 on HCAEC ROS production was assessed using a DCFDA assay. The formation of ROS was detected by loading HCAECs with the cell permeable fluoroprobe, dichlorodihydrofluorescein diacetate (H$_2$DCFDA) (Molecular Probes, Eugene, OR). Once loaded, H$_2$DCFDA is converted into a non-fluorescent, polar, non-cell permeable derivative by cellular esterases. Oxidation via reaction with ROS then convert this derivative into 2’,7’-dichlorofluorescein which is fluorescent.

HCAEC were grown to confluence on 60 mm plates and then loaded with 10 μM H$_2$DCFDA (2 mL per plate) for 30 minutes at 37°C in the dark (to avoid the effect of photobleaching). Cells were then washed with PBS containing 0.1 g/L MgCl and 0.1 g/L CaCl$_2$ and fresh medium was then added. The cells were allowed to recover for 30 minutes at 37°C in the dark and then were subjected to H/R injury, as previously described, in the presence and absence of Egfl7 (100 ng/mL) during the reoxygenation period. Cells were then washed twice with PBS then incubated with 1 mL of Accutase cell detachment medium (eBioscience, San Diego, CA) at 37°C for 2 to 10 minutes to detach the cells. The mean fluorescence intensity (MFI) of the ≥15,000 cells was then quantified using a flow cytometer (Coulter...
EPICS XL, Beckman Coulter, Brea, CA) and data analysis was performed using FlowJo software (version 7.2.5, Tree Star Inc., Ashland, OR).

2.2.7 Viability Assay

The effect of Egfl7 on cell viability was assessed using an XTT assay as per the manufacturer’s directions (Cell Proliferation Kit II (XTT), Roche Applied Science, Mannheim, Germany). XTT is a tetrazolium derivative that measures cell viability based on the activity of mitochondrial enzymes in live cells that reduce XTT to an orange formazan dye that can be quantified spectrophotometrically.

2.2.8 Neutrophil Isolation

Neutrophils were isolated from the peripheral blood of healthy donors as described elsewhere. Briefly, peripheral blood was drawn into 8 mL Vacutainer CPTs containing sodium citrate (BD Diagnostics, Franklin Lakes, NJ). After centrifugation for 25 minutes at 1700g at room temperature, the plasma and PBMC layer was discarded and the gel lock was washed twice with ice cold PBS. A 3 mL syringe attached to an 18 Ga. 1.5 inch needle was then used to collect the erythrocyte/neutrophil mixture. The cells were collected in a 50 mL conical tube and washed with 5 mL of ice cold PBS containing 2% fetal bovine serum (FBS). After centrifugation for 10 minutes at 400g at 4°C, the supernatant was aspirated off and 16 mL of RBC lysis buffer (10 mM KHCO₃, 150 mM NH₄Cl, 0.1 mM EDTA, pH 8.0) was added for 10 minutes at room temperature with intermittent vortexing. The cells were then washed with 5 mL of ice cold PBS containing 2% FBS. After centrifugation for 10 minutes at 400g at 4°C, the supernatant was aspirated off and the cells were then suspended in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% FBS, 50 U/mL penicillin G, 50 µg/mL streptomycin, 50 µg/mL gentamicin, 2.5 µg/mL fungizone, 4 mM L-glutamine and
1% of MEM nonessential amino acids, counted and checked for viability by trypan blue exclusion.

2.2.9 Neutrophil Adhesion Assay

Neutrophil adhesion to confluent HCAEC monolayers grown on 24 well plates that had been subjected to H/R injury +/- Egfl7 and/or Jagged1 was assessed using a non-static adhesion assay. Neutrophils isolated from healthy donors were resuspended in RPMI1640 medium (1x10^6 neutrophils/mL). After washing the wells containing HCAEC monolayers with PBS, 500 µL of neutrophil suspension was added to each well. The plates were incubated for 1 hour at 37°C under rotating conditions (62 rpm). Non-adherent cells were then gently washed off twice with PBS and the number of neutrophils remaining was quantified using a myeloperoxidase assay as described elsewhere. Briefly, 300 µL of a solution of 5.5 mM ortho-phenylenediamine and 4 mM H₂O₂ in a buffer containing 67 mM Na₂PO₄, 35 mM citric acid, 0.1% Triton-X100, pH 5.0, were added to each well. The reaction was stopped after 4 minutes by adding 300 µL of 1 M H₂SO₄. Absorbance at 492 nm was then measured. Standard dilutions of neutrophils were used to construct a standard curve.

2.2.10 Statistical Analysis

Statistical analysis was performed using the GraphPad Prism software (Version 5.0, GraphPad Software, La Jolla CA). Continuous data are expressed as the mean ± standard deviation. Normality of the data was determined using the method of Kolmogorov and Smirnov. A one-way analysis of variance with post-hoc pairwise comparisons between all groups was performed using Bonferroni’s correction for data determined to have a normal distribution, otherwise, a non-parametric Kruskal-Wallis test with post-hoc pairwise comparisons between all groups was performed using Dunn’s post test. Data examining the
effect of treatment at various time points or conditions were analyzed using a two-way analysis of variance.

2.3 Results

2.3.1 Hypoxia stimulates Egfl7 expression

Figure 2-1 depicts the effect of exposure to hypoxia on HCAEC Egfl7 expression after 3 and 6 hours of hypoxia. Exposure to 3h of hypoxia significantly increased Egfl7 expression (p<0.05) with a further increase after 6h of hypoxia (p<0.001) compared to the normoxic control expression level (n=6 per group).

2.3.2 Egfl7 inhibits basal ICAM-1 expression

Exposure to Egfl7 at varying doses from 0.1 ng/mL to 100 ng/mL for 6 hours reduced total cellular ICAM-1 expression in HCAECs, with ICAM-1 expression dropping maximally to 58±2% of control when a dose of 100 ng/mL was used (p<0.01, n=4 per group, Figure 2-2). Cell surface expression of ICAM-1 was significantly attenuated by incubation with Egfl7 (100 ng/mL) for 48h (43±6.7% ICAM-1 positive cells versus 71±7.2% ICAM-1 positive control cells, p=0.001, n=4 per group, Figure 2-3).

2.3.3 Egfl7 attenuates H/R induced ICAM-1 expression

Exposure to 24h of hypoxia followed by a 12h reoxygenation period resulted in increased ICAM-1 expression in HCAEC (p<0.05, n=4 per group) which was significantly attenuated by treatment with Egfl7 (100 ng/mL) during the reoxygenation period (p<0.01 compared to H/R alone, n=4 per group, Figure 2-4). Similarly cell surface expression of ICAM-1 was significantly attenuated by treatment with Egfl7 during the reoxygenation period (p<0.05, n=4 per group, Figure 2-5).
2.3.4 Egfl7 inhibits Neutrophil Adhesion to H/R Injured HCAECs

Neutrophil adherence to HCAEC monolayers subjected to 24h of hypoxia followed by a 24h reoxygenation period under normoxic conditions was significantly increased compared to control in a non-static adhesion assay (p<0.001 compared to control, Figure 2-6). Incubation with Egfl7 (100 ng/mL) throughout the H/R period blocked this increase in neutrophil adhesion (p<0.001 compared to H/R injury alone, Figure 2-6). While incubation with Jagged1 (200 ng/mL) during the H/R period attenuated the adhesion of neutrophils (p<0.01 compared to H/R injury alone, Figure 2-6), co-incubation with Egfl7 (100 ng/mL) during the H/R period caused an increase in neutrophil adhesion compared to incubation with Egfl7 alone (p<0.01 compared to H/R+Egfl7, Figure 2-6).

2.3.5 Egfl7 inhibits NF-κB nuclear translocation and DNA binding activity while it preserves IkB-α

NF-κB is active as a transcription factor when it is in the nucleus and not sequestered by IkB (the α-isof orm being the most prevalent) in the cytoplasm. Exposure to 12h of hypoxia followed by a 6h of reoxygenation period resulted in significant nuclear translocation of NF-κB (p<0.05 compared to control, n=4 per group, Figure 2-7), that was inhibited by treatment with Egfl7 (100 ng/mL) during the reoxygenation period (p<0.05 compared to H/R alone, n=4 per group). Furthermore, H/R alone resulted in a significant decrease in IkB-α level (p<0.05 compared to control, n=4 per group, Figure 2-8), which was partially abrogated by treatment with Egfl7 (100 ng/mL) during the reoxygenation period (p<0.05 compared to H/R alone, n=4 per group). These results were confirmed by quantifying NF-κB DNA binding activity in the nuclear fraction of HCAEC lysates (Figure 2-9). NF-κB (p65) DNA binding activity was increased following exposure to 24h of hypoxia and 12 h of reoxygenation (p<0.001 compared to control, Figure 2-9). Co-treatment with Egfl7 (100 ng/mL) during the
reoxygenation period significantly reduced NF-κB DNA binding activity (p<0.001 compared to H/R alone, Figure 2-9).

2.3.6 Egfl7 does not affect ROS production

ROS production in HCAECs subjected to 24 hours of hypoxia followed by 15 minutes of reperfusion was found to be significantly increased (p<0.001 compared to control, Figure 2-10). Incubation with Egfl7 (100 ng/mL) did not have any significant effect on ROS production following H/R injury (p=0.15 compared to incubation without Egfl7, Figure 2-10).

2.3.7 Egfl7 does not affect cell viability

Exposure to 24h of Egfl7 (100 ng/mL) alone did not significantly affect HCAEC viability as assessed by XTT assay (p=0.15 compared to control, Figure 2-11). Both 24h of hypoxia and 24h of hypoxia followed by 24h of reoxygenation resulted in diminished HCAEC viability (p<0.05 compared to control, Figure 2-11). Co-incubation with Egfl7 (100 ng/mL) during hypoxia had no significant effect on cell viability (p=0.15 compared to hypoxia alone, Figure 2-11), and similarly, co-incubation with Egfl7 (100 ng/mL) during H/R injury had no significant effect on cell viability (p=0.49 compared to H/R alone, Figure 2-11).
Figure 2-1. HCAEC Egfl7 protein expression in response to hypoxia. Exposure to increasing duration of hypoxia significantly increased expression of Egfl7 in HCAEC detected by western immunoblot (3h Hypoxia 140±8% of control, *p<0.05; 6h Hypoxia 385±50% of control, †p<0.001). β-actin was detected as a loading control.
Figure 2-2. HCAEC ICAM-1 protein expression in response to Egfl7. Total cellular ICAM-1 levels were significantly reduced after 6 hour incubations with varying doses of Egfl7 (n=4 per group; *p<0.05 versus control expression; †p<0.01 versus control expression). β-actin was detected as a loading control.
Figure 2-3. HCAEC ICAM-1 cell surface protein expression in response to Egfl7. Cell surface expression of ICAM-1 was suppressed in response to 6 and 48 hour incubations with Egfl7 (100 ng/mL) (n=4 per group; †p=0.06 versus 6h control; §p=0.001 versus 48h control). Both treatment with Egfl7 and duration of incubation significantly affected ICAM-1 expression (p=0.014 for time effect and p=0.0002 for treatment effect).
Figure 2-4. HCAEC ICAM-1 protein expression in response to H/R Injury +/- Egfl7. Total cellular ICAM-1 expression induced by H/R injury was significantly attenuated by incubation with Egfl7 during the reoxygenation period (n=4 per group; H/R alone 144±13% of control, †p<0.05 compared to control; H/R+Egfl7 56±15% of control, §p<0.01 compared to H/R alone). β-actin was detected as a loading control. H: Hypoxia, R: Reoxygenation.
Figure 2-5. HCAEC ICAM-1 cell surface protein expression in response to H/R Injury +/- Eglf7. Upregulated cell surface expression of ICAM-1 induced by H/R injury was significantly attenuated by incubation with Eglf7 during the reoxygenation period (n=4 per group; MFI: 24h Hypoxia/12h Reoxygenation 5.37±0.92 versus 24h Hypoxia/12h Reoxygenation with Eglf7 3.81±0.21, *p<0.05). MFI: Mean Fluorescence Intensity, H: Hypoxia, R: Reoxygenation.
Figure 2.6. Neutrophil adhesion to HCAEC monolayers subjected to H/R Injury +/- Egfl7 +/- Jagged1. Neutrophil adhesion to HCAECs subjected to 24h incubation in hypoxic conditions followed by a 24h reoxygenation period was significantly increased compared to control (Control: 20±5%, n=8; 24h H/24h R: 40±6%, n=12; *p<0.001 vs. control).

