Endothelial Injury and Repair of the Chronic Allograft Vasculopathy of Heart Allografts

by

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Abstract

Heart transplantation (HTx) is a life-saving intervention for patients with end-stage heart failure. Nonetheless, the development of Chronic Allograft Vasculopathy (CAV) limits the longevity of transplanted cardiac allografts and the survival of recipients. The pathogenesis of CAV begins with a sustained immune-mediated endothelial injury of the heart transplant supplying blood vessels. Subsequently, maladaptive vascular repair, occlusive arteriopathy, and eventual ischemia ensue. This sequence of pathological events leaves the heart allograft deprived of an adequate blood supply to fail in the long run.

Despite breakthroughs in heart failure management and immunosuppression of transplanted donor hearts, such a disease onslaught persists. A poorly defined repair response that may replicate the vascular endothelial program used during vascular development mitigates the immunological injury. Current research efforts aim at establishing a relationship between this vascular immune injury and CAV development or prognosis. Additionally, researchers investigate the embryonic vascular developmental endothelial phenotype to test its benefits in repairing the injured vasculature in the adult.

In the embryo, vascular Endothelial Cells (ECs) respond to molecular drivers of angiogenesis, such as the Vascular Endothelial Growth Factor (VEGF), by differentiating into specialized phenotypes. Such endothelial phenotypes are associated with the expression of unique genes to promote angiogenesis (e.g., the apelin gene-*APLN*) and execute various autocrine and paracrine functions. In adult blood-vessel networks, however, the role of developmental cues of angiogenesis that signal through the G protein-coupled receptors (GPCRs) to repair the established blood vessels is still elusive. Currently, no treatment exists for this occlusive CAV that injures conduit and branch arteries and is the lead cause of recipients' death, even in the first

year post-transplantation. Thus, it becomes critical to characterize targeted therapeutics to activate vascular endothelial repair (molecular) pathways to treat CAV.

We exploited a well-established humanized mouse model of heart transplantation chronic rejection that maintains good perfusion of blood to the transplanted heart allografts. We employed minor histocompatibility-mismatched heart transplants to elicit a smouldering alloimmune response against the allograft's vascular endothelium. Such a preclinical model allows a better tracking and studying of the vascular compartmental phenotypic changes post-transplantation correlated with endothelial immune injury and repair on tissue and molecular levels.

In this thesis: **First,** we tried to elucidate the role of apelin, as an endothelial proangiogenic cue, to direct vascular repair following CAV immune injury. A better-defined vascular endothelial repair pathway (i.e., an apelin-apelin GPCR (ApelinR; formerly known as APJ) signalling axis) can preserve allografts' function and longevity and vigour.

Second, we examined the potential functions of endothelial Phosphoinositide 3-Kinase beta (PI3Kβ) signalling molecule downstream of the apelin-ApelinR axis in regulating EC injury and repair responses of CAV. Hence, we can better evaluate the nature, modes of activation and outcomes of activating such a secondary molecule downstream of apelin when the immune injury progresses and vascular repair follows.

Third, we investigated the influence of inhibiting the rapid enzymatic degradation of endogenous apelin, caused by neutral endopeptidases/metalloproteases like neprilysin, in an attempt to confirm the advantages of maintaining functional levels of in-vivo apelin. This approach entails testing a clinically approved drug (i.e., Sacubitril) for trials on heart failure with reduced left

ventricular ejection fraction with a potential therapeutic translation to benefit transplant patients with immune-provoked vascular injury.

In summary, this work identifies potential therapeutic targets that can regulate vascular repair signalling pathways under current investigation and vascular inflammation following immune injury while directing pro-angiogenesis signalling. It expands our understanding of the underlying molecular and cellular mechanisms involved in vascular immune injury repair in the allograft vascular endothelium. Further, it suggests repurposing clinically approved therapeutic candidates to hinder vascular immune injury and inflammation. For example, we tested the proteolysis-resistant apelin-receptor agonist synthetic analogue (APLN-17), the Phosphoinositide 3-Kinase (PI3Kβ) selective inhibitor (GSK2636771), as well as the Sacubitril to suppress CAV progression. Future research can exploit these therapeutic candidates to treat the adverse phenotypes of vascular inflammatory diseases (e.g., autoimmune vasculitis).

Preface

This thesis is an original work by Andrew G. Masoud. The research projects, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board (study AUP number: 00001365). We maintained the animals used in these projects according to the Canadian Council for Animal Care (CCAC) guidelines under a protocol approved by the Animal Care and Use Committee of the University of Alberta. The Human Umbilical Vein Endothelial Cells (HUVECs) used in this thesis research experiments have been both collected and used under a protocol approved by the Human Research Ethics Board of the University of Alberta.

This thesis consists of a published work (Chapter 3), one in the press for reviewing towards publication in the American Journal of Transplantation (Chapter 4), and unpublished work (Chapter 5), for which we are preparing a manuscript for submission to a peer-reviewed journal.

We published Chapter 3 of the thesis as Masoud, Andrew G., et al. "Apelin directs endothelial cell differentiation and vascular repair following immune-mediated injury." *The Journal of clinical investigation* 130.1 (2020): 94-107.

Dedication

"6 Do not forsake wisdom, and she will protect you; love her, and she will watch over you. 7 The beginning of wisdom is this: Get[a] wisdom. Though it cost all you have,[b] get understanding. 8 Cherish her, and she will exalt you; embrace her, and she will honor you. 9 She will give you a garland to grace your head and present you with a glorious crown." (Proverbs 4:6-9; the New International Version of the Holy Bible)

I dedicate the current thesis to my wife, Dr. Christine Salama, my daughter Catherine Masoud, and my mother, Dr. Afifa AG. Alam, my father, Dr. Gameel TM. Saieed and my brothers; Accounting Manager Mr. Wael GT. Masoud and Professional Engineer Mr. Osama GT. Masoud.

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List of Abbreviations

ABMR: Antibody Mediated Rejection

ACE2: Angiotensin Converting Enzyme 2

ACTA2: Smooth Muscle Alpha (α)-2 Actin gene

AGTRL: Angiotensin II Receptor Like

AJs: Adherens Junctions

Akt: Protein Kinase B

ALK: Activin Receptor-Like Kinase

AMP: Adenosine Mono Phosphate

AMPK: AMP Kinase

Ang II: Angiotensin II

Ang1: Angiopoietin 1

ANOVA: Analysis of Variance

APC: Antigen-Presenting Cell

APJ: The former name of the Apelin Receptor

APLN-17: apelin-17- a proteinase-resistant synthetic apelin analogue (apelin receptor agonist) peptide

ApelinR: Apelin Receptor, (*APLNR* is the gene)

ApoE: Apolipoprotein E

AT1R: Angiotensin II type *I* Receptor

AVM: Arteriovenous Malformation

BAFF: B-cell Activating Factor belonging to the Tumour Necrosis Factor (TNF) family

BALB/C mice: Albino and Immunodeficient Laboratory-Bred strain of the house mouse

bFGF: basic Fibroblast Growth Factor

BM: Bone Marrow

BSA: Bovine Serum Albumin

CAD: Coronary Artery Disease

CALLA: Common Acute Lymphoblastic Leukemia Antigen

CAM: Chorioallantoic Membrane Assay

cAMP: cyclic Adenosine Monophosphate

CAN: Chronic Allograft Nephropathy

CAV: Cardiac Allograft Vasculopathy

CCL: C-C Motif Chemokine Ligand

CCR: C-C Chemokine Receptor

CD157: Cluster of Differentiation 157 (a myeloid cell differentiation marker)

CD271: Cluster of Differentiation 271 (a marker for mesenchymal stem cells)

CD28: Cluster of Differentiation 28 (T-cell costimulatory receptor)

CD36: Cluster of Differentiation 36 (also known as platelet glycoprotein 4, fatty acid translocase, scavenger receptor class B member 3, and glycoproteins 88, IIIb, or IV)

CD40L: Cluster of Differentiation 40 Ligand (also known as CD154, a primarily expressed protein on activated T cells and is a member of the TNF superfamily of molecules and binds to CD40 on antigen-presenting cells)

CD73: Cluster of Differentiation 73 (5-nucleotidase (5′-NT), or ecto-5′-nucleotidase)

CDH5: Cadherin 5 (also known as Vascular Endothelial (VE)-cadherin)

cIAP: cellular Inhibitor of Apoptosis Protein 1

CMV: Cytomegalovirus

COVID 19: Coronavirus Disease of 2019

Cre-ERT2: Cre recombinase fused to a triple mutant form of the human estrogen receptor

c-Rel: a Proto-oncogene and a subunit of the nuclear factor-kappa B

CTL: Cytotoxic T Lymphocytes

CTLA-4: Cytotoxic T-Lymphocyte-Associated protein 4

CXCL12: C-X-C motif chemokine 12

CXCR4: C-X-C chemokine Receptor type 4

DAMPs: Damage-Associated Molecular Patterns

DLK1: Delta Like Non-Canonical Notch Ligand 1

Dll4: Delta Like Canonical Notch Ligand 4

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

DNMAML: Dominant-Negative Mastermind-Like1 (an inhibitor of canonical Notch signalling)

DSA: Donor-Specific Antigen

EC: Endothelial Cell

ECbetaKO: Endothelial Cell-selective PI3Kbeta Knockout

ECM: Extracellular Matrix

EDG1: Endothelial Differentiation (sphingolipid) Gene 1 (sphingosine-1-phosphate receptor 1 GPCR)

EGF: Epidermal Growth Factor

EGFL7: EGF-Like Domain Multiple 7

ELA: Apela/Elabela/Toddler

EMCN: Endomucin

eNOS: endothelial Nitric Oxide Synthase

EPC: Endothelial Progenitor Cell

ERK: Extracellular Signal-Regulated Kinase

ESM1: Endothelial-Specific Molecule 1

ET: Endothelin

EVs: Extracellular Vesicles

FADD: Fas-Associated Death Domain

Fas: a cell surface death receptor that belongs to the TNF receptor superfamily (also known as the Cluster of Differentiation 95 (CD95), apoptosis antigen 1 (APO-1), APT-1, or FasR)

FasL: Fas Ligand

FBS: Fetal Bovine Serum

FOXM1: Forkhead Box protein M1

FOXO: Forkhead Transcription Factor

Foxp3: Forkhead Box P3 (also known as Scurfin)

GA: Graft Arteriopathy

G-CSF: Granulocyte Colony-Stimulating Factor

GFP: Green Fluorescent Protein

GiPCR: Giα Protein-Coupled Receptor

GPCR: G Protein-Coupled Receptor

H-2K^{b}: Murine MHC class I molecule

HAEC: Human Heart Aorta Endothelial Cells

HBSS: Hank's Balanced Salt Solution

HEY: Hairy and Enhancer-of-split-related repressor Herp, Hesr, Hrt, CHF, gridlock

HHT: Hereditary Hemorrhagic Telangiectasia

HIF: Hypoxia-Inducible factor

HLA: Human Leukocyte Antigen

HMGB1: High Mobility Group Box protein 1

hPSCs: human Pluripotent Stem Cells

HSP: Heat Shock Protein

HSPG: Heparan Sulphate Proteoglycans

HUS: Hemolytic Uremic Syndrome

HUVECs: Human Umbilical Vein Endothelial Cells

IAR: Intussusceptive Arborization

IBR: Intussusceptive Branching Remodelling

ICAM-1: Intercellular Adhesion Molecule 1

IFN: Interferon

IgG: Immunoglobulin G

IHC: Immunohistochemistry

IKB: Inhibitor of Nuclear Factor-Kappa B

IKK: Inhibitor of Nuclear Factor Kappa B Kinase

IL: Interleukin

IMG: Intussusceptive Microvascular Growth

iNOS: inducible Nitric Oxide Synthase

IP-9: Interferon-gamma-inducible Protein 9

IRI: Ischemia-Reperfusion Injury

ISHLT: The International Society for Heart and Lung Transplantation.