Incubation with Egfl7 during H/R attenuated this increase in neutrophil adhesion (24h H/24h R+Egfl7: 20±2%, n=4; p<0.001 vs. 24h H/24h R). Incubation with Jagged1 during H/R partially attenuated the increase in neutrophil adhesion (24h H/24h R+Jagged1: 32±2%, n=4; †p<0.01 vs. 24h H/24h R). Co-incubation with both Egfl7 and Jagged1 during H/R partially reversed the inhibitory effect of Egfl7 on neutrophil adhesion (24h H/24h R+Egfl7+Jagged1: 30±2%, n=4; §p<0.01 vs. 24h H/24h R+Egfl7). Egfl7: Egfl7 100 ng/mL, Jag1: Jagged1 200 ng/mL, H: Hypoxia, R: Reoxygenation.
Figure 2-7. NF-κB nuclear translocation in response to H/R Injury +/- Egfl7 in HCAECs. Western blot analysis comparing nuclear to cytoplasmic levels of NF-κB. H/R Injury resulted in nuclear translocation of NF-κB (n=4 per group; cytoplasmic/nuclear NF-κB ratio 0.93±0.01 versus control 1.40±0.12, *p<0.05; TNF-α 0.43±0.06 versus control, †p<0.01). Incubation with Egfl7 (100 ng/mL) during the reoxygenation period significantly attenuated the nuclear localization of NF-κB (cytoplasmic/nuclear NF-κB ratio 1.44±0.22 versus H/R alone 0.93±0.01, ‡p<0.05). β-actin was detected as a loading control (not shown). H: Hypoxia, R: Reoxygenation.
Figure 2-8. IkB-α levels in response to H/R Injury +/- Egfl7 in HCAECs. Western blot analysis of IkB-α, the cytoplasmic inhibitor of NF-κB. Exposure to hypoxia alone or H/R significantly decreased levels of IkB-α compared to control (n=4 per group; Hypoxia alone 34±3.5% of control, †p<0.05; H/R 26±4.6% of control, ¥p<0.05; TNF-α 28±3% of control, #p<0.05). Incubation with Egfl7 (100 ng/mL) during the reoxygenation period significantly increased the level of IkB-α compared to H/R alone (46±6.2% versus 26±4.6% of control, **p<0.05). β-actin was detected as a loading control. H: Hypoxia, R: Reoxygenation.
Figure 2-9. NF-κB (p65) DNA binding activity in response to H/R Injury in HCAECs. Exposure to either 24h hypoxia or 24h hypoxia followed by 12h reoxygenation significantly increased NF-κB DNA binding activity compared to control (156±21% of control, *p<0.05, n=4 and 298±24% of control, †p<0.001, n=4 respectively). Co-treatment with Egfl7 (100 ng/mL) during the reoxygenation phase significantly blocked NF-κB DNA binding activity stimulated by H/R injury alone (103±28% of control, p<0.001 vs. H/R alone, n=4). H: Hypoxia, R: Reoxygenation, Egfl7: Egfl7 100 ng/mL.
ROS production, as quantified by MFI of HCAEC subjected to a DCFDA assay, was increased following H/R injury (Control: 35.5±1.0, n=4; 24h H/15min R: 49.5±1.9, n=4; p<0.0001 for H/R treatment effect). Incubation with Egfl7 100 ng/mL had no significant effect on ROS production (24h H/15min R+Egfl7: 50.7±3.2, n=4; p=0.15 for Egfl7 treatment effect). The interactive effect of H/R injury and Egfl7 was also not significant (p=0.66). H: Hypoxia, R: Reoxygenation.
Figure 2-11. HCAEC cell viability in response to hypoxia or H/R Injury +/- Egfl7 assessed by XTT assay. Incubation with Egfl7 (100 ng/mL) for 24h did not significantly affect cell viability compared to control (103±2% of control, *p=0.15 compared to control; n=4 per group). Though 24h of hypoxia significantly decreased cell viability (80±4% of control, †p<0.05 compared to control), co-incubation with Egfl7 did not significantly affect this decrease in viability (74±7% of control, §p=0.18 compared to H alone). Similarly, H/R injury significantly decreased cell viability (90±5% of control, ‡p<0.05 compared to control), however, incubation with Egfl7 during the reoxygenation period did not significantly affect this decrease in viability (86±3% of control, §p=0.49 compared to H/R alone). H: Hypoxia, R: Reoxygenation.
2.4 Conclusions

We have made the following novel observations: (1) isolated HCAECs exposed to hypoxia demonstrate an increase in Egfl7 production; (2) incubation with Egfl7 suppress both total cellular and cell surface expression of ICAM-1; (3) incubation with Egfl7 during the reoxygenation period suppresses ICAM-1 production stimulated by H/R injury; (4) incubation with Egfl7 suppresses the susceptibility of neutrophil adhesion to H/R injured HCAECs; (5) incubation with Egfl7 inhibits H/R injury induced NF-κB nuclear translocation and DNA binding activity while partially preserving IκB-α levels, suggesting that it inhibits NF-κB activation; (6) incubation with Egfl7 does not affect ROS production following H/R injury; and (7) incubation with Egfl7 does not adversely affect cell viability;

In conclusion, Egfl7 inhibits HCAEC expression of ICAM-1 both basally and subsequent to H/R injury. Mechanistically, Egfl7 prevented NF-κB nuclear localization and augmented IκB-α protein levels, suggesting that it inhibits NF-κB activation – a key step in the inflammatory activation of endothelial cells. Functionally, Egfl7 potently blocked neutrophil adhesion to H/R injured HCAEC monolayers. Thus, Egfl7 may be protective against H/R injury incurred during events such as heart transplantation or after percutaneous coronary intervention or coronary artery bypass grafting for acute myocardial infarction. Augmentation of Egfl7 may be a novel therapeutic strategy to modulate the inflammatory events that prime the endothelium for the future development of atherosclerosis, in-stent restenosis, and/or transplant vasculopathy.
Chapter 3
EGFL7 ATTENUATES THE ENDOTHELIAL RESPONSE TO CALCINEURIN INHIBITION INDUCED INJURY
3.1 Introduction

Calcineurin inhibition (CNI) is cornerstone of immunosuppressive therapy following heart transplantation. CNI agents, such as cyclosporine (CyA) and/or tacrolimus (Tac), are known to cause endothelial dysfunction by decreasing nitric oxide (NO) production, increasing endothelin-1 (ET-1) production and increasing sensitivity to ET-1. Both altered NO homeostasis and exposure to ET-1 lead to the endothelial production of inflammatory cytokines and activation of inflammatory cell signaling pathways such as the NF-κB pathway. This endothelial inflammatory response includes the expression of cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), that allow circulating neutrophils to adhere to sites of endothelial injury.

As discussed in the previous chapter, we have demonstrated that Egfl7 inhibits endothelial ICAM-1 expression in response to hypoxia/reoxygenation injury. The cell signaling pathways that mediate Egfl7 actions are unclear. However, recent evidence suggests that Egfl7 may be an inhibitor of the Notch signaling pathway.

Whether CNI directly influences neutrophil adhesion to coronary artery endothelial cells has not been previously reported. Furthermore, the role of Egfl7 in the context of endothelial activation in response to CNI injury has not been previously studied. We hypothesized that exposure to CNI would upregulate neutrophil adhesion to cultured human coronary artery endothelial cells (HCAEC). We also hypothesized that Egfl7 would have a protective effect against CNI induced ICAM-1 expression, via inhibiting activation of the NF-κB signaling pathway. In addition, we hypothesized that the Notch receptor agonist, Jagged1, would inhibit the effect of Egfl7 on neutrophil adhesion, ICAM-1 production and NF-κB activation.
3.2 Methods

3.2.1 Methodology Outline

We first examined the effect of CNI on neutrophil adhesion to treated HCAECs. Cells were subjected to 48 hours of treatment with cyclosporine (0.1 to 10 µg/mL, LC Laboratories, Woburn, MA), tacrolimus (1 to 100 ng/mL, LC Laboratories, Woburn, MA), recombinant human Egfl7 (100 ng/mL, Abnova, Taiwan) or recombinant human Jagged1 (200 ng/mL, R&D Systems, Minneapolis, MN) alone or in combination. DMSO (0.01%) was used as the vehicle control for both CyA and tacrolimus, while phosphate-buffered saline (PBS) was used as the vehicle control for Egfl7 and Jagged1. A non-static neutrophil adhesion assay was then carried out as detailed below. We next examined the effect of Tac (100 ng/mL for 48 hours) in the presence and absence of Egfl7 (100 ng/mL) on ICAM-1 expression by both western immunoblotting. Additionally, the effect of Egfl7 (100 ng/mL) and/or Jagged1 (200 ng/mL) co-treatment with tacrolimus or CyA on cell surface ICAM-1 expression was determined by flow cytometry. To examine the effect of Egfl7 on activation of the NFκB signaling pathway in response to CNI injury, nuclear proteins were isolated from cells that had been subjected to CNI injury +/- Egfl7 and were subjected to an NFκB DNA binding activity assay as detailed below. Finally, cell viability was assessed using an XTT assay as per the manufacturer’s directions (Cell Proliferation Kit II (XTT), Roche Applied Science, Mannheim, Germany). XTT is a tetrazolium derivative that measures cell viability based on the activity of mitochondrial enzymes in live cells that reduce XTT to an orange formazan dye that can be quantified spectrophotometrically. Recombinant human tumor necrosis factor-α (1 ng/mL, TNF-α, R&D Systems, Minneapolis, MN) was used as a positive control in these studies.
3.2.2  Endothelial Cell Cultures

HCAECs (Lonza, Walkersville, MD) were cultured in endothelial growth medium (EGM-2 MV, Lonza, Walkersville, MD) containing endothelial growth factors, 5% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in humidified air (21% O₂, 5% CO₂, 74% N₂). Cells passaged 3 to 5 times and at 80-90% confluence were used for this study.

3.2.3  Western Immunoblotting

Western immunoblotting was performed using chemiluminescence detection. Protein samples were separated using 4% stacking and 10% running tris-glycine sodium dodecyl sulfate-polyacrylamide electrophoresis gels. Proteins were then transferred to polyvinylidene fluoride membranes. Blocking was performed in 5% milk solution for 1 hour at room temperature. A monoclonal primary antibody for ICAM-1 (R&D Systems, Minneapolis, MN) was used at a 1:5000 dilution in 1% milk. A secondary horseradish peroxidase conjugated antibody was used at a concentration of 1:5,000 (Santa Cruz Biotechnology, Santa Cruz, CA) with ECL Plus used as a substrate (GE Healthcare, UK). β-actin was detected as a loading control for all blots. X-ray films were analyzed using a Bio-Rad GS-800 calibrated densitometer and Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, CA).

3.2.4  Flow Cytometry

Flow cytometry was performed on cells grown on 60 mm plates detached and stained as follows. Cells were washed twice with PBS then incubated with 1 mL of Accutase cell detachment medium (eBioscience, San Diego, CA) at 37°C for 2 to 10 minutes to detach the cells. Once detached, 2 mL of staining buffer (1% bovine serum albumin and 20mM glucose
in PBS) was added to the cell suspension which was then divided into samples for ICAM-1 and isotype IgG staining. The samples were centrifuged at 400x g for 5 minutes at 4°C and resultant cell pellets were then resuspended in 100 µL of staining buffer containing a 1:100 dilution of either ICAM-1 monoclonal antibody or IgG\textsubscript{1} isotype control antibody (R&D Systems, Minneapolis, MN). The samples were incubated on ice for 40 minutes, washed with 1mL of staining buffer, and centrifuged at 400x g for 5 minutes at 4°C. The cell pellets were resuspended in 100 µL of staining buffer containing a 1:500 dilution of secondary antibody (Alexa Fluor 488, Invitrogen, Carlsbad, CA). The samples were incubated on ice in the dark for 25 minutes, washed with 1mL of staining buffer, and centrifuged at 400x g for 5 minutes at 4°C. The samples were then fixed in 2% paraformaldehyde and resuspended in 200 µL of staining buffer. Cell surface ICAM-1 expression on ≥15,000 cells was analyzed using a flow cytometer (Coulter EPICS XL, Beckman Coulter, Brea, CA) and data analysis was performed using FlowJo software (version 7.2.5, Tree Star Inc., Ashland, OR).