ISP: Intussusceptive Pillars

I-TAC: Interferon-inducible T-cell Alpha Chemoattractant (another name for CXCL11).

Jag1: Jagged Canonical Notch Ligand 1

JNK: c-Jun N-terminal Kinases (stress-activated protein kinases that belong to the Mitogen-Activated Protein Kinases-MAPKs)

kDa: kiloDalton

KLF4: Krüppel-Like Factor 4

Kras: Kirsten rat sarcoma viral oncogene homolog

LNAME: N(ω)-Nitro-L-Arginine Methyl Ester

LPS: Lipopolysaccharide

LT-β: Lymphotoxin beta

MAC: Membrane Attack Complex

Mac-2: also known as Galectin-3 (a marker of type 1 inflammatory macrophages)

MAPK: Mitogen-Activated Protein Kinase

MHC: Major Histocompatibility Complex

MHECs: Mouse Heart Endothelial Cells

MI: Myocardial Infarction

MMPs: Matrix Metalloproteinases

MPA: Mycophenolic Acid

mRNA: messenger Ribonucleic Acid

mTOR: Mammalian Target of Rapamycin

mTORC: mTOR Complex

NEMO: NF-κB Essential Modulator

NEPi: Neprilysin Inhibitors

NF-kB: Nuclear Factor-Kappa B

NICD: Notch Intracellular Domain

NIK: NF-κB-Inducing Kinase

NK: Natural Killer

NLRP: Nucleotide-binding oligomerization domain, Leucine-rich Repeat and Pyrin domain-containing

NO: Nitric Oxide

NOS: Nitric Oxide synthase

Nrarp: Notch-regulated ankyrin-repeat protein

P100: a component of the large precursor proteins of NF-κB2

P110: Phosphoinositide 3-Kinase catalytic subunit

P38: p38 mitogen-activated protein kinase

P50: Nuclear Factor-Kappa B subunit 1(NF-κB1)

P52: a subunit of NF-κB

P85: Phosphoinositide 3-kinase regulatory subunit

PAH: Pulmonary Arterial Hypertension

PAI-1: Plasminogen Activator Inhibitor 1

PBS: Phosphate Buffered Saline

PCNA: Proliferating Cell Nuclear Antigen

PCR: Polymerase Chain Reaction

PD: Programmed Death

PDGF: Platelet-Derived Growth Factor

PDGFR: Platelet-Derived Growth Factor Receptor

PDGFRB: Platelet-Derived Growth Factor Receptor beta

PD-L1: Programmed Death-Ligand 1

PECAM: Platelet Endothelial Cell Adhesion Molecule

PGE2: Prostaglandin E2

PI3K: Phosphotidylinositide-3 Kinase

PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha

PIK3CB: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Beta

PIK3CD: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta

PIK3CG: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Gamma

PIP2: Phosphatidylinositol (4,5) bisphosphate

PIP3: Phosphatidylinositol (3,4,5) triphosphate

PKC: Protein Kinase C

PlGF: Placental Growth Factor

Polyc9: protein complement component 9

PRRs: Pattern Recognition Receptors

PTEN: Phosphatase and Tensin homologue deleted on chromosome 10

qPCR: Quantitative Polymerase Chain Reaction

Rag: Recombination-activating genes (essential for the rearrangement and recombination of the genes encoding immunoglobulin and T cell receptor molecules)

RANK: Receptor Activator of NF-κB

RANKL: Receptor Activator of NF-κB Ligand

Ras: Rat Sarcoma virus gene

RCTs: Randomized Controlled Trials

RDC1: Rhodopsin-like receptor 1 **(**a putative G-protein coupled receptor, also known as Chemokine Receptor 7-CXCR7)

RelA/P65: REL Proto-Oncogene, NF-KB Subunit (REL)-Associated protein

RIP1: Receptor Interacting Protein 1(also known as RIPK1)

ROS: Reactive Oxygen Species

RTK: Receptor Tyrosine Kinase

S1P: Sphingosine 1-Phosphate

S1P1: S1P Receptor 1

S6K1: Ribosomal protein S6 Kinase beta 1

SDF1: Stromal cell-Derived Factor 1

SH: Src Homology

SHIP: Src Homology 2 (SH2) domain-containing inositol polyphosphate 5-phosphatase

siNS: short interference Non-Silencing

siRNA: small interfering RNA

SM22: (Calponin-related) Smooth Muscle-Specific protein 22

SMAD: homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the Caenorhabditis elegans protein Sma

SMCs: Smooth Muscle Cells

STAT: Signal Transducer And Activator of Transcription

TACI: Transmembrane Activator, Calcium Modulator and Cyclophilin Ligand Interactor

TAK 1: Transforming growth factor β-Activated Kinase 1

TET2: Tet methylcytosine dioxygenase 2

TGF-β: Transforming Growth Factor β

Th: T helper

Tie-2: Tyrosine kinase with immunoglobulin-like and EGF-like domains 2

TIMP2: Tissue Inhibitor of Metalloproteinases 2

TLRs: Toll-Like Receptors

TMA: Thrombotic Microangiopathy

TNFR: Tumour Necrosis Factor-Alpha Receptor

TNFα: Tumor Necrosis Factor α

TRADD: Tumor Necrosis Factor Receptor type 1-Associated DEATH Domain protein

TRAF: TNF Receptor Associated Factors

Tregs: Regulatory T cells

TxA2: Thromboxane A2

TβRII: Transforming growth factor β Receptor 2 (TGF-βR2)

VCAM: Vascular Cell Adhesion Molecule

VEGF: Vascular Endothelial Growth Factor

VEGFR: Vascular Endothelial Growth Factor Receptor

Vhl: Von Hippel-Lindau gene

VPF: Vascular Permeability Factor

VSMCs: Vascular Smooth Muscle Cells

vWF: von Willebrand Factor

Wnt: Wingless-related integration site gene

Chapter 1 : Introduction

1 Thesis Outline

This thesis studies potential therapeutic targets to modulate vascular immune injury repair to understand better the molecular interactions underpinning the vascular repair process and reacting to immune-provoked vascular endothelial injury signals. The **first chapter** gives a thorough overview of coronary vascular circulation development with an emphasis on the studied or proposed molecular pathways to orchestrate vascular immune injury and repair, angiogenesis (i.e., creating new blood vessels from established ones), inflammation, and solid organ transplantation rejection modelling in experimental research animals. The **second chapter** discusses the materials and methods employed to carry out the present research work. The **third chapter** outlines how vascular immune injury following heart transplantation affects transplant rejection outcomes. Further, it highlights how apelin, as a critical pro-angiogenic endothelial repair peptide, modulates vascular repair in a nitric oxide-dependent manner within the apelin-ApelinR G Protein-coupled Receptor (GPCR) angiogenic pathway. In addition, we investigated the effects of enzymatic degradation-resistant apelin synthetic analogue (APLN-17) treatment on rescuing the cardiac allografts and normalizing vascular injury indices or readouts in this chapter. **The fourth chapter** looks at the role of endothelial Phosphoinositide 3-Kinase Beta (PI3Kβ) as a messenger molecule signalling downstream of the apelin-ApelinR pathway in immune-mediated vascular injury and repair in mouse heart allografts. This research also sheds light on the role of PI3Kβ in influencing the activity of the vascular inflammatory Nuclear Factor Kappa B (NF-κB) pathway and the endothelial mechanisms that protect the transplanted cardiac allografts from immunological and inflammatory insults. This study has the potential to define a novel vascular signalling pathway that includes PI3Kβ as a critical regulator of immune-modulated vascular

injury protection, which has substantial translational and therapeutic implications in patients with immune-mediated vascular disease pathologies. **The fifth chapter** delves more into the effects of protecting apelin from endogenous degradation by neprilysin. Thus, we administered Sacubitril, a neprilysin inhibitor, to mice that harbour heart allografts to track vascular injury and repair after this intervention. Testing Sacubitril in CAV progression can change current care practice for organ transplantation patients by repurposing a clinically approved drug for heart failure treatment (i.e., Sacubitril) to hinder vascular immune injury and promote vascular health and repair in chronic transplant rejection as well in patients experiencing other vascular immune diseases.

The sixth chapter, titled: "General Discussion, Conclusions, Limitations and Future Directions," entails a thorough review of the most pertinent novel findings of the current study in correlation to the most updated literature information in the fields of solid organ transplantation, Chronic Allograft Vasculopathy (CAV), vascular immune injury repair, vascular inflammation, vascular angiogenic signalling pathways (e.g., the Receptor Tyrosine Kinase (RTK) and the GPCR), and vasoactive peptides stabilization (e.g., apelin). In addition, in keeping with vascular functional integrity towards therapeutic targets' development for CAV repair, novel GPCR-mediated vascular repair pathways employed by endothelial tip cells in vascular development and within the contexts of vascular injury in the adult vasculature undergoing repair were characterized. Eventually, we discussed the potential translational limitations of the current study's biological findings to contribute to clinical CAV management and the future directions and extensions of the recent research within the present study.

1.1 Objectives

1- Characterization of the role of apelin in vascular injury and repair in heart allografts

Aims: We aimed to test whether apelin directs vascular repair following immune-mediated vascular injury through gain or loss-of-function approaches.

2- Characterization of the role of PI3Kβ in vascular injury and repair of CAV in mouse heart allografts.

Aims: We sought to examine the effects of manipulating the endothelial PI3Kβ activity in conferring protection from immune-mediated vascular injury as a redundant convergence signalling node downstream of the apelin-ApelinR signalling pathway.

3- Characterization of the role of Sacubitril in vascular injury and repair of transplantation allograft vasculopathy in heart allografts.

Aims: We sought to confirm the beneficial effects of augmenting apelin's abundance and maintaining its function by inhibiting its endogenous degradation caused by neprilysin. Thus, we can appraise the full potential of apelin to preserve a more protected heart allograft with promoted endothelial cell repair phenotype following immune-mediated vascular injury.