3.2.5 Neutrophil Isolation

Neutrophils were isolated from the peripheral blood of healthy donors as described elsewhere.\textsuperscript{350} Briefly, peripheral blood was drawn into 8 mL Vacutainer CPTs containing sodium citrate (BD Diagnostics, Franklin Lakes, NJ). After centrifugation for 25 minutes at 1700g at room temperature, the plasma and PBMC layer was discarded and the gel lock was washed twice with ice cold PBS. A 3 mL syringe attached to an 18 Ga. 1.5 inch needle was then used to collect the erythrocyte/neutrophil mixture. The cells were collected in a 50 mL conical tube and washed with 5 mL of ice cold PBS containing 2% fetal bovine serum (FBS). After centrifugation for 10 minutes at 400g at 4°C, the supernatant was aspirated off and 16 mL of RBC lysis buffer (10 mM KHCO\textsubscript{3}, 150 mM NH\textsubscript{4}Cl, 0.1 mM EDTA, pH 8.0) was added for 10 minutes at room temperature with intermittent vortexing. The cells were then
washed with 5 mL of ice cold PBS containing 2% FBS. After centrifugation for 10 minutes at 400g at 4°C, the supernatant was aspirated off and the cells were then suspended in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% FBS, 50 U/mL penicillin G, 50 µg/mL streptomycin, 50 µg/mL gentamicin, 2.5 µg/mL fungizone, 4 mM L-glutamine and 1% of MEM nonessential amino acids, counted and checked for viability by trypan blue exclusion.

3.2.6 Neutrophil Adhesion Assay

Neutrophil adhesion to confluent HCAEC monolayers grown on 24 well plates that had been subjected to CNI treatment +/- Egfl7 and/or Jagged1 was assessed using a non-static adhesion assay. Neutrophils isolated from healthy donors were resuspended in RPMI1640 medium (1x10⁶ neutrophils/mL). After washing the wells containing HCAEC monolayers with PBS, 500 µL of neutrophil suspension was added to each well. The plates were incubated for 1 hour at 37°C under rotating conditions (62 rpm). Non-adherent cells were then gently washed off twice with PBS and the number of neutrophils remaining was quantified using a myeloperoxidase assay as described elsewhere.³⁵¹, ³⁵² Briefly, 300 µL of a solution of 5.5 mM ortho-phenylenediamine and 4 mM H₂O₂ in a buffer containing 67 mM Na₂PO₄, 35 mM citric acid, 0.1% Triton-X100, pH 5.0, were added to each well. The reaction was stopped after 4 minutes by adding 300 µL of 1 M H₂SO₄. Absorbance at 492 nm was then measured. Standard dilutions of neutrophils were used to construct a standard curve.

3.2.7 NF-κB DNA Binding Activity Assay

Nuclear extracts were obtained from cell pellets using an NE-PER extraction kit (Thermo Scientific, Rockford, IL) as per the manufacturer’s directions. NFκB DNA binding activity
within the nuclear fraction was determined using an NFκB (p65) transcription factor activity assay kit (Cayman Chemical Company, Ann Arbor, MI) as per the manufacturer’s directions.

3.2.8 Viability Assay
The effect of Egfl7 on cell viability was assessed using an XTT assay as per the manufacturer’s directions (Cell Proliferation Kit II (XTT), Roche Applied Science, Mannheim, Germany). XTT is a tetrazolium derivative that measures cell viability based on the activity of mitochondrial enzymes in live cells that reduce XTT to an orange formazan dye that can be quantified spectrophotometrically.

3.2.9 Statistical Analysis
Statistical analysis was performed using the GraphPad Prism software (Version 5.0, GraphPad Software, La Jolla CA). Continuous data are expressed as the mean ± standard deviation. Normality of the data was determined using the method of Kolmogorov and Smirnov. A one-way analysis of variance with post-hoc pairwise comparisons between all groups was performed using Bonferroni’s correction for data determined to have a normal distribution, otherwise, a non-parametric Kruskal-Wallis test with post-hoc pairwise comparisons between all groups was performed using Dunn’s post test.

3.3 Results
3.3.1 Egfl7 inhibits HCAEC ICAM-1 expression in response to CNI
Exposure to Tac (100 ng/mL) for 48 hours (n=4/group) did not significantly affect total cellular ICAM-1 expression in HCAECs (82±18% of control, p>0.05), however, membrane fraction ICAM-1 was significantly increased (149±10% of control, p<0.01) by Tac treatment (Figure 3-1). Co-treatment with Egfl7 (n=4/group) significantly attenuated Tac induced ICAM-1 level in the membrane fraction (97±5% of control, p<0.01 vs Tac alone, Figure 3-1).
Cell surface HCAEC ICAM-1 expression, as detected by flow cytometry, was stimulated by both CyA and Tac treatment (Figures 3-2 and 3-3, n=6/group). Treatment with Egfl7 alone or concurrently with CyA or Tac (n=4 to 8/group) significantly blocked cell surface ICAM-1 expression (Figures 3-2 and 3-3). While combined treatment with Jagged1 did not effect ICAM-1 expression under control conditions, it significantly attenuated the ability of Egfl7 to block CNI stimulated cell surface ICAM-1 expression (n=4/group, Figure 3-4).

3.3.2 Egfl7 inhibits Neutrophil Adhesion to HCAEC following CNI treatment

Exposure of HCAEC to 48h of treatment with CyA or Tac (n=8/group) increased neutrophil adhesion (Figure 3-5). Incubation with either Egfl7 or Jagged1 (n=12/group) alone had no significant effect on neutrophil adhesion (Figure 3-5). HCAEC co-treatment with Egfl7 significantly attenuated Tac (Figure 3-6) and CyA (Figure 3-7) (n=8 to 16/group) stimulated neutrophil adhesion. Combined treatment with Jagged1 (n=4 to 8) significantly attenuated the ability of Egfl7 to block neutrophil adhesion to HCAEC treated with Tac (10 to 100 ng/mL, Figure 3-6) or CyA (1 to 10 µg/mL, Figure 3-7).

3.3.3 Egfl7 blocks CNI induced HCAEC NF-κB Activation

Exposure to 6h of CyA (10 µg/mL, n=4/group) significantly increased NF-κB (p65) DNA binding activity in the nuclear lysates of HCAEC (128±2% of control, p<0.001, Figure 3-8), which was significantly attenuated by treatment with Egfl7 (86±3% of control, p<0.001 vs. CyA alone, Figure 3-8, n=4/group). Combined treatment with Jagged1 (n=4/group) significantly attenuated the ability of Egfl7 to inhibit CyA stimulated NF-κB activity (105±4% of control, p<0.05 vs. CyA+Egfl7, Figure 3-8).
3.3.4 Egfl7 does not affect HCAEC viability

Treatment with CyA (10 µg/mL) or Tac (100 ng/mL) for 48h (n=8/group) significantly decreased HCAEC viability as measured using an XTT assay (A450-A650: Control 0.83±0.03 vs. CyA 0.67±0.03, p<0.05; vs. Tac 0.70±0.05, p<0.05, Figure 3-9). Exposure to 48h of Egfl7 (100 ng/mL) alone (n=8/group) or in combination with either CyA (10 µg/mL) or Tac (100 ng/mL) did not significantly affect HCAEC viability (Figure 3-9).
Figure 3-1. HCAEC ICAM-1 protein expression in response to Tac and Egfl7. Total cellular ICAM-1 protein expression from whole cell lysates and membrane fractions in response to incubation with Tac+/-Egfl7 measured by western blot analysis. Membrane fraction ICAM-1 levels were significantly increased after 48 hour incubation with Tac (100 ng/mL, 149±10% of control, *p<0.01 vs. control expression, n=4) which was attenuated by co-treatment with Egfl7 (100 ng/mL, 97±5% of control, †p<0.01 vs Tac alone. Tac 100: Tac 100 ng/mL, Egfl7: Egfl7 100 ng/mL.
Figure 3-2. HCAEC cell surface ICAM-1 expression in response to Tac and Egfl7 measured by flow cytometry. Cell surface ICAM-1 expression was significantly increased by 48h treatment with Tac 100 ng/mL (304±21% of control, p<0.001, n=6). Treatment with Egfl7 (100 ng/mL) in combination with Tac (100 ng/mL) reduced cell surface ICAM-1 expression (215±15% of control, p<0.001 vs. Tac alone, n=8).
Figure 3-3. HCAEC cell surface ICAM-1 expression in response to CyA and Egfl7 measured by flow cytometry. Cell surface ICAM-1 expression was significantly increased by 48h treatment with CyA 10 µg/mL (215±13% of control, p<0.001, n=6). Treatment with Egfl7 (100 ng/mL) alone (65±3% of control, p<0.01 vs. control, n=8) or in combination with CyA (10 µg/mL) reduced cell surface ICAM-1 expression (148±5% of control, p<0.001 vs. CyA alone, n=8).
Figure 3-4. HCAEC cell surface ICAM-1 expression in response to CNI +/- Egfl7 +/- Jagged1 measured by flow cytometry. Treatment with Jagged1 (200 ng/mL, n=4/group) when combined with CyA (10 µg/mL) and Egfl7 (100 ng/mL) for 48h significantly blocked the inhibitory affect of Egfl7 on cell surface ICAM-1 expression (193±3% of control, p<0.01 vs. CyA+Egfl7). Treatment with Jagged1 (200 ng/mL, n=4/group) when combined with Tac (100 ng/mL) and Egfl7 (100 ng/mL) for 48h significantly blocked the inhibitory affect of Egfl7 on cell surface ICAM-1 expression (245±6% of control, p<0.05 vs. Tac+Egfl7).
Figure 3-5. Neutrophil adhesion to HCAEC monolayers subjected to CNI. Neutrophil adhesion to HCAEC subjected to 48h incubation with Tac (1-100 ng/mL) or CyA (1-10 µg/mL) was significantly increased compared to control (Control: 20±5%, n=8; Tac 1 ng/mL: 33±10%, n=8; Tac 10 ng/mL: 41±5%, n=8; Tac 100 ng/mL: 34±2%, n=8; CyA 1 µg/mL: 38±3%, n=8; CyA 10 µg/mL: 37±3%, n=8; *p<0.001 vs. control). Egfl7: Egfl7 100 ng/mL, Jag1: Jagged1 200 ng/mL, Tac 1: Tac 1 ng/mL, Tac 10: Tac 10 ng/mL, Tac 100: Tac 100 ng/mL, CyA 0.1: CyA 0.1 µg/mL, CyA 1.0: CyA 1.0 µg/mL, CyA 10: CyA 10 µg/mL.
Figure 3-6. Neutrophil adhesion to HCAEC monolayers subjected to Tac +/- Egfl7 +/- Jagged1. Co-treatment with Egfl7 (100 ng/mL, n=8 to 16/group) for 48h significantly inhibited neutrophil adhesion to HCAEC incubated with Tac (1-100 ng/mL). Treatment with Jagged1 (200 ng/mL, n=4/group) when combined with Tac (10-100 ng/mL) and Egfl7 (100 ng/mL) for 48h significantly blocked the inhibitory affect of Egfl7 on neutrophil adhesion. Egfl7: Egfl7 100 ng/mL Jag1: Jagged1 200 ng/mL, Tac 1: Tac 1 ng/mL, Tac 10: Tac 10 ng/mL, Tac 100: Tac 100 ng/mL.
Figure 3-7. Neutrophil adhesion to HCAEC monolayers subjected to CyA +/- Egfl7 +/- Jagged1. Co-treatment with Egfl7 (100 ng/mL, n=8 to 16/group) for 48h significantly inhibited neutrophil adhesion to HCAEC incubated with CyA (1-10 µg/mL). Treatment with Jagged1 (200 ng/mL, n=4/group) when combined with CyA (1-10 µg/mL) and Egfl7 (100 ng/mL) for 48h significantly blocked the inhibitory affect of Egfl7 on neutrophil adhesion. Egfl7: Egfl7 100 ng/mL Jag1: Jagged1 200 ng/mL, CyA 0.1: CyA 0.1 µg/mL, CyA 1.0: CyA 1.0 µg/mL, CyA 10: CyA 10 µg/mL.
Figure 3-8. NF-κB (p65) DNA binding activity in response to CyA in HCAEC. Incubation with CyA (10 µg/mL) for 6h significantly increased NF-κB DNA binding activity (128±2% of control, *p<0.001, n=4). Co-treatment with Egfl7 (100 ng/mL) significantly blocked NF-κB DNA binding activity stimulated by CyA (86±3% of control, p<0.001 vs. CyA alone, n=4). Treatment with Jagged1 (200 ng/mL, n=4/group) when combined with CyA (10 µg/mL) and Egfl7 (100 ng/mL) for 6h significantly blocked the inhibitory affect of Egfl7 on NF-κB DNA binding activity (105±4% of control, p<0.05 vs. CyA+Egfl7). CyA: CyA 100 µg/mL, Egfl7: Egfl7 100 ng/mL, Jag1: Jagged1 200 ng/mL.
Figure 3-9. HCAEC cell viability in response to CNI +/- Egfl7 assessed by XTT assay. Incubation with Egfl7 (100 ng/mL) for 48h did not significantly affect cell viability compared to control (A450-A650: 0.84±0.03 vs. 0.83±0.03, p>0.05, n=8/group). Though 48 h of treatment with either CyA (10 µg/mL) or Tac 100 ng/mL significantly decreased cell viability compared to control (A450-A650: Control 0.83±0.03 vs. CyA 0.67±0.03, *p<0.05; vs. Tac 0.70±0.05, *p<0.05, n=8/group), co-incubation with Egfl7 (100 ng/mL) did not significantly affect this decrease in viability.
3.4 Conclusions

We have made the following novel observations: (1) isolated HCAECs exposed to CNI demonstrate an increase in cell surface ICAM-1 expression; (2) isolated HCAECs exposed to CNI treatment demonstrate an increased adhesiveness to neutrophils; (3) incubation with Egfl7 suppresses both neutrophil adhesion to, and ICAM-1 production by HCAECs stimulated with CNI injury; (4) incubation with Egfl7 inhibits CNI induced HCAEC NF-κB activation; (5) co-incubation with Jagged1 inhibited the effects of Egfl7 on HCAEC adhesiveness, ICAM-1 production and NF-κB activation in response to CNI injury and (6) Egfl7 does not affect HCAEC cell viability that is adversely affected by CNI injury.

In conclusion, our study reveals the novel observation that Egfl7 is a potent inhibitor of neutrophil adhesion to HCAECs subsequent to CNI induced injury. Mechanistically, Egfl7 blocked NFkB pathway activation and ICAM-1 expression, suggesting that it has significant anti-inflammatory properties. Since Jagged1 blocked the effect of Egfl7, NOTCH receptor antagonism may contribute to the mechanism of action of Egfl7. Thus, we conclude that Egfl7 may be protective against calcineurin inhibition induced endothelial injury incurred after cardiac transplantation, and thus may modulate events that lead to the development of cardiac allograft vasculopathy.
Chapter 4
PHARMACOLOGIC STRATEGIES USING RAPAMYCIN,
CILOSTAZOL OR BOSENTAN ATTENUATE NEOINTIMAL
HYPERPLASIA IN A MURINE MODEL OF VASCULAR
INJURY
4.1 Introduction

Immunosuppression with calcineurin inhibition using agents such as cyclosporine and tacrolimus has facilitated the clinical application of transplantation. Although CNI therapy has made graft survival in cardiac transplantation possible, it comes at the cost of endothelial dysfunction. Ramzy et al. evaluated the effects of cyclosporine exposure on vasomotor function in a rodent model of transplant vascular injury.\textsuperscript{20} They demonstrated that CyA treatment results in the impairment of endothelial dependent vasorelaxation in thoracic aortic segments. Furthermore, CyA led to an increased sensitivity to exogenous endothelin-1 via an upregulation of ET\textsubscript{A} receptors on the vascular wall.

Rapamycin, a macrocyclic immunosuppressant and product of \textit{Streptomyces hygroscopicus}, inhibits cellular proliferation stimulated by growth factor–driven signal transduction in response to alloantigens.\textsuperscript{234} In animal models, rapamycin has also demonstrated a potent ability to attenuate the development of transplant vasculopathy although the mechanism of action remains unclear.\textsuperscript{237,237} With regard to endothelial function, as compared to CyA, Ramzy et al demonstrated that rapamycin does not impair endothelial dependent vasorelaxation nor does it alter sensitivity to endothelin-1 induced vasospasm.\textsuperscript{239} Although rapamycin portends endothelial functional benefits over CyA, which may be an impetus for its use early after transplantation, it has the adverse side effect of impairing wound healing that severely limits its use immediately post-transplantation and often results in cessation of its use.\textsuperscript{240,241} The efficacy of delayed initiation of rapamycin therapy on preventing endothelial dysfunction and subsequent development of neointimal hyperplasia post-transplantation is unclear. The discontinuation of rapamycin therapy on the development of neointimal hyperplasia is also currently unknown.
Cilostazol is a specific inhibitor of cAMP phosphodiesterase III that inhibits platelet aggregation and smooth muscle cell proliferation. Fujinaga et al. have reported that local application of cilostazol to a rat anastomotic stricture model of free artery graft stenosis results in significant inhibition of neointimal hyperplasia. Clinically, a number of trials have demonstrated a benefit of systemic (oral) use of cilostazol after coronary stent (drug eluting and bare-metal) implantation in reducing the incidence of in-stent restenosis.

Thus, there is data from both animal and clinical studies that suggest an inhibitory effect of cilostazol on neointimal hyperplasia. The effect of cilostazol on neointimal hyperplasia after vascular injury in the context of immunosuppression, as in the post-transplant setting, is currently unknown. We believe that in the context of CNI induced endothelial dysfunction, cilostazol may attenuate the neointimal hyperplasia that occurs with CAV.

Bosentan is a potent ET\textsubscript{A/B} receptor antagonist. Previous studies employing a porcine model of orthotopic heart transplantation suggest that supplementing a blood perfusate with 100 µM of bosentan improves load independent indices of myocardial function following 6 hours of allograft storage. Furthermore, bosentan conferred significant endothelial protection as evidenced by preservation of vasomotor function. Previous cellular studies demonstrated that bosentan exposure reduces cardiomyocyte ET-1 production leading to improved tolerance to ischemia. The protective effects of bosentan may extend beyond the cardiomyocyte and may have a direct beneficial effect on endothelial function and homeostasis. As mentioned previously, one finding in rodent models of vascular injury is an enhanced sensitivity to exogenous ET-1 with CyA, thus we believe that ET-1 antagonism with bosentan has potential to attenuate the detrimental effects of CNI therapy on endothelial function.
We sought to evaluate the effect of CNI and rapamycin treatment on neointimal hyperplasia in a murine model of femoral arterial injury known to reliably generate a neointima. Additionally we sought to evaluate the effect of cilostazol and bosentan on the development of neointimal hyperplasia in the context of CNI treatment. Finally, we sought to determine the expression patterns of ICAM-1 and Egfl7 (as described in the preceding chapters) in the murine femoral artery before and after injury. We specifically chose a non-transplant model for these experiments to evaluate the direct effects of these therapies in the absence of potentially confounding immune-related or I/R mediated injury mechanisms.

The objectives of this study were to test the following hypotheses: (1) Immunosuppression with either CyA or tacrolimus will exacerbate the development of neointimal hyperplasia compared to rapamycin; (2) Delayed initiation of rapamycin therapy will confer some benefit against the development of neointimal hyperplasia compared to control, but will be inferior to early initiation of rapamycin after vascular injury; (3) Discontinuation of rapamycin therapy will result in the progression of neointimal hyperplasia and (4) treatment with cilostazol or bosentan will attenuate the development of neointima in mice treated with CNI.

### 4.2 Methods

#### 4.2.1 Animals and Drugs

All experimental protocols were approved by our institutional animal care committee. Animal care conformed to the Canadian Council on Animal Care guide to the care and use of experimental animals. (Volume 1, 2nd edition. 1993).

Adult male C57BL/6 mice (25-30g) were administered the drug of interest (vehicle/saline control, CyA: 5 mg/kg (Norvartis International AG, Basel, Switzerland), tacrolimus: 1 mg/kg (Astellas Pharma Inc., Tokyo, Japan), rapamycin: 1.5 mg/kg (LC Laboratories, Woburn, MA,
USA), cilostazol: 10 mg/kg (Sigma-Aldrich Canada Ltd., Oakville, ON), daily via intraperitoneal injection or bosentan: 100 mg/kg (Actelion Pharmaceuticals Canada Inc., Laval, QC), daily via oral gavage) alone or in combination and were administered daily from the day of arterial injury until sacrifice, 3 weeks later. In cross-over studies, animals were given CyA for 10 days followed by rapamycin for 10 days and vice versa. For rapamycin withdrawal studies, rapamycin was given for 3 weeks and then sacrifice occurred 3 or 6 weeks later. The doses of all drugs corresponded to clinically relevant doses and have been used previously by our group and others. 20, 186, 188, 239, 332.

4.2.2 Wire Injury Protocol

Femoral artery wire injury was performed as described by Sata et al. 362 Briefly, on the day of femoral artery wire injury, the animals were anaesthetized using 3-5% inhaled isoflurane. The groin hairs were clipped and local anaesthetic (1% lidocaine hydrochloride without epinephrine) was infiltrated into the skin overlying the left femoral vessels. A skin incision was made to expose the left femoral vessels. Utilizing a surgical dissecting microscope (Zeiss OPMI-6 surgical microscope, Carl Ziess AG, Germany) a small branch artery between the rectus femoris and vastus medialis muscles was isolated. This exposed branch artery was dilated by topical application of one drop 1% lidocaine hydrochloride. Microscissors were used to open the branch artery transversely through which a 0.38 mm straight spring wire was inserted up into the femoral artery for at least 5 mm toward the iliac artery (see Appendix 1 for images). The wire was left in place for 1 minute and then removed. The branch artery was then tied off and the skin incision was sutured closed.
4.2.3 Tissue Harvest Procedure

On the day of sacrifice, the animals were anaesthetized using isoflurane. The chest, abdomen and groins were shaved. The groins and heart were exposed (via median sternotomy). The femoral artery distal to the site of injury was then divided. A total volume of 5 mL of normal saline was then injected into the left ventricle to flush the arterial system. The femoral artery was then carefully excised and embedded en bloc in optimal cutting temperature compound. The animal was then sacrificed by exsanguination under general anesthesia.

4.2.4 Histological Staining

Cross-sections (5 µm thickness) of femoral arteries were stained with haematoxylin and eosin. Additional 5 µm frozen sections were air dried and fixed in acetone. Endogenous peroxidase and biotin activities were blocked respectively using glucose oxidase (Vector Laboratories, Burlington, ON) and avidin/ biotin blocking kit (Thermo Fisher Scientific Inc., Fremont, CA). After blocking for 15 min with 10% normal serum from the species from which the secondary antibody was obtained, sections were incubated accordingly at room temperature overnight with primary antibodies against Egfl7 (diluted 1/300, rabbit polyclonal anti-Egfl7 antibody, Abnova, Taiwan) or ICAM-1 (diluted 1/100, goat polyclonal anti-ICAM-1 antibody, R&D Systems, Minneapolis, MN). For the slides incubated with anti-Egfl7 antibody, this was followed by an incubation with a biotinylated secondary antibody (Vector Laboratories, Burlington, ON) for 30 min at room temperature. This was followed by incubation with a horseradish peroxidase-conjugated ultrastreptavidin labeling reagent (ID Labs Inc., London, ON). After washing in TBS, colour development was done with freshly prepared NovaRed solution (Vector Laboratories, Burlington, ON). Finally, sections were counterstained lightly with Mayer’s hematoxylin, dehydrated in alcohols, cleared in xylene and mounted in Permount (Fisher Scientific Co., Ottawa, ON).
The slides were then viewed on an upright microscope (Olympus BX51, Olympus Canada Inc., Markham, ON) and digital images of the specimen were captured using a microscope camera (Olympus DP71, Olympus Canada Inc., Markham, ON). Planimetric analysis was performed (see Appendix 2 for details) to determine the intima-to-media ratio of arterial cross-sections using ImageJ software (ImageJ version 1.44c, National Institutes of Health, Bethesda, MD, USA). The intima-to-media ratio of at least three cross-sections of the artery harvested from each animal was used to calculate a mean intima-to-media ratio for each individual animal. Cross-sections that did not appear to be transverse sections of the artery, or where part of the arterial wall was missing or distorted were not analyzed. Similarly, cross-sections where the internal or external elastic lamina could not be identified were not analyzed.