1.2 Contribution to The Knowledge

In this work, first, we exploited human and mouse heart transplants to study endothelial cell repair in chronic indolent immune-mediated vascular disease. The currently used mouse heart transplantation model reiterates many of the features found in chronic transplant vasculopathy of humans. Such features can manifest among solid organ and bone marrow transplant recipients. We identified the endothelial-gene biomarkers, which establish reparative differentiation of the endothelial cells in the arterial and microvessel compartments of the heart allograft vessels. Further, we found that the induction of endothelial genes (e.g., apelin) marking the repair response is conserved among mice and humans, using two independent tissue banks from human heart transplants and a third from hearts post-myocardial infarction. The endothelial repair gene (i.e., apelin) critically defends the vasculature against immune injury. Apelin-deficient heart transplants from donor knockout mice develop worse vascular damage versus wild-type hearts. We showed that apelin significantly promotes endothelial cell repair. Furthermore, apelin protects the repairing vessels from immune cell surveillance by inducing eNOS activity to repel immune cell adhesion to the endothelium. Moreover, the treatment with a synthetic apelin receptor agonist (APLN-17) analogue robustly protects the vasculature and inhibits immune cell infiltration of mouse heart allografts.

We believe these findings serve as foundational research work to prompt exploration of the clinical utility of determining reparative endothelial biomarker expression in various vascular diseases, including transplantation and autoimmune vasculitis. Further, these data reveal that native apelin production has not saturated the repair pathway, at least in the mouse. Hence, further research must explore the effects of apelin receptor agonist treatment in promoting vascular repair in transplant vascular injury and elsewhere.

Apelin reagents are currently under development as inotropes for heart failure applications. Moreover, they may be re-purposed to promote vascular repair.

Second, we examined the role of the signalling molecule downstream of the apelin-ApelinR, PI3Kβ, in endothelial cells to characterize better the mechanistic pathway through which apelin serves its functions in directing endothelial repair following immune injury.

We found that the inactivation of PI3Kβ significantly regulated the NF-kB-dependent expression of proinflammatory chemokine and adhesion molecules in the vascular endothelium of mice and humans. This afforded protection to the graft in vivo against obliterative vasculopathy.

We show that a repurposed PI3Kβ inhibitor drug, currently in trials for salvage cancer chemotherapy, similarly protected the graft vasculature.

We believe our findings identify a novel mechanism of pro-inflammatory molecule regulation in endothelial cells. These findings suggest PI3Kβ inhibitors may be helpful in other clinically meaningful vascular inflammatory diseases such as atherosclerosis and autoimmune vasculitis.

Third, we tested the importance of keeping a functional level of endogenous apelin by inhibiting its enzymatic neprilysin-mediated degradation (i.e., administering Sacubitril through daily oral gavage after establishing a vascular CAV injury in vivo). Such an approach can change the current care practice for patients undergoing solid organ transplantation by repurposing a clinically approved drug (i.e., Sacubitril) to overcome immune-provoked vascular injury and its subsequent CAV development post-transplantation.

2. Vascular Development

Embryos grow and sustain their essential life functions through vascular development, a complex biological process (Daniel & Abrahamson, 2000). Embryonic angiogenesis, which is creating

new blood vessels from established ones (Hueper & Russell, 1932; Risau, 1995), is still appealing to study to understand better the molecular foundations of such an intricate process (Ferrara et al., 2003). Thus, researchers can develop novel therapies for various vascular diseases in adults. The embryonic cardiovascular system is the first organ to develop (Ferrara et al., 1996; Risau, 1997). Endothelial cells (ECs), generated from the mesoderm, cover the luminal surface of the circulatory system in interaction with blood (Adair & Montani, 2010; Risau, 1995). Initially, hemangioblasts form the mesodermal stem cells that differentiate into angioblasts and hematopoietic stem cells (Risau, 1995). Such angioblasts are still missing the complete set of typical EC markers.

After that, vasculogenesis creates blood vessels from angioblasts (Risau, 1995, 1997; Schmidt et al., 2007) within extraembryonic and intraembryonic tissues (Risau, 1997; Schmidt et al., 2007). The process of vasculogenesis entails endothelial cell-mural cell communication and extracellular matrix (ECM) interactions with these cells' receptors. These interactions that orchestrate both vasculogenesis and angiogenesis are controlled spatiotemporally by the growth factors' signalling to create blood islands whereby angioblasts give rise to ECs (Xu & Cleaver, 2011), as well as by thrombospondins, as endogenous inhibitors of angiogenesis (Adams & Lawler, 2011; Good et al., 1990).

2.1 Coronary Arterial and Microvascular Circulation Development

It is critical to cultivate a detailed understanding of coronary arterial and microvascular circulation development to support research approaches in overcoming complex cardiovascular diseases with what they bring of morbidity and mortality to patients in North America (Blair et al., 2013) and worldwide (Go et al., 2014). Promoting repair in injured coronary arteries is still an unmet need (Rubanyi, 2013). Hence, better knowledge of cellular and molecular mechanisms and

pathways of coronary vascular development during embryogenesis is deemed effective as a research strategy (Potente et al., 2011; Schaper, 2009).

Embryonic vascular angiogenesis initiates the first steps for coronary arterial plexus formation with subsequent remodelling (Chen et al., 2014; Kattan et al., 2004; Vrancken Peeters et al., 1997). Initially, in embryogenesis, cardiac development starts with a thin layer of the developing myocardium with access to plenty of oxygen and nutrients from blood coming to its lumen without the need for establishing a vascular blood supply. After that, however, the heart size increases and demands more oxygen and nutrient supplies. Thus, an immature coronary vascular plexus develops on the ventricles to meet these developmental requirements (Red-Horse et al., 2010; Zeini et al., 2009). Such vascular networks commence multiple steps of branching and expansion to form an established anastomosis with the aorta to initiate blood flow (Waldo et al., 1990). Subsequently, remodelling creates an established hierarchy of blood vessels and capillaries to provide the myocardium with sufficient oxygenation (Vrancken Peeters et al., 1997; Waldo et al., 1990).

In the adult, the established coronary vascular network features nonmyocyte cell types that are significantly involved in many biological processes such as the myocardial blood supplying and the cardiomyocyte metabolic reprogramming along with the resistance to ischemia caused by the Vascular Endothelial Growth Factor-B (VEGF-B)-induced vascular growth (Kivela et al., 2014; Pinto et al., 2016). Structurally, the inner side of the coronary vessels features a highly specialized endothelial cell type lining that can function as an anti-thrombotic unit while sensing the surrounding microenvironment for biomechanical stimuli like shear stress signals generated by the flow of blood (Baeyens et al., 2016). Furthermore, these populations of coronary endothelial cells are heterogeneous. This observation has been evident since embryogenesis,

where the endothelial transcriptional continuum (Quijada et al., 2021) decides on the developing phenotype specialization to whether it will be supplying blood (i.e., arterial) or draining blood (i.e., venous) (Fish & Wythe, 2015). A classic example of endothelial heterogeneity is the unique and specialized cluster of retinal endothelial cells (i.e., the tip-cell phenotype) that respond to proangiogenic cues like VEGF during development (Carmeliet, 2000). Thus, reiterating such developmental phenotypes of specialized endothelial cells with their generated unique proangiogenic signals became the seed of many clinical research projects to improve vascular perfusion following cardiovascular ischemia (Giacca & Zacchigna, 2012).

2.2 Sprouting Angiogenesis

As a morphogenetic multi-step fine-tuned process, the sprouting form of angiogenesis emerges to initiate a unique vascular endothelial molecular phenotype. This vascular endothelial phenotype can promote vascular development, organogenesis, and reproduction or modulate inflammation, diabetes, and wound resolution within pathological settings (Carmeliet, 2000).

In response to the established VEGF signal gradients at the angiogenic front of a sprouting blood vessel, the leading ECs acquire a specialized molecular, cellular phenotype, designated as tip cells, with higher motility, migration, and invasion of the surrounding extracellular matrix (De Smet et al., 2009; Eelen et al., 2020; Gerhardt et al., 2003). The trailing stalk ECs stay behind the tip cells with higher proliferation potential to sustain vascular stability during growth (De Smet et al., 2009). Subsequent pericyte recruitment to the developing tip cells promotes vascular maturation (Bergers & Song, 2005).

To better coordinate this process of EC specialization, EC dynamic selection emerges to govern the molecular EC phenotype allocation with a unique genetic profile for each EC type (Bentley et al., 2008). This EC selection is orchestrated mainly by the Notch signalling pathways (Jakobsson

et al., 2009; Kangsamaksin et al., 2014), and competition is established among these ECs to commence a distinct phenotype in "in vitro" angiogenesis models (Bentley et al., 2008). Initially, VEGF interacts with the VEGF Receptor 2 (VEGFR2) in the leading ECs to increase the Delta Like Canonical Notch Ligand 4 (Dll4) expression, which subsequently stimulates Notch cascades in nearby ECs. Later, Notch activation hinders VEGFR2 expression while increasing VEGFR1 abundance with diminished VEGF binding ability (Hellstrom, Phng, Hofmann, et al., 2007; Jakobsson et al., 2009; Kangsamaksin et al., 2015). This sequence of events lays the foundations for the stalk EC phenotype to promote vascular elongation and stabilization with well-formed lumens. Many studies confirmed the correlation between Notch stimulation and the lead tip ECs selection rates (e.g., the murine retinal angiogenesis model) (Hellstrom, Phng, Hofmann, et al., 2007; Jakobsson et al., 2009). Additionally, Notch1 specific deletion from ECs or via gene-wide deletion approaches yielded mortality among embryos owing to defective vascular remodelling during angiogenesis (Krebs et al., 2000; Limbourg et al., 2005). In the same direction, other genetic deletion approaches to study the impact of Dll4 on vascular system establishment in association with the Notch signalling confirmed that the Dll4 function as a vital component of the Notch-Dll4 signalling axis (Hellstrom, Phng, Hofmann, et al., 2007; Krebs et al., 2004; Suchting et al., 2007).

On the one hand, the tip cell vascular endothelial phenotype expresses various unique gene markers such as the apelin (*Apln*), the Endothelial cell Specific Molecule 1 (*Esm1*), Delta-Like Ligand 4 (*Dll4*), Platelet-Derived Growth Factor subunit B (*Pdgfb*), Vascular Endothelial Growth Factor Receptor 2 (*Vegfr2*), and the C-X-C Motif Chemokine Ligand 12-CXCL12 (also known as Stromal cell-Derived Factor 1-SDF1) Receptor (*Cxcr4*) (del Toro et al., 2010). On the other hand, the stalk EC exhibits a higher enrichment for the Transforming Growth Factor Beta-induced gene

(*Tgfbi*), also known as Betaig-3 (βIg-H3) in the Extracellular Matrix (ECM), to inhibit angiogenesis(Lee et al., 2021), the von Willebrand factor (*vWF*), and the *Vegfr1* genes (W. Chen et al., 2019; Hellstrom, Phng, Hofmann, et al., 2007).

The proangiogenic cues aid the tip endothelial cell, leading the angiogenic sprout to acquire motility and elaborate soluble and matrix-associated molecules to cross-talk with adjacent trailing endothelial cells and neighbouring vascular smooth muscle cells (del Toro et al., 2010; Eilken & Adams, 2010b).