4.2.5 Statistical Analysis

Statistical analysis was performed using the GraphPad Prism software (Version 5.0, GraphPad Software, La Jolla CA). Continuous data are expressed as the mean ± standard deviation. Normality of the data was determined using the method of Kolmogorov and Smirnov. A one-way analysis of variance with post-hoc pairwise comparisons between all groups was performed using Bonferroni’s correction for data determined to have a normal distribution, otherwise, a non-parametric Kruskal-Wallis test with post-hoc pairwise comparisons between all groups was performed using Dunn’s post test. A Fisher’s exact test was used when comparing proportions.
4.3 Results

4.3.1 Effects of Immunosuppressants on Neointima Formation

Wire injury, as performed in this study, was able to stimulate the formation of a significant neointima by 3 weeks after injury (Figure 4-1 and Appendix 3). Treatment with either CyA (5 mg/kg/day) or tacrolimus (1 mg/kg/day) for 3 weeks following wire injury did not significantly affect the formation of this neointima (Figure 1). In contrast, treatment with rapamycin (1.5 mg/kg/day) completely inhibited the formation of any neointima at 3 weeks following injury (Figure 4-1).

4.3.2 Effect of Rapamycin Treatment Withdrawal on Neointima Formation

Cessation of daily rapamycin (1.5 mg/kg/day) treatment for 21 days following a 3 week treatment period after wire injury resulted in the development of a neointima, p<0.001 (Figure 4-2). This neointima formation occurring over 21 days, which was suppressed during the initial 3 week rapamycin treatment period (Figure 4-2), was significantly less than that formed in the control group (Figure 4-2, Control I:M ratio 2.51±0.18, n=9 vs. 0.75±0.12, n=4, p<0.001). By 6 weeks following withdrawal of rapamycin treatment, all mice (5/5) were found to have occluded wire injured femoral arteries compared to no occlusions (0/8) amongst a control group of wire injured mice sacrificed at the same time-point (9 weeks) following wire injury, p<0.001 (Figure 4-3).
4.3.3 Effect of Rapamycin Treatment Conversion on Neointima Formation

Initial treatment with 10 days of rapamycin (1.5 mg/kg/day) followed by 10 days of CyA (5 mg/kg/day) after wire injury did not significantly affect neointima formation compared to a control (injured but untreated) group (Figure 4-4). Conversely, initial treatment with 10 days of CyA (5 mg/kg/day) followed by 10 days of rapamycin (1.5 mg/kg/day) significantly attenuated neointima formation compared to control (Figure 4-4) and compared to the strategy of initial treatment with rapamycin followed by CyA (Figure 4-4, 10d Rapa/10d CyA I:M ratio 2.22±0.47, n=4 vs. 10d CyA/10d Rapa I:M ratio 0.98±0.52, n=4, p<0.01).

4.3.4 Effects of Cilostazol on Neointima Formation

Treatment with cilostazol (10 mg/kg/day) for 3 weeks following wire injury blocked the formation of a neointimal compared to control, p<0.001 (Figure 4-5). Cilostazol treatment also blocked the formation of a neointima when administered in combination with CyA (5 mg/kg/day), p<0.001, or tacrolimus (1 mg/kg/day), p<0.001 (Figure 4-5).

4.3.5 Effect of Bosentan on Neointima Formation

Treatment with bosentan (100 mg/kg/day) for 3 weeks following wire injury attenuated the formation of a neointimal compared to control, p<0.001 (Figure 4-6). Bosentan treatment also attenuated the formation of a neointima when administered in combination with CyA (5 mg/kg/day), p<0.05 or tacrolimus (1 mg/kg/day), p<0.01 (Figure 4-6).

4.3.6 Presence of ICAM-1 and Egfl7 in Femoral Arteries Before and After Wire Injury

In the uninjured, native murine femoral artery, ICAM-1 was found to be absent from the medial layer of the vessel bounded by the internal and external elastic lamina (Figure 4-7, top
panel, middle image). Coincidental to this observation was the presence of Egfl7, which was found to be prominently located within the same medial layer that was devoid of ICAM-1 (Figure 4-7, top panel, right image). After wire injury, ICAM-1 was also found to be absent from the medial layer of vessels (Figure 4-7, bottom panel, middle image). Additionally, ICAM-1 was not found to be present in the neointima of these injured vessels. As in the uninjured arteries, Egfl7 was limited to the medial layer, but also was found within the neointima, again in the areas devoid of ICAM-1 (Figure 4-7, bottom panel, right image). These observations of ICAM-1 and Egfl7 localization were unchanged by treatment with either CyA or tacrolimus (not shown).
Three weeks following wire injury, femoral arteries from untreated animals (control) developed a significant neointima compared to uninjured femoral arteries (I:M ratio: Control 2.51±0.18, n=9 vs. Uninjured 0.34±0.06 n=4, *p<0.001). Treatment with CyA (5 mg/kg/day) or tacrolimus (1 mg/kg/day) for 3 weeks following injury did not significantly affect neointima formation compared to control (I:M ratios: CyA 2.11±0.32, n=9, p>0.05 compared to control; tacrolimus 2.31±0.17, n=6, p>0.05 compared to control). Treatment with rapamycin (1.5 mg/kg/day) for 3 weeks following injury significantly blocked the formation of a neointima compared to control (I:M ratio 0.33±0.04, n=6, †p<0.001). CyA: cyclosporine, I:M: intima to media.
Figure 4-2. Effect of rapamycin treatment withdrawal on neointima formation. Three weeks following cessation of a three week period of rapamycin (1.5 mg/kg/day) treatment after wire injury, neointima formation was observed (I:M ratios: Rapa+21d WD 0.75±0.12, n=4 vs. Rapa 0.33±0.04, n=6, p<0.01). Rapa: rapamycin, WD: withdrawal, I:M: intima to media.
Figure 4-3. Late occlusion of injured arteries after rapamycin treatment withdrawal. Representative histology of C57BL/6 mice femoral arteries in cross-section stained with haematoxylin and eosin 6 weeks after cessation of rapamycin (1.5 mg/kg/day) treatment that had been administered for 3 weeks following wire injury. All (5/5) mice were found to have occlusion of their femoral arteries compared to none in the control group (0/8), p<0.001.
Figure 4-4. Effect of rapamycin treatment conversion on neointimal formation. Following wire injury, mice were treated with either 10 days of rapamycin (1.5 mg/kg/day) followed by 10 days of CyA (5 mg/kg/day) or vice versa. Treatment with rapamycin followed by CyA did not affect the formation of neointima when compared to control (I:M ratio: 10d Rapa/10d CyA 2.22±0.47, n=4, p>0.05 vs. control). Treatment with CyA followed by rapamycin significantly attenuated the formation of a neointima (I:M ratio: 10d CyA/10d Rapa 0.98±0.52, n=4, p<0.01 vs. control). CyA: cyclosporine, Rapa: rapamycin, I:M: intima to media.

<table>
<thead>
<tr>
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<th>Intima to Media Ratio</th>
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<tbody>
<tr>
<td>Uninjured</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
</tr>
<tr>
<td>10d Rapa / 10d CyA</td>
<td>2.22±0.47</td>
</tr>
<tr>
<td>10d CyA / 10d Rapa</td>
<td>0.98±0.52*</td>
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</tbody>
</table>

Figure 4-4 illustrates the effect of rapamycin treatment conversion on neointimal formation. Following wire injury, mice were treated with either 10 days of rapamycin (1.5 mg/kg/day) followed by 10 days of CyA (5 mg/kg/day) or vice versa. Treatment with rapamycin followed by CyA did not affect the formation of neointima when compared to control (I:M ratio: 10d Rapa/10d CyA 2.22±0.47, n=4, p>0.05 vs. control). Treatment with CyA followed by rapamycin significantly attenuated the formation of a neointima (I:M ratio: 10d CyA/10d Rapa 0.98±0.52, n=4, p<0.01 vs. control). CyA: cyclosporine, Rapa: rapamycin, I:M: intima to media.
Figure 4-5. Effect of cilostazol treatment on neointima formation. Treatment with cilostazol (10 mg/kg/day) for 3 weeks following wire injury blocked the formation of a neointimal compared to control (I:M ratio: 0.27±0.08, n=6, *p<0.001 vs. control). Cilostazol treatment also blocked the formation of a neointima when administered in combination with CyA (5 mg/kg/day) (I:M ratio: 0.29±0.09, n=4, †p<0.001 vs. CyA alone) or tacrolimus (1 mg/kg/day) (I:M ratio: 0.26±0.08, n=6, §p<0.001 vs. tacrolimus alone). CyA: cyclosporine, Tac: tacrolimus, I:M: intima to media.
Figure 4-6. Effect of bosentan treatment on neointima formation. Treatment with bosentan (100 mg/kg/day) for 3 weeks following wire injury blocked the formation of a neointimal compared to control (I:M ratio: 1.88±0.18, n=10, *p<0.001 vs. control). Bosentan treatment also blocked the formation of a neointima when administered in combination with CyA (5 mg/kg/day) (I:M ratio: 1.79±0.16, n=8, †p<0.05 vs. CyA alone) or tacrolimus (1 mg/kg/day) (I:M ratio: 1.85±0.27, n=8, §p<0.01 vs. tacrolimus alone). CyA: cyclosporine, Tac: tacrolimus, I:M: intima to media.
Figure 4-7. Expression of ICAM-1 and Egfl7 in the neointima formed after wire injury. 
(Top panel) Representative sections of uninjured murine femoral artery stained with H&E (left), IHC staining for ICAM-1 (middle) and IHC staining for Egfl7 (right). (Bottom panel) Representative sections of murine femoral artery 3 weeks following wire injury, stained with H&E (left), IHC staining for ICAM-1 (middle) and IHC staining for Egfl7 (right). H&E: haematoxylin and eosin, IHC: immunohistochemical.
4.4 Conclusions

We have made the following novel observations in a model of neointimal hyperplasia following mechanical wire injury: (1) treatment with either CyA or tacrolimus does not affect neointima formation; (2) withdrawal of rapamycin treatment results in a delayed neointima formation accompanied by a late occlusion of wire injured vessels; (3) early treatment with rapamycin followed by conversion to CyA does not affect neointima formation, however, delayed initiation of rapamycin confers some attenuation of neointima development; (4) cilostazol blocks the formation of neointima even with CNI co-treatment and (5) bosentan modestly attenuates neointima formation in the context of CNI therapy. Additionally, we observed that ICAM-1 is suppressed in the media and neointima of arteries where Egfl7 expression is detected.

In conclusion, our study reveals the novel findings that delayed rapamycin initiation after vessel injury may have some residual benefit in preventing neointimal hyperplasia, whereas abrupt cessation of rapamycin treatment initiated early after injury may ultimately lead to vessel occlusion and thus may warrant caution. In addition, co-treatment with cilostazol in the context of CNI therapy has the potential to prevent neointima formation, while co-treatment with bosentan may only modestly attenuate neointima formation. Thus, we conclude that these strategies of delayed initiation of rapamycin therapy, and co-treatment with cilostazol and/or bosentan may modulate the events that lead to the development of cardiac allograft vasculopathy and warrant further investigation in transplant models.
Chapter 5
DISCUSSION
5.1 Introduction

Over the past few decades, the treatment of cardiovascular disease has undergone dramatic change with the development of new pharmacologic strategies that target various cell signaling and metabolic pathways. Unfortunately, despite the significant improvements that these strategies have yielded, conditions such as heart failure continue to increase in incidence.\(^1\),\(^2\) Heart failure continues to present a therapeutic challenge as patients typically experience a continual decline in heart function over years, with some ultimately reaching end-stage heart failure. Treatment options for end-stage heart failure refractory to medical management remain limited and include mechanical circulatory support or heart transplantation in those who are considered appropriate candidates.

It is important to recognize that those few patients who are fortunate enough to receive a heart transplant are not cured. Their heart failure is replaced by a host of other ailments and potential complications that are associated with heart transplantation and the immunosuppression that it requires. Ultimately, it is these by-products of transplantation that limit median survival after heart transplantation to between 10 and 13 years.\(^4\) Amongst the causes of death following transplantation, which include primary graft failure, malignancy and infection, CAV continues to afflict transplanted hearts and its incidence following transplantation has not shown clinically significant improvement in recent years (Figure 1-1).\(^4\)

CAV is characterized by intimal hyperplasia within the coronary vessels that is thought to be a response to vascular injury secondary to I/R injury during transplantation, injury from immunosuppression, as well as persistent antibody-mediated rejection.\(^{16,18,203}\) Previous studies have demonstrated that endothelial injury, dysfunction and inflammation are central...
to the development of CAV.\textsuperscript{10, 18, 31, 77, 203} Immunosuppression with CNI therapy has been shown to cause endothelial dysfunction in both in vivo animal models and human subjects.\textsuperscript{20, 24, 184-187} Similarly, I/R injury has been shown to incite endothelial inflammation and subsequent dysfunction.\textsuperscript{28-35} Clearly, CNI therapy and I/R injury are two major targets for therapeutic strategies that can alleviate the endothelial injury they cause that leads to the subsequent development of CAV.

Recently, proliferation signal inhibitors such as rapamycin and everolimus have been used as a therapeutic strategy to both prevent and slow the progression of established CAV.\textsuperscript{235, 236} Although rapamycin and everolimus have been demonstrated to have efficacy with regard to the prevention and treatment of CAV, these drugs have adverse side effects that complicate their use. Rapamycin in particular is known to interfere with wound healing and thus is often avoided immediately post-transplantation.\textsuperscript{240, 241} Furthermore, its use is associated with the occurrence of pericardial and pleural effusions as well as gastrointestinal related adverse effects that frequently result in premature cessation of therapy. The effects of delayed initiation or withdrawal of rapamycin therapy on the development of intimal hyperplasia after vascular injury have not been clearly defined. This information is important in determining whether there is a rationale for using rapamycin late after transplant and whether early withdrawal of therapy has any negative effects. We hypothesized that delayed initiation of rapamycin therapy would impair its ability to attenuate neointimal hyperplasia in a murine model of arterial injury. We also hypothesized that withdrawal of rapamycin therapy would lead to progressive neointimal hyperplasia in this model.

Novel potential therapeutic strategies to counter the endothelial dysfunction caused by CNI therapy and to inhibit intimal hyperplasia that underlies the pathophysiology of CAV include
using the endothelin-1 antagonist, bosentan, and the PDE3 inhibitor, cilostazol. Ramzy et al. have previously demonstrated that bosentan attenuates CyA induced vasomotor dysfunction in an in vivo model of CNI exposure.\textsuperscript{186} Multiple groups have demonstrated that cilostazol, in both animal models and clinical studies, has an inhibitory effect on intimal hyperplasia.\textsuperscript{326-329, 340-348} Whether bosentan and/or cilostazol can attenuate the development of intimal hyperplasia in the presence of ongoing CNI related exposure has not been previously reported. This information is important in determining whether there is a rationale for using bosentan and/or cilostazol as potential therapeutic strategies to prevent CAV. We hypothesized that the use of bosentan or cilostazol would attenuate neointimal hyperplasia in a murine model of arterial injury.

As mentioned previously, endothelial injury resulting in endothelial inflammation and dysfunction as a result of I/R and CNI therapy, is thought to be central to the development of CAV. It follows that attenuating so-called endothelial “activation” from these two injury mechanisms may have a significant impact on the prevention of subsequent CAV. Recently, Egfl7, a novel protein that is expressed specifically by endothelial cells, has been noted to be upregulated upon arterial injury after mechanical balloon injury and chemical injury from ferric chloride.\textsuperscript{242, 243, 249} Furthermore, it is upregulated in the neonatal rat brain following global hypoxia and downregulated in endothelial cells subjected to hyperoxic injury.\textsuperscript{252} Overexpression of Egfl7 in endothelial cells has been associated with anti-apoptotic intracellular events.\textsuperscript{251} Whether Egfl7 has a role in modulating the inflammatory phenotype associated with endothelial activation after injury has not been previously investigated. This information is important in determining whether there is a rationale for targeting or exploiting Egfl7 as a potential therapeutic strategy to attenuate the endothelial inflammatory response to I/R and/or CNI related injury. We hypothesized that Egfl7 would be protective
against both H/R injury (in a cellular model of I/R injury) and CNI induced injury, and that it would attenuate cell adhesion molecule expression, NF-κB pathway activation and neutrophil adhesion to injured endothelial cells.

Our investigations demonstrated that Egfl7 is a potent inhibitor of endothelial ICAM-1 expression, NF-κB activation, and neutrophil adhesion to coronary endothelial cells subjected to H/R or CNI induced injury. We also demonstrated that both bosentan and cilostazol are capable of attenuating neointimal hyperplasia in a murine model of arterial injury. In addition, we demonstrated that rapamycin withdrawal is associated with a blunted development of neointimal hyperplasia but is also associated with late vessel occlusion. Finally, we observed that delayed initiation of rapamycin therapy retains some efficacy in inhibiting the development of neointimal hyperplasia. These results suggest that Egfl7 may be a novel target for intervention to attenuate the endothelial inflammatory response to injury and that bosentan and/or cilostazol may be potential therapeutic strategies that may inhibit the development of CAV. These results will need to be confirmed with in vivo studies in transplant models to determine whether they have a role in preventing CAV.

5.2 Experimental Models

5.2.1 Cell Culture Model

HCAECs between passages 3 to 5 were used in all of our cellular studies to ensure their morphology and phenotypic qualities were maintained. The cobblestone morphology and oval shape of the endothelial cells were identified by routine light microscopy. Endothelial cell culture use as a model to test the response of endothelial cells to various forms of injury and stimuli has been well established.
For experiments aimed at examining the endothelial response to I/R injury, we utilized a cell culture model of H/R injury to simulate the environmental conditions of the endothelium during transplantation. During transplantation, the donor heart is subjected to a period of static storage in an effectively hypoxic environment. In our model, endothelial cells were subjected to a hypoxic environment (0.1% O$_2$) that represents a PO$_2$ of less than 8 mmHg. Since endothelial cells have a significant tolerance for hypoxia, they do not become hypoxic until the PO$_2$ is less than 30 mmHg, thus a PO$_2$ of less than 8 mmHg was intentionally chosen to ensure that significant endothelial injury occurred. Reperfusion of the donor heart was simulated in our model by changing of the culture medium and reoxygenation by restoration of a normoxic environment (21% O$_2$) for the endothelial cell cultures. Thus, we believe that the H/R methods used in our in vitro experiments closely mimics the conditions of I/R injury sustained by the donor heart endothelium in vivo.

5.2.2 Animal Model
A model of neointimal hyperplasia utilizing C57BL/6 mice as previously described by Sata et al. was used in our animal studies. This model had been used by previous investigators to reliably reproduce neointimal hyperplasia that reaches maximal severity by 3 to 4 weeks following injury. This model involves the insertion of a straight spring guidewire (0.38 mm diameter) into the femoral artery by way of a large muscular side branch, with resultant denudation and distention of the artery (wire/artery diameter ratio 2.0±0.1). This method allows subsequent antegrade flow down the femoral artery and excludes the effect of flow restriction on neointimal formation that confounds other similar models.

We intentionally chose this model of neointimal hyperplasia as a non-transplant model of vascular injury to exclude the effects of I/R injury and immune-mediated vascular rejection.
as we evaluated the direct effects of immunosuppressive therapies in combination with the potential beneficial effects of bosentan and/or cilostazol on neointimal hyperplasia. We also chose a mouse model to facilitate future studies of interest in genetic knockout animals. Our overall goal was to identify successful therapeutic strategies in this model that could then undergo further assessment in a transplant model such as a heterotopic heart transplant model or an aortic interposition model.

5.3 Egfl7 Modulation of the Endothelial Inflammatory Response to Injury

Accumulating evidence suggests that Egfl7 may play a protective role in maintaining blood vessel integrity and specifically may have the potential to modulate the endothelial response to injury. Endothelial injury that occurs following insults such as ischemia/reperfusion and CNI therapy results in decreased endothelial NO production, superoxide anion production, increased ET-1 production and sensitivity to ET-1. These disturbances, in turn, stimulate the expression of inflammatory cytokines, such as IL-6 and cell adhesion molecules such as ICAM-1, which allow circulating neutrophils to migrate to sites of endothelial injury where they subject local tissues, including the endothelium, to further injury. Furthermore, ischemia/reperfusion and CNI related injuries are mechanisms of injury that have been implicated in the development of transplant coronary disease following heart transplantation. Given the potential of Egfl7 to be a novel regulator of the coronary endothelial response to injury, we sought to determine the role that it plays in regulating the coronary endothelial response to both H/R and CNI induced injury in isolated human coronary artery endothelial cells.
We have made the following novel observations: (1) isolated HCAECs exposed to hypoxia
demonstrate an increase in Egfl7 production; (2) incubation with Egfl7 suppress both total
cellular and cell surface expression of ICAM-1; (3) incubation with Egfl7 during the
reoxygenation period suppresses both neutrophil adhesion to, and ICAM-1 production by
HCAECs stimulated by H/R injury; (4) incubation with Egfl7 suppresses both neutrophil
adhesion to, and ICAM-1 production by HCAECs stimulated with CNI injury; (5) incubation
with Egfl7 inhibits H/R injury induced NF-κB nuclear translocation and partially preserves
IκB-α levels; (6) incubation with Egfl7 inhibits CNI induced HCAEC NF-κB activation; (7)
co-incubation with Jagged1 inhibited the effects of Egfl7 on HCAEC adhesiveness, ICAM-1
production and NF-κB activation in response to CNI injury; (8) incubation with Egfl7 does
not adversely affect cell viability.

5.3.1 Effect of Hypoxia on Egfl7 Production

Our study demonstrated that hypoxia stimulates HCAEC Egfl7 production in a seemingly
dose-dependent manner. This observation is in keeping with those reported by Gustavsson et
al., who reported a 40% increase in Egfl7 gene expression in the brains of neonatal rats that
had been subjected to 3 hours of hypoxia. Furthermore, Xu et al. in both animal and
isolated cell culture models of hyperoxic injury demonstrated that endothelial cell Egfl7
expression is downregulated by hyperoxic injury, suggesting that Egfl7 expression is
modulated according to environmental oxygen conditions. They also reported that Egfl7
overexpression in endothelial cells inhibited hyperoxia-induced cytochrome-c release and
caspase-3 activation, and reduced the expression of the pro-apoptotic protein Bax while
increasing the expression of the anti-apoptotic protein Bcl-xL. These findings suggest that
Egfl7 may have a protective role in endothelial cell biology. Given these prior protective
effects, our observation that hypoxia is a stimulant for HCAEC Egfl7 production suggests that Egfl7 may play a role against endothelial injury during hypoxia.

Although we confirmed that Egfl7 does not adversely affect cell viability, we were unable to demonstrate a protective effect against decreased cell viability caused by H/R injury. However, we treated cells with Egfl7 during the H/R injury; pretreatment prior to H/R injury may modulate apoptotic protein expression similar to the observations of Xu et al.\textsuperscript{251} Despite this lack of observable effect on cell viability, the fact that we observed significant increases in Egfl7 expression at both 3h and 6h of exposure to hypoxia is important since the approximate timeframe of the ischemic insult encountered with heart transplantation during the period of allograft storage and transport is between 3 and 6 hours. Accumulation of Egfl7 during this period may have an important anti-inflammatory protective role during the reperfusion phase of I/R injury. Intervention in organ donors aimed at stimulating Egfl7 prior to donor heart cardioplegic arrest may be a way to exploit the protective effects of Egfl7.

5.3.2 Effect on NF-κB Pathway Activity

Our study demonstrated that Egfl7 inhibits nuclear translocation of NF-κB that occurs upon H/R and CyA injury in HCAECs. It is well established that NF-κB plays a key role in regulating the expression of endothelial cell adhesion molecules such as ICAM-1.\textsuperscript{105} H/R injury results in the production of reactive oxygen species and these intermediates are involved in NF-κB activation and binding of activated NF-κB to its associated DNA binding site.\textsuperscript{365} CNI treatment has been previously demonstrated to have a proinflammatory effect on endothelial cells and can also upregulate NF-κB expression and activity.\textsuperscript{366-368} In unstimulated and uninjured cells, NF-κB is sequestered in the cytoplasm in an inactive state
by IκB isoforms, IκB-α being the most abundant isoform. Upon stimulation, IκB is phosphorylated and degraded, which releases NF-κB and allows it to translocate to the nucleus to act as an active transcription factor. Nuclear translocation of NF-κB in H/R injured endothelial cells has been implicated in the enhanced transcription-dependent expression of cell adhesion molecules because the promoter regions of the genes for these molecules contain NF-κB binding sites that are essential for the expression of these proteins on endothelial cells. Thus, our finding that Egfl7 inhibits nuclear translocation and DNA binding activity of NF-κB upon H/R or CNI injury suggests that it inhibits NF-κB activation, which may be the mechanism by which Egfl7 inhibits ICAM-1 expression. Furthermore, we found that Egfl7 partially maintains levels of IκB-α in H/R injured HCAEC, suggesting that stabilization of IκB-α protein may be one of Egfl7’s downstream effects that protects against NF-κB activation. However, since we were unable to completely block the degradation of IκB-α in our H/R studies with Egfl7, there may be another significant pathway that mediates Egfl7’s inhibitory affect on NF-κB nuclear translocation.