Predominantly, apelin is an endothelial cell-derived peptide agonist for the apelin G-protein-coupled receptor (Cox et al., 2006; del Toro et al., 2010). We can best characterize apelin for its effect as a potent inotropic agent for cardiac myocytes (Charo et al., 2009; Scimia et al., 2012). However, apelin is induced in the tip endothelial cell directly by tissue hypoxia and indirectly by VEGF and signals to trailing stalk ECs that express the apelin receptor (Eyries et al., 2008; Pi et al., 2017). Apelin loss in the developing embryo is associated with subtle defects in vascularization (Charo et al., 2009; Kasai et al., 2008). In the adult, apelin is required for tip EC sprouting during regenerative angiogenesis after tailfin amputation in zebrafish (Eyries et al., 2008) and plays a role in myocardial remodelling after infarction (Wang et al., 2013a).

Deletion of the *Esm1* gene in mice, coding for the soluble matrix secreted protein of dermatan sulphate proteoglycan or endocan from tip ECs (Rocha et al., 2014) to provoke cell growth expansion and invasion (Abu El-Asrar et al., 2015; Kali & Shetty, 2014), yielded hindered vascular growth with impaired reactivity to VEGF-A signalling, vascular permeability, and the biological abundance of VEGF (Rocha et al., 2014). Additionally, the *Esm1* gene silencing hindered cancer cell survival with impacted protein kinase B (AKT) phosphorylation (Kang et al., 2012; Kang et al., 2011).

Further, EC-specific depletion of the *Pdgfb* gene hindered the recruitment of pericytes to blood vessels with yielded vascular defects in placental, heart, and renal glomerular systems (Bjarnegard et al., 2004). Moreover, mutations in the coding domain for exon number 6 in the PDGFB growth factor protein ($Pdgfb^{ret}$) resulted in a defective ability to interact with the extracellular Heparan Sulphate ProteoGlycans (HSPGs) (Lindblom et al., 2003). Consequently, this malfunctioning PDGFB protein impaired further pericytes attraction to enclose ECs during vascular development, even though PDGFB under these conditions is still active biologically (Lindblom et al., 2003), indicative of the intricate roles of tip cell marker genes to orchestrate vascular development (Abramsson et al., 2007; Ostman et al., 1991; Vazquez-Liebanas et al., 2022).

2.3 Intussusceptive Angiogenesis (segmentation)

Segmentation is a subtype of remodelling in blood vessels to help with their expansion (V. Djonov et al., 2000; Patan et al., 1992). Such a concept emerged as an initial idea in 1950 (Short, 1950) and further investigation in 1968 came in agreement with this concept (Caduff et al., 1986). This non-sprouting angiogenesis involves accelerated blood vessel segment formation through ingrowth of processes from ECs and mural cells within the arterial cavity while recruiting mononuclear Bone Marrow (BM)-derived cells without involving active EC proliferation (Mentzer & Konerding, 2014). Following annealing, these structures give rise to circumferential Intussusceptive Pillars (ISPs) towards a final mesh-like form of the vascular system (Burri & Tarek, 1990; Caduff et al., 1986). Thus, loops shape two distinct vascular structures within dilated vessel segments that demonstrate slowed blood flow and decreased shear stress (Gattinoni et al., 2020; Lee et al., 2010).

Noteworthy, intussusception is multifaceted, and it entails numerous cells involvement, including ECs, mural, and immune cells, to orchestrate the remodelling of vascular tissues (Ackermann et al., 2014), tumour neovascularization progression (Ackermann et al., 2012) as well as the vascular inflammatory disorders (Ackermann et al., 2014).

Research investigations highlighted intussusceptive angiogenesis due to EC-mural cell communication (Armulik et al., 2005; Hall, 2006).

The C-X-C motif chemokine 12 (also known as SDF1)/C-X-C Chemokine Receptor Type 4-CXCR4 axis emerged as a crucial regulator of vascular segmentation in response to tissue hypoxia (Dimova et al., 2019; Taylor et al., 2010). To illustrate, the pharmacological inhibition approaches, such as the blocking antibodies that antagonize the CXCR4, hindered the vascular intussusceptive activity, resulting in fewer vascular pillars in the chicken area vasculosa (Dimova et al., 2019). Another supporting finding of the involvement of the SDF1/CXCR4 axis in intussusceptive angiogenesis implies that the expression of SDF1 and CXCR4 is more prevalent in pulmonary tissues of COVID-19 patients (Ackermann et al., 2020). Furthermore, this finding was consistent in inflamed colonic tissues (Ackermann et al., 2013; Konerding et al., 2010) and cancers (Ackermann et al., 2012), demonstrating inflammatory-induced angiogenesis.

From a mechanical perspective, numerous factors may impact the development of intussusceptive pillars. Specifically, the changes in blood flow dynamics in arterial branches, as shown in the chick chorioallantois membranes (Auerbach et al., 1974; Ribatti et al., 2001), might accelerate intussusceptive angiogenesis (V. Djonov et al., 2000).

Further, shear stress forces variations in ECs and VSMCs can start metabolic sequelae that can restructure the cytoskeleton and the cellular gap-junction modifications (Fang et al., 2019).

Endothelial cells may sense changes in shear stress forces and transduce them via molecules like the Platelet Endothelial Cell Adhesion Molecule 1 (PECAM1, also known as CD31), enhancing angiogenesis cues, Nitric Oxide Synthase (NOS) and the expression of other vascular adhesion molecules (Chatterjee, 2018).

To better identify the forms of intussusceptive angiogenesis, we can classify it into three types: Intussusceptive Arborization (IAR) (V. Djonov et al., 2000), Intussusceptive Microvascular Growth (IMG), and Intussusceptive Branching Remodeling (IBR) (Burri & Djonov, 2002). These three forms work in harmony to establish organ-tailored angioarchitecture.

Intussusceptive microvascular development reflects pillars' establishment and subsequent extension, resulting in increased capillary surface area with primitive and relatively disordered capillary networks (Burri, 1992). On the other hand, intussusceptive arborization requires the creation of assembled pillars outlining straight capillary segments and microvessel structures (V. G. Djonov et al., 2000). These coalesced pillars establish microvascular blocks toward a well-formed vascular network (V. G. Djonov et al., 2000).

Notably, remodelling aims at meeting the local hemodynamic needs. Hence, remodelling drives the generation of transluminal pillars around arterial or venous bifurcation locations (Kurz et al., 2003). Subsequent eccentric pillar creation and growth at the bifurcation site frequently leads to a form of IBR known as intussusceptive vascular pruning. Thus, IAR and IBR are essential for developing an optimized local organ angioarchitecture since they relate to less complex vascular networks through trimming (Makanya et al., 2009).

2.4 Vascular Endothelial-Smooth Muscle Cell Cross-talk

The endothelial-mural vascular cell interplay is critical for vascular functions within physiological and pathological settings (Lilly, 2014). In healthy contexts, vascular smooth muscle cells (VSMCs) phenotype is contractile to enable vascular dynamicity, crucial for controlling blood flow in smaller resistance arteries (Fisher, 2010). The interaction between the endothelium and the mural cells of the vascular wall in health and diseases partially determines the arterial reaction to stimulants (Li et al., 2018).

Notably, such vascular endothelial-mural interplay keeps the vessels' functions, integrity, and blood flow. This interplay utilizes direct or indirect engagement forms. The latter features the production of chemicals or Extracellular Vesicles (EVs) outside the cells within the matrix (Wagenseil & Mecham, 2009). Interestingly, many of these investigated conserved EVs became an attractive research topic for studying to target various human disease pathologies that rely on EC-VSMC EV-modulated communication for novel therapeutics' development (Caby et al., 2005; Chaput et al., 2005; Choi et al., 2013; D'Souza-Schorey & Clancy, 2012; Deatherage & Cookson, 2012; Deng et al., 2015; Hergenreider et al., 2012; Mullier et al., 2014; S et al., 2013; Sarlon-Bartoli et al., 2013; Shedden et al., 2003; Simpson et al., 2009). Subsequently, a sustained release of vasoactive chemicals from the ECs is necessary so smooth muscle cells continue to be reactive and contract whenever needed (Wagenseil & Mecham, 2009). However, when the endothelium is injured, there is a disruption in the interaction between ECs and VSMCs, which causes VSMC alterations, a matrix over deposition extracellularly, as well as a state of inflamed milieu (Bennett et al., 2016; Davignon & Ganz, 2004; Frismantiene et al., 2018; Jia et al., 2017).

Regarding the paracrine mode of interaction, the functions of VSMCs are significantly modulated by the EC state (Fulton et al., 1999; Navab et al., 1988). Within physiological settings, the

paracrine EC-VSMC interaction features the production of endothelial vasoactive substances that transit to smooth muscle cells to promote vascular development (Li et al., 2018; Lilly, 2014). It creates a more relaxed blood vessel with activated nitric oxide production (Rajendran et al., 2013). On the other hand, endothelial cell dysfunction reduces nitric oxide production and excretion (Schwartz et al., 1990).

Considering other molecular modulators, ECs also produce additional vasoconstriction substances like Endothelin (ET), Thromboxane A2, Angiotensin Converting Enzyme (ACE), and Angiotensin. These mediators are essential for VSMC vasoconstriction (Alonso & Radomski, 2003; Enseleit et al., 2001; Sandoo et al., 2010).

Indeed, the bidirectional communication between ECs and VSMCs is intricate (Balcells et al., 2010; Billaud et al., 2014; Nogueira-Ferreira et al., 2014). This communication might involve other patterns of interactions like the myoendothelial gap junctions as well as many other forms of communication (Amabile et al., 2013; Climent et al., 2015; Curtis et al., 2013; Gosak et al., 2014; Gridley, 2010; Kizub et al., 2013; Loyer et al., 2014; Nogueira-Ferreira et al., 2014; Sandow et al., 2012).

ECs also use mechanical force stimuli to influence the development of smooth muscle cells. Distinctly, endothelial cells released mediators following mechanical strain to suppress the expansion of VSMCs are controlled significantly by variabilities in vascular hydrostatic pressure, laminar flow, and shear stress (Gimbrone et al., 1999; Resnick et al., 2003). Further, the process of arteriogenesis features the establishment of arteries or arterioles with SMC lining independent of hypoxia (Buschmann & Schaper, 2000; Schaper & Buschmann, 1999) during vascular development (Carmeliet, 1999, 2000; Carmeliet & Conway, 2001; Schaper, 2001; Van Royen et al., 2001). The shear stress (mostly with its rate being increased) emerged as a stimulant for

arteriogenesis. This effect became validated following the administration of vasodilators or via ligating the femoral artery in animal models (Arras et al., 1998; Egginton et al., 2001; Schaper, 2001; Scholz et al., 2000; Van Royen et al., 2001). Arteriogenesis was mediated to a large extent by the Transforming Growth Factor-β (TGF-β) as well as the Monocyte Chemoattractant Protein 1 (MCP-1), independent of the VEGF-mediated angiogenesis context (Hoefer et al., 2002; van Royen et al., 2002). Moreover, PECAM1 emerged as a mechanoresponsive cell-cell junctional molecule that allows for Extracellular Regulated protein Kinase1/2 (ERK1/2) activation following shear stress (Fujiwara et al., 2001; Kano et al., 2000).

Another modulator of the paracrine interaction between ECs and VSMCs is the PDGFB. It attracts more VSMCs to expand and migrate across the vascular compartments during embryogenesis. This sequence of events helps develop a functional vascular wall (Hellstrom et al., 1999). However, many conditions determine how smooth muscle cells react to PDGFB from developed endothelium. For instance, when the endothelium becomes defective, shear stress starts to be translated as a biological response that features a diffuse PDGF-BB overexpression signal (Andrae et al., 2008; Palumbo et al., 2000). Such a process drives smooth muscle cell expansion and migration across the vascular compartments and TGF-1 engagement as a regulator.

Notably, TGF-1 transcription in ECs increases by a shear stress signal that becomes mechanically transduced to control smooth muscle cell toggling between the different phenotypes (Han, Sakamoto, et al., 2019). These biomechanical stimuli link and support the paracrine communication between the two vascular cell compartments (i.e., the ECs and the VSMCs).

Furthermore, hyperhomocysteinemia-induced VSMC proliferation and migration become enhanced by abnormal PDGF secretion from ECs (Fu et al., 2018). Such a milieu enhances

VSMC activation and expansion (Fu et al., 2018). Interestingly, intimal expansion arterial ligation models demonstrated the outcomes of manipulating the PDGF-B in specializing the VSMC type and its activation (Li et al., 2011; Pompili et al., 1995).

In addition, various paracrine molecules modulate the Sphingosine 1 Phosphate (S1P) signalling from VSMCs to ECs (Igarashi & Michel, 2009; Kono et al., 2004; Yanagida & Hla, 2017). During vascular development, the significance of the S1P-S1P GPCR (S1P1) axis became evident by deleting the S1P1 receptor through gene-wide approaches (Allende et al., 2003; Liu et al., 2000) or specifically in ECs (Allende et al., 2003), which resulted in embryonic lethality due to impaired pericyte coverage and significant bleeding in these embryos. Further, coculturing ECs with VSMCs and testing for the loss of the S1P1 receptor on the endothelial cells led to substantial abnormalities in VSMC coverage. Such an effect became modulated via the smooth muscle cell Tissue Inhibitor of MetalloProteinases-2 (TIMP-2) signalling that yielded defective cellular junctions (Coussin et al., 2002; Mascall et al., 2012).

The apelin-ApelinR signalling also represents a quintessential paracrine communication axis between ECs and VSMCs. The ECs predominantly produce apelin (Zhao et al., 2016). Further, apelin and its receptor are concurrently abundant in VSMCs (Li et al., 2008) to orchestrate VSMC expansion, growth, migration and calcification (Li et al., 2008; Luo et al., 2018). Such VSMC proliferation (enhanced with a switch to the dedifferentiated synthetic type of VSMCs) acts as an employed mechanism to respond to vascular damage with repair in atherosclerosis, as an example (Chistiakov et al., 2015; Lyon et al., 2011).

Further, VSMC-specific deletion of Phosphoinositide 3-Kinase beta (PI3Kβ), but not PI3Kα, resulted in an inadequate pericyte coverage during angiogenesis with hindered vascular maturation (Figueiredo et al., 2020). This observation indicates the importance of the paracrine

angiogenic cues when interacting with the GPCRs in orchestrating vascular development and angiogenesis (Figueiredo et al., 2020). Additionally, the unopposed PI3K activity resulted in vascular over maturation following Phosphatase and Tensin Homolog (PTEN) deletion (Figueiredo et al., 2020).

The preceding examples explain the critical roles paracrine mediators play and signalling axes (e.g., the GPCRs signalling) between ECs and VSMCs in vascular development and disease settings. Some research articles provided a detailed review of the paracrine EC-VSMC signalling. They highlighted the tissue-specific vascular-bed signalling net effects. Further, they emphasized vascular repair within the therapeutic angiogenesis contexts (Potente et al., 2011; Rafii et al., 2016).

In terms of EC-VSMC direct contact communication, microscopic investigations have shown close contact locations in blood arteries where EC and VSMCs interact (Mendez-Barbero et al., 2021; Sandow et al., 2012). These direct contact points promote metabolic and electrical coupling pathways and signalling molecule trafficking between the two vascular cell compartments (Mendez-Barbero et al., 2021). Biologically, these contact sites allow for the direct exchange of chemicals and ions between nearby cells, essential to maintaining vascular integrity (Gaengel et al., 2009).

3. Vascular Injury

Early research on the reaction of arteries to mechanical injury in the 1970s and 1980s indicated that vascular damage thickens the blood vessel along with leukocytes and platelets migration to areas of injury (Ross et al., 1977). Infiltrating leukocytes and damaged vascular cells produce cytokines and growth factors, which induce VSMCs to migrate into the intima, proliferate, and yield a thickened fibroblastic intima (Bowen-Pope et al., 1985; Ferns et al., 1991; Fingerle et al.,

1989; Reidy, 1985; Reidy & Schwartz, 1981; Shimokado et al., 1985). Examples of such vascular intimal hyperplasia mediators following vascular EC injury include the proinflammatory cytokines (e.g., IFN-γ, TNF-α and IL-1β) (Rectenwald et al., 2000), factors produced from the infiltrating macrophages during allograft vascular injury (e.g., TGF-β, PDGFB, Macrophage-Induced Gene-MIG as a provoked monokine following IFN-γ stimulation, SDF1/CXCR4, Macrophage Migration Inhibitory Factor-MIF, and Chemokine Ligand 5-CCL5/RANTES) (Zernecke & Weber, 2010), the IFN-γ-Inducible Protein-10 (IP-10) and other signalling molecules like the mammalian (also called mechanistic) Target Of Rapamycin Complex 1 (mTORC1) (Z. Chen et al., 2004; El-Hamamsy et al., 2005; Mills et al., 2012; Moses et al., 2003; Nuhrenberg et al., 2008; Schober et al., 2004; Schober et al., 2003; Schwarz et al., 2009; Taflin et al., 2011; Zernecke & Weber, 2010). In addition to growth factors synthesis from adjacent cells, apoptosis of ECs promotes VSMC aggregation via various matrix proteins, inhibiting VSMC apoptosis (Raymond et al., 2004; Soulez et al., 2012).

Vascular ECs can encounter oxidative, inflammatory and immune stressors that activate many death signals (von Rossum et al., 2014). Distinctly, activated Granzyme/Perforin death signalling or Fas Ligand-mediated death can yield a pathological maladaptive repair response with intimal thickening following endothelial cell injury (Choy et al., 2004; Choy et al., 2003). Additionally, inflammatory cytokines and complement systems can activate the ECs to trigger leukocyte accumulation with vascular inflammation and antibody-mediated changes with augmented antigen presentation (Cravedi et al., 2013; Russell et al., 1994; Terasaki & Cai, 2005). Furthermore, downstream net effector molecule production, such as Nitric Oxide (NO), can be modulated by the initial inflammatory cytokine activation (Tripathi et al., 2007). Such an effect can control the prognosis of vascular response-to-injury outcomes. In VSMCs, FasL-mediated

cell death (a type-II transmembrane protein belonging to the TNF family ligand, also known as CD95L or CD178), Endothelin-1 (ET-1), and the inducible Nitric Oxide Synthase (iNOS)-mediated NO de-sensitization act to modulate the Medial damage. Furthermore, these factors modulate arteriosclerotic intimal thickening and pathological vasoconstriction (Ferns & Avades, 2000; von Rossum et al., 2014).

Injury to medial smooth muscle fibres may cause or worsen intimal thickening (Kocher et al., 1991). In particular models of arterial vascular rejection, apoptosis of VSMC ensued, and reduction of $CD8^+$ T lymphocytes hindered VSMC apoptosis with less expansion of the intima (Legare et al., 2000; Martinet et al., 2011). Interferons (IFNs) boost VSMCs' reactivity to FasL by moving its corresponding receptor to the cell surface (Sata et al., 2000). Intimal thickening in allograft arteries may be triggered by the production of SDF-1, leading to mesenchymal stem cell migration and proliferation into the intima (Doring et al., 2014; Du et al., 2012).

Conspicuously, cell damage causes the production of alarmins, as first responders, which drive inflammation and immunological activation (Bianchi, 2007). Of note, alarmins represent constitutively-expressed intracellular immunomodulating self molecules that stimulate ECs, VSMCs, and Antigen Presenting Cells (APCs) by binding the Pattern Recognition Receptors (PRRs) as endogenous correspondents to the Pathogen-Associated Molecular Patterns (PAMPs) (Bianchi, 2007). Examples include High Mobility Group Box-1 protein (HMGB1), IL-1α, IL-33, Heat Shock Proteins (HSPs) and others to modulate allogeneic responses (Matta et al., 2017; Oppenheim & Yang, 2005). They also encourage APCs to flock to sites of vascular injury (von Rossum et al., 2014). As a result, they may contribute to vascular injury immunopathology by initiating or propagating immune responses (von Rossum et al., 2014). Distinctly, alarmins can

recruit leukocytes either directly, to a large extent, through the Giα Protein-Coupled Receptor (GiPCR) or indirectly via provoking chemoattractant chemokine activities (Yang et al., 2009).

During vascular immunological responses, cytokines activate the endothelium, allowing leukocyte recruitment to tissues (von Rossum et al., 2014). At this point, morphological changes progress at the ECs with enhanced permeability for proteins in plasma (Pober & Sessa, 2007). As a result of these vascular changes, immune responses arise and become localized. Organ transplants feature heightened inflammation and immunity with innate immune activation following the ischemia injury and antibody and adaptive immune cell targeting of the graft in alloimmune injury (von Rossum et al., 2014). As a result, the cytokines generated alter the function and phenotype of vascular cells, leading blood vessels to remodel (Mitchell & Libby, 2007).

4. Vascular Endothelial Repair

Maintaining a healthy vascular homeostatic state necessitates successful vascular repair to recover the preceding vascular injury (Lee & Slutsky, 2010; London et al., 2010; Minamino & Komuro, 2006; Zhao et al., 2006).

There are many immune-mediated vascular disease pathologies (e.g., atherosclerosis, Pulmonary Arterial Hypertension (PAH), Thrombotic MicroAngiopathy (TMA), and Hemolytic Uremic Syndrome (HUS) that can cause mortality in North America (Joshi et al., 2009). Such diseases correlate with vascular endothelial dysfunction following immune, inflammatory, and ischemic injuries. Intensive research tried to develop alternative therapeutics to cure such diseases by stimulating neovascularization and blood vessel construction through various experimental approaches (Potente et al., 2011). Thus, it is essential to better understand the molecular traits of

vascular ECs during specialization and differentiation to tip-cell phenotypes within the vascular endothelial repair.

Endothelial tip-cell differentiation traits are essential to modulate vascular development, angiogenesis, morphology and branching with vascular abnormalities in knockout mice (del Toro et al., 2010; Helker et al., 2020; Strasser et al., 2010).

Further, tip cell differentiation features the production of soluble paracrine signals (e.g., PDGF-BB and apelin) to signal to pericytes (Bergers & Song, 2005; Sweeney et al., 2016). While essential for microvascular healing, these signals can yield unexpected responses in arterial vascular repair, resulting in maladaptive neointimal growth (Owens et al., 2004). Key components of the neo-angiogenesis pathway aid in healing injured microvessels and arterial endothelium (Ucuzian et al., 2010).