5.3.3 Effect on ICAM-1 Expression

We determined that exposure to Egfl7 led to decreased basal total cellular and cell surface ICAM-1 expression. ICAM-1 is basally expressed in endothelial cells, but cell surface levels normally remain low. Upon endothelial activation by stimulation from injury or exposure to inflammatory mediators, ICAM-1, as well as other cell adhesion molecules such as VCAM-1, P-selectin and E-selectin are translocated to the cell surface. These adhesion molecules play a critical role in interacting with circulating neutrophils causing them to roll on and eventually adhere to endothelial cells. Neutrophils can then transmigrate between activated endothelial cells to sites of injury where they further propagate tissue injury and inflammation. Our finding that Egfl7 suppresses basal endothelial ICAM-1 levels suggests
that it may be capable of attenuating the intensity of acute cell surface expression of adhesion molecules upon injury by reducing the basal intracellular stores of ICAM-1. As such, we sought to examine this hypothesis in cell culture models of both H/R and CNI induced injury.

Our study demonstrated that Egfl7 inhibited total cellular and cell surface expression of ICAM-1 expression in response to H/R injury when cells were exposed to Egfl7 during the reoxygenation phase of the injury. Fukushima et al. have previously reported that inhibition of ICAM-1 and/or P-selectin using antibody therapy immediately prior to reperfusion in a rat model of myocardial I/R injury was successful in attenuating infarct size and reducing the accumulation of leukocytes in the ischemic area. Additionally, Ma et al. demonstrated that administration of an anti-ICAM-1 antibody 10 minutes prior to reperfusion in a feline model of myocardial I/R injury resulted in a significant decrease in myocardial necrosis, enhanced endothelium-dependent vasorelaxation of isolated LAD coronary arteries, and a significant inhibition of neutrophil adhesion to the I/R injured coronary artery endothelium compared to controls. Thus, our finding that Egfl7 limits HCAEC expression of ICAM-1 following H/R injury leads us to hypothesize that it may also be protective against myocardial I/R injury which will need to be evaluated in an in vivo model.

We also determined that exposure to CNI increased cell surface ICAM-1 expression in HCAECs and that co-treatment with Egfl7 significantly inhibited this effect. Whether CNI treatment leads to the expression of CAMs such as ICAM-1 in endothelial cells was previously unclear, since there are conflicting data in the literature. For example, Gallego et al. have reported that CyA increases cell surface expression of ICAM-1, VCAM-1 and E-selectin on the cell surface of HUVECs. Conversely, Rafiee et al. have demonstrated that CyA decreases the expression of the CAMs on HIMECs. Whether these differences in
CyA modulated CAM expression are due to cell type or dose effects are unclear. Since we used HCAECs in our studies, our findings of increased ICAM-1 expression stimulated by CNI treatment are most relevant to the coronary endothelium after transplantation. Our finding that Egfl7 suppresses endothelial ICAM-1 levels stimulated by CNI suggests that this may be part of the mechanism by which Egfl7 inhibits neutrophil adhesion to HCAEC subjected to CNI treatment.

Interestingly, in our murine model of neointimal hyperplasia, we made the observation that ICAM-1 was absent from the media and neointima where Egfl7 was primarily found. This relationship was consistent amongst control (untreated) animals and those that received CNI therapy. This in vivo finding is obviously a correlation that needs to be studied further. Whether Egfl7 directly inhibits ICAM-1 expression in this model needs to be evaluated further in Egfl7 knockout mice.

5.3.4 Effect on Neutrophil Adhesion

We observed that exposure to H/R injury or CNI increases neutrophil adhesion to HCAECs. Neutrophils are a subtype of leukocyte that are recruited to sites of activated endothelial cells and are believed to be the primary source of ROS and other inflammatory products that subsequently contribute to endothelial injury and dysfunction.\textsuperscript{26, 111, 128} As mentioned previously, CAMs such as ICAM-1 play a key role in facilitating neutrophil adhesion to endothelial cells.\textsuperscript{148, 162-165} Evidence from animal models of myocardial I/R injury have demonstrated that following reperfusion there is a rapid accumulation of neutrophils in the area at risk with neutrophil adhesion to coronary endothelium within minutes.\textsuperscript{111, 153, 154} In these animal models, neutrophil adhesion has been associated with a progressive decline in coronary endothelial function.\textsuperscript{111}
Inflammatory cells, such as neutrophils, are also thought to be key in promoting and sustaining the arteriopathy that results from chronic CyA treatment.\textsuperscript{373,374} CyA has been previously demonstrated to increase the adhesion of leukocytes to vascular endothelium under physiologic flow conditions, with the upregulation of cell adhesion molecules including ICAM-1, VCAM-1, and E-selectin, implicated in this process.\textsuperscript{374} Furthermore, both CyA and Tac increase the binding of dendritic cells to endothelial cells, again with ICAM-1 and VCAM-1 implicated in this process.\textsuperscript{375}

Thus, our findings that both H/R injury and CNI treatment increase neutrophil adhesion to HCAEC is in keeping with previous studies. Importantly, our observation that Egfl7 has the potent ability to block neutrophil adhesion to HCAEC in the face of H/R or CNI injury suggests that Egfl7 should be investigated further for its potential utility in attenuating vascular injury and endothelial dysfunction caused by neutrophil recruitment. Whether exogenous Egfl7 can block neutrophil adhesion to activated endothelium \textit{in vivo} remains to be determined.

5.3.5 \hspace{1em} \textbf{Mechanism of Egfl7 Effect}

Elucidating the mechanism of Egfl7’s effects on endothelial cell function is a critical component to determining how best to exploit its anti-inflammatory properties. Egfl7’s ability to block neutrophil adhesion to injured endothelial cells in our studies is likely due to suppression of CAM expression. We have demonstrated that Egfl7 potently inhibits ICAM-1 expression, likely due at least in part to attenuated NF-κB activation in response to H/R and CNI stimuli. However, the direct mechanism by which Egfl7 elicits NF-κB pathway inhibition is unclear. NF-κB pathway activation is driven by a number of stimuli including oxidative stress and ROS.\textsuperscript{91,92,98,101-103} In our studies, we found that Egfl7 does not affect
ROS production stimulated by H/R injury in endothelial cells, suggesting that an alternate pathway is involved in mediating Egfl7 anti-inflammatory effects.

**Effect of Notch pathway stimulation on Egfl7 signaling**

We determined that the inhibitory effects of Egfl7 on H/R and CNI stimulated neutrophil adhesion to HCAEC, and CNI stimulated ICAM-1 expression and NF-κB activation were blocked by co-treatment with the Notch receptor agonist, Jagged1. Interestingly, blockade of Notch signaling has been previously shown to decrease macrophage expression of ICAM-1, while Notch1 upregulation has been shown to increase stimulated macrophage expression of ICAM-1.

Schmidt et al. have reported the novel finding that Egfl7 antagonizes Notch-specific signaling in neural stem cells, and moreover, demonstrated that Egfl7 binds to Notch receptors and competitively inhibits binding of Jagged1 to these receptors. More recently, Nichol et al. have shown that Egfl7 is a competitive inhibitor of Delta-like4 binding to Notch4 and that the expression of the Notch target gene, Hey1, is significantly down-regulated in transgenic mice that overexpress Egfl7. Our observation that co-treatment with Jagged1 was able to reverse the anti-inflammatory effects Egfl7 are in keeping with these prior studies that suggest that Egfl7 is a competitive inhibitor of Notch receptor binding.

Interestingly, there is a complex cross talk that occurs between Notch and NF-κB signaling and there is evidence that Notch pathway activation results in NF-κB nuclear retention and activation. Our finding that the effects of Egfl7 are inhibited by Jagged1 suggest that Egfl7 may be attenuating Notch signaling, which in turn, may repress the nuclear retention of
NF-κB and explain the inhibition of NF-κB activity that we observed. Whether the Notch signaling pathway is activated by H/R or CNI stimuli in endothelial cells remains to be elucidated. If Notch pathway inhibition is confirmed to be the signaling mechanism by which Eglf7 exerts its downstream anti-inflammatory effects, then Notch pathway inhibition itself may become a target for intervention.

5.4 Inhibition of Neointimal Hyperplasia following Mechanical Injury

CAV is characterized by a diffuse, concentric fibrous intimal hyperplasia that primarily affects the arteries, arterioles, and capillaries of the donor heart. It is this intimal hyperplasia that we sought to attenuate in a murine model of neointimal hyperplasia using novel therapeutic strategies in the context of CNI immunosuppression. Since the mechanism driving the development of CAV is complex and multifactorial, we sought to isolate the specific effects of these strategies in this non-transplant model. Furthermore, we sought to evaluate the effects of early withdrawal and delayed initiation of current strategies to attenuate CAV using the proliferation signal inhibitor, rapamycin.

We have made the following novel observations: (1) withdrawal of rapamycin treatment results in a delayed neointima formation accompanied by a late occlusion of wire injured vessels; (2) early treatment with rapamycin followed by conversion to CyA does not affect neointima formation, however, delayed initiation of rapamycin confers some attenuation of neointima development; (3) cilostazol blocks the formation of neointima even with CNI co-treatment and (4) bosentan modestly attenuates neointima formation in the context of CNI therapy.
5.4.1 Effects of Rapamycin Withdrawal and Delayed Initiation

Rapamycin is a macrocyclic immunosuppressant that inhibits cellular proliferation stimulated by growth factor–driven signal transduction in response to alloantigens. It is used in patients after heart transplantation as an alternative immunosuppressant to CNI therapy, particularly in patients with established CAV. With regard to endothelial function, as compared to CyA, Ramzy et al. demonstrated that rapamycin does not impair endothelial dependent vasorelaxation nor does it alter sensitivity to endothelin-1 induced vasospasm. Unfortunately, it has the adverse side effect of impairing wound healing that severely limits its use immediately post-transplantation and often results in cessation of its use. Thus, the use of rapamycin is desirable because it does not adversely affect endothelial function, however, its initiation is optimally timed after wound healing has completed.

We determined that abrupt cessation after 3 weeks of rapamycin therapy in a murine model of neointimal hyperplasia resulted in a mild generation of neointima compared to a complete blockade of neointimal hyperplasia with continued rapamycin therapy. Moreover, in the murine model used in our experiments, there was a consistent total arterial occlusion observed 6 weeks after cessation of rapamycin treatment in all animals examined. The early suppressed neointima formation after cessation of rapamycin therapy may be due to residual rapamycin remaining in mice as it undergoes elimination, however, given the short half life (~ 4 to 6 hours) of rapamycin in mice (as compared to ~ 60 hours in humans), this is unlikely. A more likely explanation is that rapamycin suppresses the viability of circulating progenitor cells that normally would home to the site of vascular injury and participate in the formation of the neointima. After rapamycin therapy has been discontinued, there is likely a period of time where these circulating progenitor cells are being regenerated and are not
present to participate in neointima formation. This hypothesis remains to be examined \textit{in vivo}.

Of greater concern was the universal arterial occlusion that we observed 6 weeks after cessation of rapamycin treatment. Whether this is due to a rebound effect involving pro-thrombotic or pro-coagulant factors is unclear. This phenomenon may be due to 2 factors, (1) a delay in re-endothelialization of the wire injured arterial segment after rapamycin cessation, combined with (2) a normalization of hemostatic factors that were previously inhibited by rapamycin. Thus, the denuded wire injured arterial segment that is highly thrombogenic faces a normalized system of hemostatic factors (platelets, fibrin, etc.), which sets the stage for late thrombosis. Indeed, Rampino et al. have reported a platelet-independent defect in hemostasis associated with rapamycin use that normalized after suspension of rapamycin treatment.\textsuperscript{378} Similarly, thrombocytopenia has been reported with rapamycin use in transplant recipients.\textsuperscript{379} Alternatively, there may be an as yet undetermined pro-thrombotic phenotype expressed within the regenerating endothelium/neointima that causes late thrombosis. Our observation of late vessel occlusion after rapamycin cessation also seemingly recreates the phenomenon of late drug-eluting stent thrombosis that occurs with stents that elute drugs such as rapamycin to prevent in-stent restenosis, a form of neointimal hyperplasia. It may be that when these stents cease in their drug elution, that they undergo thrombosis by a mechanism similar to that observed in our model. Further study will be required to elucidate this mechanism.