Vascular EC regeneration takes many forms, such as tissue-resident EC initiation of regeneration through integrating a plethora of specialized molecular signalling pathways, secondary messengers, and net influential transcription factors (Evans et al., 2021). Further, the EC junctional annealing emerged to maintain vascular integrity (Komarova et al., 2017). Additionally, researchers studied the Endothelial Progenitor Cells (EPCs) for their involvement in vascular repair (Isner et al., 2001; Walter et al., 2005; Walter et al., 2002). Further literature updates about the EPCs' role in vascular repair will be highlighted in the following sections below. Lastly, we can review other cellular paracrine factors' contributions to modulating vascular repair in these articles (Evans et al., 2021; Y. D. Zhao et al., 2014).

4.1 Tissue-resident ECs in Vascular Repair

To start with, tissue-resident specialized ECs were deemed responsible for vascular repair following injury without the involvement of any exogenous Bone Marrow (BM)-derived or circulating progenitor cells (Itoh et al., 2010; McDonald et al., 2018; Singhal et al., 2018). Essentially, the genetic lineage tracing approach confirmed this foundational finding (Itoh et al., 2010; Liu et al., 2019; McDonald et al., 2018).

For example, Itoh and colleagues (Itoh et al., 2010) highlighted the vital exclusive role of tissue-resident EC stretching, expansion, and movement across the location of injury in repairing the damage caused by photo-chemical insults using Tie2Cre-GFP mice to label live ECs. These results were further confirmed in mouse aortic EC injury in vivo with subsequent lineage tracing (Cdh5Cre-ERT2) (McDonald et al., 2018). Moreover, another study revealed that a specific population of pulmonary tissue ECs are significantly engaged in EC repair in mouse pulmonary vascular injury models (Kawasaki et al., 2015). Another supporting study by Singhal and associates (Singhal et al., 2018) discovered that hepatic tissue EC regeneration became exclusively provoked by local tissue ECs (Cdh5Cre-ERT2). However, progenitor cells were integrated into injured vessels only when irradiation destroyed local ECs. Still, these progenitors failed to develop further into ECs (Singhal et al., 2018).

Worth highlighting that characterizing the lining endothelial-cell regeneration in large adult arteries remained puzzling until McDonald et al. explored many exciting features of aortic EC renewal. This team exploited in vivo EC denudation and tracked the repair process through various approaches like live-cell labelling, tracing and transcriptional traits' characterization in the studied ECs (McDonald et al., 2018). Distinctly, this research elaborated on the origin of ECs during vascular regeneration from locally-residing ECs next to the injured ones with variable

magnitudes of proliferation on the transcriptional (molecular) level (McDonald et al., 2018). Further, the observed transcriptional phenotype of aortic ECs next to the injured EC populations during vascular regeneration was unique and dissimilar to endothelial angiogenic sprouting (McDonald et al., 2018). Most importantly, studying the tracked genes in response to denudation of aortic ECs highlighted the roles of the Forkhead box protein M1 (FoxM1) and the family of regulator genes and proto-oncogenes (MYC) during the regeneration process. This observation became supported by confirmatory loss-of-function experiments like gene knockout mice utilization (McDonald et al., 2018).

It is worth referring to a subset of cells of interest known as mesangioblasts (Slukvin & Kumar, 2018). These multipotent cells frequently exhibit endothelial and pericyte markers while closely associated with blood vessels (Iwasaki et al., 2011; Slukvin & Kumar, 2018). These markers include PDGFB, Nerve Growth Factor Receptor (CD271), 5'-Nucleotidase Ecto (NT5E, also known as CD73), Endomucin (EMCN), Delta Like Non-Canonical Notch Ligand 1 (DLK1), and many more to enable the eventual formation of vessel-like structures (Iwasaki et al., 2011; Laurenzana et al., 2015; Slukvin & Kumar, 2018). Despite the notion that mesangioblasts' role in regenerating the heart was widely studied (Galli et al., 2005; Iwasaki et al., 2011), their participation in repairing vascular ECs remains elusive.

4.2 Vascular EC Reannealing for EC Barrier Repair and Integrity

Reannealing of EC junctions to keep a solid and functional EC barrier is considered a pillar for the vascular EC repair response following injury, whereby endothelial healing entails reestablishing endothelial connections to restore endothelial barrier stability (Evans et al., 2021).

Gap junctions, tight junctions, and Adherens Junctions (AJs) are some examples of inter-endothelial junctions (Komarova et al., 2017). Tight junctions create linkages among ECs on a

level of molecules, resulting in well-sealed EC boundaries. However, the homotypic attachment was not mediated by the gap junctions. They instead generate channels that allow electrical and, to some extent, the chemical interplay among adjoining ECs (Giannotta et al., 2013; Komarova et al., 2017). Junctions between ECs preserve the endothelial continuity and limit fluid, protein, and blood cell flow to maintain endothelial semipermeable characteristics. The permeability of the endothelial barrier to mechano-chemical factors in both standard and abnormal situations becomes predominantly controlled via the functions of AJs. VEGF activation of its receptor (VEGFR2) modulated vascular permeability after a cyclic stretch with dissociated VEGFR2 and VE-cadherin (also known as CDH5) in ECs (Tian et al., 2016). Further, VEGFR2 activation augmented thrombin-induced vascular permeability following cyclic stretch, with VEGFR2 knockdown attenuating the EC permeability following cyclic stretch and concomitant thrombin stimulation (Tian et al., 2016). Other studies also highlighted the VEGF effects on junctional remodelling within contexts of EC proliferation or the impact of VE-cadherin on VEGFR2 regulation through clathrin to control EC growth in correlation to contact inhibition (Grazia Lampugnani et al., 2003; Lampugnani et al., 2006).

The AJ complex consists of β-catenin, VE-cadherin, p120-catenin, and α-catenin. The association of VE-cadherin's component attaches endothelial cells to form junctions, while members of the cytoplasm connect β-catenin and p120-catenin. Subsequently, actin-associated protein α-catenin interacts with β-catenin. Post-translational changes in such proteins govern endothelial permeability via mediating vascular EC junction plasticity (Giannotta et al., 2013; Komarova et al., 2017). N-cadherin modulates the attraction of VE-cadherin towards AJs, hence maintaining vascular vigour (Kruse et al., 2019).

Indeed, vascular EC junctions orchestrate vascular homeostasis (Fromel & Fleming, 2015; Lwaleed et al., 2015), angiogenesis (Szymborska & Gerhardt, 2018), repair (Tesfamariam, 2016; Zou et al., 2016), tumour progression (Choi & Moon, 2018; Hida et al., 2018) and inflammation (Vestweber, 2012). Such effects indicate the importance of keeping EC junctional integrity to empower EC regeneration or repair.

4.3 Examples of The Studied Signaling Pathways for Vascular Repair

The canonical VEGF signalling pathway in ECs became extensively investigated to address its correlational modulation of vascular repair. Ostendorf and associates (Ostendorf et al., 1999) discovered the impact of suppressing VEGF on hampering the glomerular renewal of ECs, plus the increased mortality in rat models of nephritis without affecting proteinuria. VEGF therapy improved endothelium regeneration in a rabbit carotid artery of the endothelial mechanical injury model (Callow et al., 1994). VEGF receptor overexpression inhibited neointimal proliferation in a model of coronary stenting, indicating the role of VEGF in repair following vascular damage (Buchwald et al., 2006). Moreover, adenoviral VEGF therapy boosted EC healing of arterial denudation in research animals. However, VEGF inhibition had the opposite effect (Hutter et al., 2004). In several investigations, VEGF emerged as a critical factor for EC proliferation, recovery, and healing (Evans et al., 2021).

Besides, the Phosphoinositide 3 kinases (PI3Ks) integrate various angiogenic cues and growth factors to modulate EC proliferation, survival, metabolism, polarity and angiogenesis (Cantley, 2002; Jiang & Liu, 2009; Morita et al., 1999). Section 7 of Chapter 1 in this thesis features a detailed discussion of PI3Ks in vascular endothelial injury repair. Briefly, the PI3Kγ-FoxM1 pathway was deemed vital for EC recovery from induced inflammatory injury (Huang et al., 2016). Distinctly, in 2016 a study by Huang and associates (Huang et al., 2016) identified

EC FoxM1 activation during pulmonary damage in the sepsis model to be PI3K gamma catalytic subunit (p110γ) dependent in the mouse. Despite comparable levels of peak lung damage, global *Pik3cg* gene mutant animals displayed deficient EC expansion and chronic inflammation following sepsis induction (Huang et al., 2016).

Additionally, restored FoxM1 expression in the endothelium in global *Pik3cg* gene knockout experimental animals boosted repair and recovery (Huang et al., 2016). Endothelial cells isolated from the lung of individuals suffering from acute respiratory distress syndrome featured a decreased p110γ level. This study's authors (Huang et al., 2016) systematically discovered the activation of this regeneration axis in the whole-body vasculature. Such deficient repair in blood vessels became evident in the *Pik3cg* gene knockout venules of mice's cremasteric muscles. It has been corrected via FoxM1 expression using a transgenic approach on these knockout mice. A mechanistic link emerged from this study (Huang et al., 2016) to explain the FoxM1 induction by PI3Kγ through the nuclear outward translocation and phosphorylation of the FOXO transcription factor. Furthermore, in this study (Huang et al., 2016), SDF1 was confirmed as a potential ligand upstream of GPCRs to stimulate PI3Kγ within the inflammatory sepsis-induced pulmonary vascular injury model (Huang et al., 2016).

5. Chronic Allograft Vasculopathy

Heart transplantation is the definitive treatment for end-stage heart failure patients to prolong life. More than 5,000 annual heart transplants took place worldwide to save advanced-heart-disease patients' lives (Stehlik et al., 2018). Despite the recent advances in immune-suppression therapies that mitigate alloreactive immune responses against the allograft, considerable rates of early acute cellular rejection and late chronic cell- and antibody-mediated graft injury are still prominently present (Lund et al., 2015a). Chronic Allograft Vasculopathy (CAV) is a progressively occlusive

accelerated disease (Avery, 2003; Billingham, 1992) and represents the primary limiting factor for long-term graft survival following heart transplantation without definitive treatment (Lund, Khush, Cherikh, Goldfarb, Kucheryavaya, Levvey, Meiser, Rossano, Chambers, Yusen, & Stehlik, 2017). The International Society for Heart and Lung Transplantation has reported that 33% of transplanted patients encountered CAV and graft failure within five years following transplantation (Yusen et al., 2016). Thus, identifying the potential CAV therapeutic targets is essential.