We determined that delayed initiation of rapamycin therapy after 10 days of cyclosporine treatment retained some efficacy of rapamycin to attenuate neointimal hyperplasia after wire injury. In contrast, conversion to cyclosporine after 10 days of rapamycin did not yield any
significant attenuation of neointima formation compared to control. This finding suggests that delaying the initiation of rapamycin therapy for a short period of time after transplantation to allow for wound healing is acceptable. It is likely that neointimal hyperplasia occurs in a sigmoidal fashion with a slow early progression, followed by a rapid phase that plateaus at a maximal intensity, previously documented to be 3 to 4 weeks after injury in the murine model that we used. Thus, as long as rapamycin therapy is initiated early during the slow growth phase, much of the neointimal hyperplasia is blocked.

5.4.2 Effect of Bosentan

Bosentan is a potent competitive antagonist of the ET\(_A\) and ET\(_B\) receptors that is currently used clinically in the treatment of pulmonary hypertension. Previous studies in animal models have demonstrated that treatment with bosentan confers endothelial protection against both I/R injury following transplantation and CyA induced vasomotor dysfunction. Bosentan has been previously demonstrated to inhibit neointimal hyperplasia in both a collared carotid artery model in rabbits and an organ culture model of intimal hyperplasia in human saphenous vein. We have demonstrated that bosentan attenuates neointimal hyperplasia in a murine model of wire injury, when administered alone or in combination with CNI therapy.

Although we observed a statistically significant reduction in neointima formation with the use of bosentan, the magnitude of the effect was approximately 15 to 20 percent reduction which can be considered moderate at best. Interestingly, Marano et al. observed a similar weak effect of bosentan at inhibiting neointima formation in rabbit carotid arteries that had undergone endothelial denudation by balloon injury. In comparison, they observed a much greater beneficial effect of bosentan on carotid arteries that had undergone collaring.
Thus, it seems that bosentan may exert a greater effect on neointimal hyperplasia in response to flow mediated factors as opposed to endothelial injury. Our observation of bosentan’s effect, though not great in magnitude, does demonstrate a biologic effect of bosentan in the face of CNI therapy that has been previously shown to cause endothelial dysfunction. These findings suggest that it will be meaningful to evaluate whether concomitant use of bosentan and CNI therapy leads to inhibition of CAV in an in vivo model of transplant vasculopathy.

5.4.3 Effect of Cilostazol

Cilostazol is a specific inhibitor of PDE3 that inhibits platelet aggregation, and smooth muscle cell constriction and proliferation. Clinically, cilostazol is used for the treatment of peripheral arterial occlusive disease to alleviate the symptom of intermittent claudication. Previous studies in animal models of neointimal hyperplasia have demonstrated that locally administered cilostazol is effective at inhibiting the formation of a neointima. We have demonstrated that systemic administration of cilostazol blocks the formation of a neointima in a murine model of wire injury, when administered alone or in combination with CNI therapy.

Our results are in keeping with those observed by Fujinaga et al. who reported that local application of cilostazol to a rat anastomotic stricture model of free artery graft stenosis resulted in significant inhibition of neointimal hyperplasia. We evaluated systemic administration of cilostazol as opposed to local administration as systemic administration would be relevant to its use in patients after heart transplantation, as opposed to local administration which would have relevance in the setting of coronary drug-eluting stents. Furthermore, we demonstrated that cilostazol remains effective at attenuating neointimal hyperplasia when used in combination with CNI therapy, further supporting its clinical
relevance in the post-transplant setting. Clinically, cilostazol’s use has been curtailed by a concern of a potential for increased cardiac-related mortality in patients with a history of congestive heart failure.\textsuperscript{382, 383} However, this concern would not be particularly relevant in patients after heart transplantation, given that their previously diseased and failing hearts would have been replaced during transplantation. Thus, given our findings, we believe that cilostazol should be evaluated as an adjunct to current CNI based immunosuppressive strategies to prevent the intimal hyperplasia that underlies the pathophysiology of CAV.

The mechanism of cilostazol’s potent inhibitory effect on neointima formation remains to be investigated. Fujinaga et al. have suggested that cilostazol may decrease smooth muscle cell proliferation and the production of the extracellular matrix protein, tenasin-C.\textsuperscript{327} Others have observed that cilostazol treatment may suppress NF-κB, CAM expression, inflammatory cytokine secretion and leukocyte adhesion to endothelium.\textsuperscript{330-339} Whether these mechanisms are affected by systemic administration of cilostazol and are involved in the inhibition of neointimal hyperplasia require further investigation. Furthermore, the effect of cilostazol on CNI-induced endothelial injury is currently unknown and also requires further investigation.

5.5 Study Limitations

Our study provides evidence that Egfl7 inhibits coronary endothelial adhesiveness to neutrophils and ICAM-1 expression in \textit{in vitro} models of I/R and CNI induced injury, likely thorough inhibition of NF-κB. Since the physiologic level of Egfl7 is not currently known, we likely have utilized a supraphysiologic dose of Egfl7 that may not be entirely representative of what occurs \textit{in vivo}. The potential beneficial effects of Egfl7 on attenuating I/R and CNI induced injury are tentative and require further study in an \textit{in vivo} model of
transplantation. Although our study demonstrated attenuated ICAM-1 expression in response to Egfl7, it does not exclude effects on other cell adhesion molecules and inflammatory signaling pathways.

Our study provides evidence that withdrawal of rapamycin therapy results in late arterial occlusion after wire injury in a murine model of neointimal hyperplasia. We also found that bosentan and cilostazol attenuate neointima formation on the background of CNI therapy in this model. Since we assessed neointimal hyperplasia in a non-transplant model, these results may not be entirely relevant to the post-transplant setting and require further study. Furthermore, we assessed neointimal hyperplasia within the femoral artery as opposed to coronary arteries. The response of the coronary artery to the drugs used in our study is likely similar to those of the femoral artery, however, it is possible that some differences may be present. In addition, we did not assess the effects of combination therapy of CNI and either MMF or azathioprine (commonly used clinically after heart transplantation) with our drugs of interest. Finally, although we utilized doses of drugs shown to be relevant in mice in previous studies, we cannot exclude the possibility that there may be a dose relationship to some of the results that we report.

5.6 Future Areas of Research

Our studies have identified Egfl7 as an anti-inflammatory signaling agent that prevents endothelial activation under the stresses of H/R and CNI exposure in vitro. Clearly, these anti-inflammatory properties will have to be investigated in vivo, to determine whether Egfl7 can be used as a therapeutic agent to modulate the endothelial response to injury. Both Egfl7 knockout and over-expressing mice have been constructed and can be used to study the effect
of Egfl7 signaling on endpoints such as neointimal hyperplasia, endothelial function, neutrophil adhesion and even allograft vasculopathy in heterotopic transplant models.

We have provided evidence that Egfl7 may exert its effects by functioning as a Notch signaling pathway antagonist. This potential mechanism of Egfl7’s action needs to be investigated further to determine whether Egfl7’s beneficial effects can be elicited by Notch signaling pathway interruption itself, for which small molecule inhibitors currently exist. Thus, inhibition of the Notch signaling pathway itself is an area of future investigation to determine whether this therapeutic strategy has any role in preventing endothelial activation, dysfunction and inflammation.

We have demonstrated that cessation of rapamycin therapy is associated with some residual inhibition of neointimal hyperplasia, but with later arterial occlusion. This alarming observation needs to be studied further in an in vivo transplant model of vasculopathy to determine whether rapamycin withdrawal is truly associated with arterial occlusion. If so, then anti-thrombotic strategies following rapamycin cessation may need to be considered. We also demonstrated that delayed initiation of rapamycin therapy was able to attenuate the developing of a neointima in our mouse model of wire injury. Although this finding does provide some reassurance that delaying rapamycin therapy to allow wound healing is acceptable within the goal of preventing CAV, this strategy requires further confirmation in an in vivo model of transplant vasculopathy.

Finally, we have provided evidence that bosentan, and more potently, cilostazol, are both effective in attenuating the development of a neointima in a murine model of wire injury, even in the context of CNI therapy. These data provide preliminary evidence that the adjunctive use of bosentan and/or cilostazol in the post-transplant setting of
immunosuppression could be novel therapeutic strategies to prevent CAV. However, these strategies need to be tested in an in vivo model of allograft vasculopathy such as a heterotopic heart transplant model or an aortic interposition model to verify their efficacy before embarking on clinical studies.

5.7 Summary of Original Contributions

The investigations presented herein evaluated the role of Egfl7 in modulating the endothelial response to H/R and CNI related injury. We also investigated the effects of withdrawal and delayed initiation of rapamycin therapy on neointimal hyperplasia. In addition, we evaluated the role of bosentan and cilostazol as novel therapeutic strategies to inhibit neointimal hyperplasia on the background of CNI therapy. Egfl7 was found to have potent anti-inflammatory properties including inhibition of NF-κB pathway activation, ICAM-1 expression and neutrophil adhesion to injured endothelium. Withdrawal of rapamycin therapy was found to result in a delayed neointima formation, however, also led to later vessel occlusion. Both cilostazol and bosentan were found to attenuate neointimal hyperplasia in isolation as well as during co-treatment with CNI therapies. The results of our studies have yielded the following original contributions to the literature:

1. Egfl7 attenuates the endothelial response to H/R injury in HCAECs.
   a) Isolated HCAECs exposed to hypoxia demonstrate an increase in Egfl7 production.
   b) Egfl7 suppresses both total cellular and cell surface expression of ICAM-1.
   c) Egfl7 exposure during the reoxygenation period suppresses ICAM-1 production stimulated by H/R injury.
d) Egfl7 suppresses the susceptibility of neutrophil adhesion to H/R injured HACECs.

e) Co-incubation with Jagged1 inhibited the effects of Egfl7 on HCAEC adhesiveness in response to H/R injury.

f) Egfl7 inhibits H/R injury induced NF-κB nuclear translocation and DNA binding activity while partially preserving IκB-α levels.

g) Egfl7 does not affect ROS production following H/R injury.

h) Egfl7 does not adversely affect cell viability that is adversely affected by H/R injury.

2. Egfl7 attenuates the endothelial response to CNI injury in HCAECs.

   a) HCAECs exposed to CNI demonstrate an increase in cell surface ICAM-1 expression.

   b) HCAECs exposed to CNI treatment demonstrate an increased adhesiveness to neutrophils.

   c) Egfl7 suppresses both neutrophil adhesion to, and ICAM-1 production by HCAECs stimulated with CNI injury.

   d) Egfl7 inhibits CNI induced HCAEC NF-κB activation;

   e) Co-incubation with Jagged1 partially inhibited the effects of Egfl7 on HCAEC adhesiveness, ICAM-1 production and NF-κB activation in response to CNI injury.

   f) Egfl7 does not affect HCAEC cell viability that is adversely affected by CNI injury.

3. Timing of rapamycin therapy affects neointimal hyperplasia and vessel patency.
a) Withdrawal of rapamycin treatment results in a delayed neointima formation accompanied by a late occlusion of wire injured vessels.

b) Early treatment with rapamycin followed by conversion to CyA does not affect neointima formation.

c) Delayed initiation of rapamycin confers some attenuation of neointima development.


a) Cilostazol completely blocks the formation of neointima even with CNI co-treatment.

b) Bosentan modestly attenuates neointima formation in the context of CNI therapy.
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Appendices

Appendix 1: Murine Femoral Wire Injury Model

- Straight spring guidewire inserted through a muscular side-branch of the femoral artery
- Dilated/injured proximal femoral artery
- Side-branch tied off
- Antegrade flow down the distal femoral artery
Appendix 2: Planimetric Analysis of Femoral Artery Cross-sections

Intima-to-Media ratio
= Intima Area/Media Area
= (Internal Elastic Lamina Area - Lumen Area)/(External Elastic Lamina Area - Internal Elastic Lamina Area)
Appendix 3: Representative H&E Stained Femoral Artery Cross-sections

Uninjured Femoral Artery vs. Injured Femoral Artery

Histology of C57BL/6 mice femoral arteries in cross-section stained with haematoxylin and eosin. (Left) uninjured native femoral artery at baseline; (Right) femoral artery demonstrating neointimal hyperplasia 3 weeks after wire injury. Black arrow: Internal elastic lamina; Red arrow: External elastic lamina.