On histological examination, CAV of the transplanted hearts' arteries becomes characterized by diffuse, occlusive, and concentric intimal constriction with luminal stenosis of coronaries. In addition, it featured conventional atherosclerotic components in this disease context (Tan et al., 2007). CAV injury starts to develop following a variety of complicated interplaying etiological assaults. CAV becomes predisposed by older age, obesity, hyperglycemia, hyperlipidemia, ischemic heart disease, and the Cytomegalovirus (CMV) (Chih et al., 2016; Delgado et al., 2015). Immune-induced allograft damage is the most critical modulator of CAV (Costello et al., 2013a). The alloimmune response targets the transplant coronary artery endothelium that expresses the foreign Human Leukocyte Antigen (HLA). Subsequently, the endothelium becomes identified by host T-lymphocytes to activate vascular cell adhesion molecules with inflammatory cellular recruitment (Pober et al., 2014; Zhang et al., 2000). In 1979, Dvorak and associates conducted a classic experiment highlighting microvascular EC as a vital target for immune cell injury. This experiment exploited vascularized skin allografts in humans for the first time (Dvorak et al., 1979). Indeed, the whole vascular tree of the allograft is compromised, from the coronary artery to the microvessels, according to a recent assessment of failed heart grafts explanted for re-transplantation of the recipient (Loupy et al., 2016). In mouse models (Kitchens et al., 2007;

Nagano et al., 1997; Uehara et al., 2005) and human heart transplants with vasculopathy, immune responses dominated by interferon production and mononuclear cell infiltration of the graft arteries became more evident (van Loosdregt et al., 2006). Vascular ECs regulate vessel tone, platelet activation, leukocyte adhesion, and VSMC proliferation to maintain vascular homeostasis in the normal state (Deanfield et al., 2007). On the other hand, CAV-injured ECs have different activities and trigger various pathological reactions, including excessive tissue repair, vascular cell proliferation, fibrosis, and tissue remodelling (Chih et al., 2016). As a result of the advancing graft ischemia, the implanted allograft deteriorates over time (Kloc & Ghobrial, 2014).

5.1 CAV Development

CAV represents a multifaceted pathological condition fueled by immunologic and nonimmunologic events. This condition is still under intensive research despite the accomplishments in interpreting the several mechanistic drivers of such a complex process (Lu et al., 2011). CAV is separated from atherosclerotic plaque pathogenesis by vascular events affecting arteries and veins' intima, media, and adventitia (Rahmani et al., 2006).

T lymphocytes that detect graft antigens expressed on ECs are vital for CAV progression (Al-Lamki et al., 2008; Moreau et al., 2013). As a part of the innate immune response, dendritic cells may transmigrate into the graft in response to antigen recognition or chemokines produced by the endothelial cell surface in reaction to the Damage-Associated Molecular Patterns (DAMPs) (Ochando et al., 2006). Other stimulants of the endothelial cells include IgG donor-specific alloantibodies or host natural antibodies (Kummer et al., 2020). In turn, secreted IL-18 from stimulated ECs helped expand the peripheral T helper cell populations and provoked the production of alloantibodies after ischemia/reperfusion injury (Liu et al., 2020). These antibody responses become mediated by complement system proteins that form Membrane Attack

Complexes (MACs) to form pores in the endothelial cell plasma membrane with eventual EC lysis (Wehner et al., 2007). Infections such as CMV can also provide antigens that stimulate T cells (Grattan et al., 1989; Reinke et al., 1994; Rubin, 2001; Streblow et al., 2007; von Willebrand et al., 1986; Walker et al., 2009). Cytokines generated by activated endothelial cells, such as interleukin (IL)-1, IL-15, and IL-18, enhance the T-cell response (Krishnaswamy et al., 1999; Liu et al., 2020; Xie et al., 2020).

Furthermore, generated cytokines from stimulated T cells at the blood vessels (e.g., IFN-γ and possibly others) induce VSMC proliferation. Additionally, they activate infiltrating NK cells and macrophages to produce additional mitogens that drive intimal smooth muscle cell proliferation and matrix deposition (Sprague & Khalil, 2009). The result is that the vessel intima expands, and the lumen eventually becomes obliterated (Figure 1.1) (Mitchell & Libby, 2007).

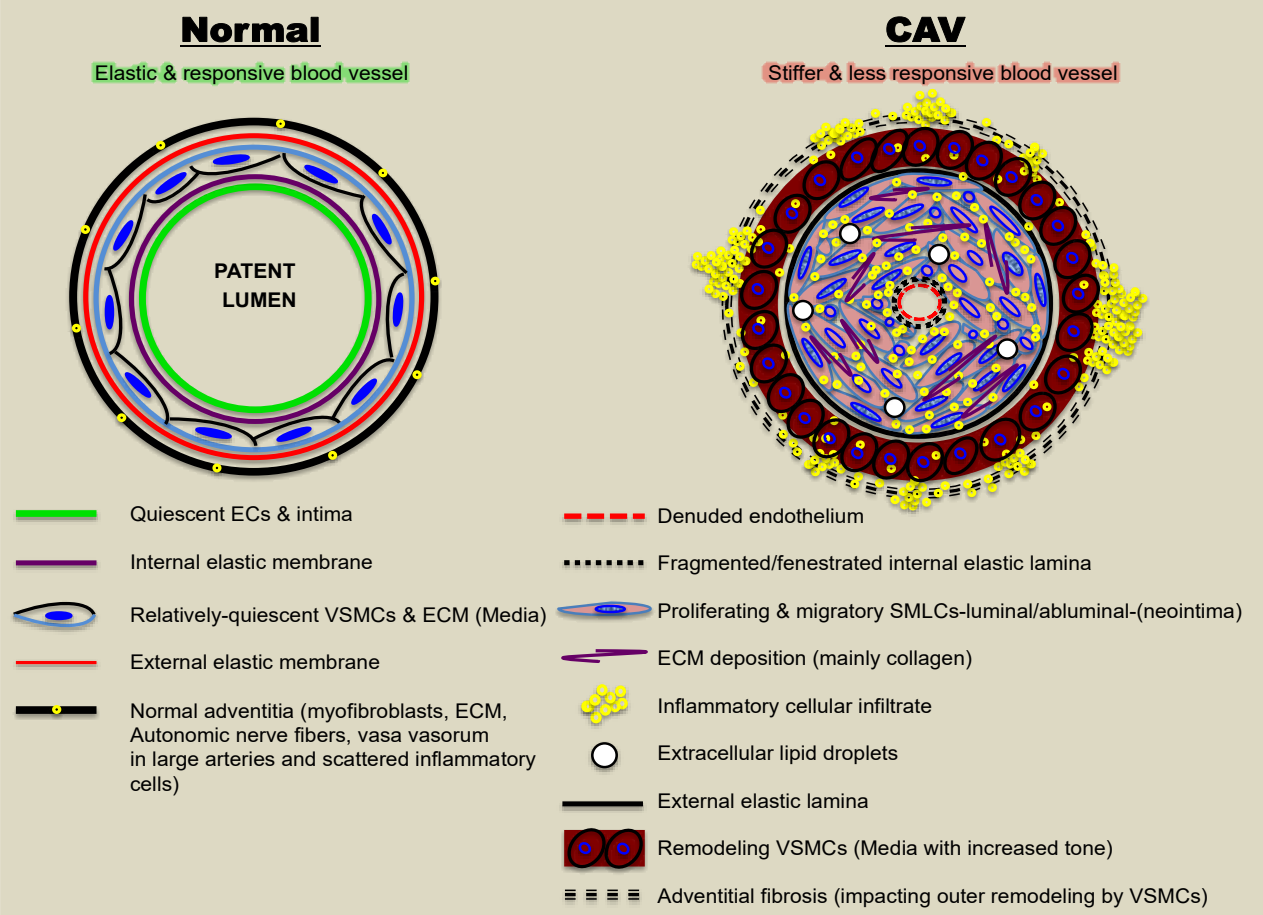

Figure 1.1: Chronic Allograft Vasculopathy (CAV) development.

A simple depiction shows vascular changes in CAV, where immune injury starts by targeting the graft-nourishing vascular-tree Endothelial Cells (ECs), causing denuding EC loss and subsequent maladaptive repair. The classical inside-out vascular remodelling theory (W. Chen et al., 2018; Kloc & Ghobrial, 2014) indicates that the endothelium of the tunica intima at the luminal vascular side secretes inflammatory cytokines and various immune mediators to recruit leukocytes to stimulate macrophages occupying the intimal surface. Then, ECs become injured. In turn, Vascular Smooth Muscle Cells (VSMCs) in the tunica media become activated and directed towards a more proliferative phenotype. This phenotype helps form the neointima with fibro-intimal thickening, collagen, matrix deposition, and eventual vascular stiffening and

obliteration. A newer outside-in theory proposes a different direction of CAV pathogenesis events. It advocates the tunica adventitia as the starting point of vascular inflammatory response whereby inward dissemination towards the intima occurs. The stimulated macrophages toggle the adventitial progenitor stem cells' phenotype to the smooth muscle cell with enhanced proliferation and in-migration to constitute the neointima (Kloc & Ghobrial, 2014). **(Picture is created by Microsoft PowerPoint)**

5.2 CAV Maladaptive Repair

Endothelial cells tend to induce a repair response following the persistent alloimmune injury of the graft endothelium to overcome this vascular insult (Van Belle et al., 1998). The failure of the implanted grafts becomes evident by the development of maladaptive vascular repair and tissue fibrosis (Jiang et al., 2014). Various in-depth investigations of cellular signalling pathways in vascular ECs and adjacent VSMCs have hypothesized different underlying reasons for the maladaptive repair response over the years (Bradley et al., 2018; Gimbrone & Garcia-Cardena, 2016; Lacolley et al., 2017; Owens et al., 2004; Perry & Okusa, 2016; Shu et al., 2019; Sun et al., 2013; Wu et al., 2016).

"Vascular maladaptive repair response represents an inefficient delayed-type-hypersensitivity response against donor ECs and medial SMCs," Libby et al. and colleagues suggested two decades ago (Libby et al., 1995; Libby & Tanaka, 1994). They hypothesized that when the alloimmune response fails to eliminate donor vascular wall cells, more medial SMC recruitment occurs, occluding the supplying artery lumen. Medial SMCs modulate the vascular tone and calibre to maintain hemodynamic stability. This effect becomes evident in physiological vascular contexts by featuring a contractile nature (Alexander & Owens, 2012). However, some VSMCs can switch to non-contractile phenotypes, such as migratory, proliferative, secretory, or

osteogenic phenotypes, contributing significantly to arterial remodelling (Gerthoffer, 2007). Phenotype switching is triggered by vascular stress factors such as inflammation, oxidation, hemodynamic shear stress, and exposure to Angiotensin II (Birukov, 2009). Other paracrine stimuli can also drive phenotypic switching (e.g., the transforming growth factor-beta (TGF-β) and the fibroblast growth factor) (Birukov, 2009). Genetic alteration of the contractile phenotype can also induce this phenotypic switching (Alexander & Owens, 2012). Indeed, many mechanisms are involved in VSMC phenotype switching. Examples include the changes in VSMC cytoskeleton, the detachment of adhesion molecules from the Extracellular Matrix (ECM), the polymerization of actin, and the protrusion of lamellipodia at the leading edge following ECM degradation by Matrix MetalloProteinases (MMPs) (Willis et al., 2004). Collectively, these mechanisms allow VSMCs to move through the ECM toward chemoattractant stimuli (Willis et al., 2004). As a result of the MMP activation, VSMC proliferation, hyperplasia, and migration, the graft vascular artery wall thickens, stiffens, and elastin degrades, resulting in graft ischemia (van Varik et al., 2012).

5.3 Insights on CAV Pathogenesis

The initial idea of CAV pathogenesis became centred around cell-mediated responses (Abrahimi et al., 2015). Graft alloantigens, particularly the Human Leukocyte Antigen Class I and Class II (HLA, also known as Major Histocompatibility Complex-MHC I and II), are predominantly expressed on graft ECs and serve as targets for host T and B cell activation (Salomon et al., 1991; Tellides et al., 2000). T-cell populations in the vessel wall of clinical CAV specimens are enriched for IFN-γ and Transforming Growth Factor-β (TGF-β)-producing lymphocytes (Hagemeijer et al., 2008). Notably, IFN-γ is produced mainly by $CD4^+$ Th1 cells, $CD8^+$ Cytotoxic T Lymphocytes (CTLs), and Natural Killer (NK) cells (Hagemeijer et al., 2008).

Further, TGF-β correlates with $CD4^+$ T Regulatory Lymphocytes (T regs), which are not detectable in the vessel wall based on other markers (Hagemeijer et al., 2008).

In mouse heart transplantation models, genetic ablation of IFN-γ or IFN-γ antibody neutralization reduced vasculopathy but not acute graft rejection (Nagano et al., 1997). Further, IFN-γ induced smooth muscle cell intimal enlargement when human artery segments became exploited as aortic interposition grafts in immunocompromised mice (Tellides et al., 2000; Wang et al., 2007a; Wang et al., 2004). When the same mice are administered allogeneic T cells to the artery graft, neutralizing IFN-γ inhibited intimal expansion (Wang et al., 2004), but neutralizing TGFβ abolished medial sparing without lowering intimal growth (Lebastchi et al., 2011). These findings suggest that IFN-γ is the leading cause of CAV progression (Tellides & Pober, 2007). $CD4^+$ T cells and NK cells appear to cooperate in the generation of IFN-γ in mouse models (Uehara et al., 2005).

While cellular and humoral immunity reactions seem to function as distinct responses, they may work synergistically (Clark & Kupper, 2005). Distinctly, one dose of human alloantibody stimulates complement deposition of polyC9 on EC in immune-inhibited mice receiving human coronary artery grafts but is inadequate to trigger intimal enlargement when the artery becomes transplanted into a second mouse host (Pober et al., 2021). Transplanting duplicate artery segments into a second host with circulating human T cells from an allogeneic donor to the artery, on the other hand, boosts T-cell recruitment, activation, and IFN-γ production, resulting in drastically amplified CAV alterations in the artery graft (Qin et al., 2016).

Notably, complement becomes essential for the increased T-cell responses generated by the Donor-Specific Antigen (DSA) but not for the basal T-cell responses. ECs internalize PolyC9, which stimulates several signalling cascades, including the creation of IL-1. This sequence of

events causes the endothelial cell to produce adhesion molecules and chemokines that promote T-cell recruitment ("Correction to: Complement Membrane Attack Complexes Assemble NLRP3 Inflammasomes Triggering IL-1 Activation of IFN-gamma-Primed Human Endothelium," 2021; Xie et al., 2019). ECs that have been activated by complement also express IL-15 and IL-18, which induce T-cell activation (Liu et al., 2020; Xie et al., 2020). B lymphocytes in CAV arteries are evident in the adventitia in formations that resemble tertiary lymphoid organs. These cells emerged as a source of DSA, aided by alloreactive T follicular helper cells. Still, recent cloning and expression of the immunoglobulins produced by these B cells show that they are instead polyreactive antibody makers (Chatterjee et al., 2018).

In terms of innate immunity cells in CAV, macrophages may be a source of growth factors that contribute to intimal VSMC proliferation, and macrophage removal lowers the degree of CAV-like lesions in mouse heterotopic heart models (Kitchens et al., 2007). In humanized immune system mouse models, macrophages appear unnecessary for creating CAV lesions. Furthermore, because the nature of the macrophages found within the intima is poorly understood, it is plausible that they help inhibit T-cell activities.

Noteworthy, other non-immune contributory factors in CAV may work in harmony to potentiate the currently established immune factors. For instance, Ischemia-Reperfusion Injury (IRI) (Eltzschig & Eckle, 2011; Jacobs et al., 2010; Patel et al., 2021; Van Raemdonck, 2010), donors' brain death (Mehra et al., 2004), and Cytomegalovirus infection (Graham et al., 2009; Walker et al., 2009).

5.4 Vascular ECs in CAV

The recurrent EC damage generated by ischemia-reperfusion and rejection events is considered stimulatory for remodelling and VSMC expansion in CAV (Ross, 1999). The whole arterial

vascular tree of the allograft becomes injured in CAV, starting from the large coronaries to microvessels. Following damage, intimal endothelial repopulation can take place while cells from the donor and recipient are available (Atkinson et al., 2004; Colvin-Adams et al., 2013; Glaser et al., 2002; Hillebrands et al., 2001; Hu et al., 2002; Quaini et al., 2002; Simper et al., 2003). The turnover of ECs is predominant in tiny epicardial and intramyocardial vessels during CAV (Minami et al., 2005). This effect might lead to altered vascular EC characteristics, making the intima more prone to allogeneic insults with subsequent vasculopathy.

5.5 Vascular SMCs in CAV

CAV neointimal lesions are mostly $ACTA2^{+}$ (smooth muscle α-actin) cells (Atkinson et al., 2004). These cells can emerge from specialized VSMCs from the donor artery medium (Atkinson et al., 2004; Glaser et al., 2002; Hruban et al., 1993; Quaini et al., 2002). VSMCs are unusual in that they maintain the potential to expand in reaction to pathogenic factors. While such a reaction is essential for development or repair, it participates in abnormal remodelling of the vessels.

Notably, donor tissue's non–self–produced antigens in transplantation cause innate and adaptive immunological alloreactivity. The attracted T lymphocytes to allografts become triggered through antigen presentation to release the canonical intimal stimulator for thickening, the IFN-γ (Pober et al., 2014; Tellides & Pober, 2007). Even though IFN-γ is sufficient to elicit Graft Arteriopathy (GA) in animal models (Tellides et al., 2000), it has no direct effect on VSMC expansion (Tellides et al., 2000). Several animal research has revealed that VSMC apoptosis occurs alongside and exacerbates intimal hyperplasia in GA (Andriambeloson et al., 2001; Bigaud et al., 1999; Hirsch et al., 1998; Legare et al., 2000; Li et al., 2012; Moldovan et al., 1998; Nejat et al., 2008). In human specimens, there is a link between apoptosis, also known as medial thinning, and CAV degree (Dong et al., 1996; Huibers et al., 2014; Johnson et al., 1989; Liu & Butany,

1992; Lu et al., 2011). The method by which IFN-γ promotes VSMCs to differentiate and facilitate the creation of CAV lesions remains unknown. As reported (R. Liu et al., 2013; Ostriker et al., 2021), Tet Methylcytosine Dioxygenase 2 (TET2) regulates the VSMC phenotype. TET2 transcriptional activity, remarkably evaluated through the 5-Hydromethylcytosine (5-hmC), was suppressed in restenosis following femoral artery injury in the mouse (R. Liu et al., 2013; Ostriker et al., 2021).

5.6 CAV Therapies

With varying effectiveness, therapeutic approaches addressing both immunological and non-immune pathologic pathways implicated in CAV were examined. Current clinical CAV therapeutics focus on implementing various preventive interventions such as Cytomegalovirus (CMV) infection prevention, rejection avoidance, vascular risk factor control, and specialized treatment approaches that limit disease progressions, such as statins and mTOR inhibitors (Bellumkonda & Patel, 2020; Klimczak-Tomaniak et al., 2020; Stehlik & Kemeyou, 2019). Percutaneous revascularization for localized obstructive coronary artery stenosis and allograft re-transplantation are also becoming more popular. These multimodal therapies have improved the outcomes for people with CAV over time (Tremblay-Gravel et al., 2017).

The chronic allograft vasculopathy evolution declines following treatment with MycoPhenolic Acid (MPA) and mTOR inhibitors (e.g., sirolimus and everolimus) (Arora et al., 2018; Eisen et al., 2005; Vigano et al., 2007). The MPA inhibits inosine monophosphate dehydrogenase, essential for purine generation in lymphocytes (Jonsson & Carlsten, 2001). Compared to azathioprine, the MPA reduced coronary intimal enlargement on ultrasonography (Eisen et al., 2005; Kobashigawa et al., 2013). Further, mTOR inhibitors blocked lymphocyte stimulation by suppressing the mTOR serine/threonine kinase, interleukin 2, and the cell cycle shift from the G1

phase to the S phase (Powell et al., 1999). Additionally, mTOR inhibitors decreased the proliferation of fibroblasts and smooth muscle cells, which are responsible for coronary intimal hyperplasia in CAV (Keogh et al., 2004).

Additionally, statins emerged as effective agents in preventing CAV (Katznelson et al., 1998; Weis et al., 2001). Statin treatment decreased the CAV occurrence and hemodynamically significant rejection while enhancing survival in Randomized Clinical Trials (RCTs) (Kobashigawa et al., 2005; O'Rourke et al., 2004; See et al., 2003; Wenke et al., 2003). As a result, statins are suggested for all cardiac transplant patients and generally start early post-operative.

Considering approaches that implement revascularization through stenting, one trial of everolimus-dispensing stents found that 1-year revascularization rates were 17% for target arteries and 26% for non-target arteries (R. Cheng et al., 2017). Prospective studies reveal that drug-eluting stents had fewer in-stent restenosis than bare-metal stents, but they have the same associated CAV outcomes, including mortality and Myocardial Infarction (MI) (Dasari et al., 2011).

Re-transplantation is considered the last-ditch treatment approach. Nevertheless, it features various limitations because of donor organ scarcity and worse results than de novo cardiac transplantation. Re-transplantation usually becomes recommended for individuals with CAV. They have a severe illness (corresponding to the International Society for Heart and Lung Transplantation-ISHLT grade of CAV3) and allograft malfunction (Khush et al., 2018). International registry analysis supported this standard of practice, revealing comparable results from re-transplantation and medication management. However, a survival benefit for patients

with CAV-associated systolic graft dysfunction was described as a left ventricular ejection fraction of 45 percent and lower (Goldraich et al., 2016).

6. Vascular Angiogenic Ligand-Receptor Pathways

6.1 VEGF/VEGFR

The VEGF group consists of VEGF-A to -E (encoded virally), the Placental Growth Factor (PlGF) (reviewed in this article by (Ferrara & Adamis, 2016), the VEGF-F from the venom of snakes, as well as the Endocrine Gland-derived Vascular Endothelial Growth Factor (EG-VEGF) (Duffy et al., 2004; Samson et al., 2004; Tjwa et al., 2003; Yamazaki & Morita, 2006).

VEGFA, referred to as VEGF, represents a critical pillar in modulating angiogenesis and vasculogenesis. Additionally, it coordinates programmed cell death (i.e., apoptosis), cell proliferation, the permeability of blood vessels as well as immune-cell recruitment to inflammatory and vascular injury locations (Duffy et al., 2004; Ferrara, 2004; Hoeben et al., 2004; Koch & Claesson-Welsh, 2012; Neufeld et al., 1999; Senger et al., 1983; Takahashi & Shibuya, 2005).

VEGF-C and -D are principally involved in governing the formation of the lymphatic system (Alitalo et al., 2005), but they will not be the focus of this thesis discussion.

Via messenger RNA (mRNA) alternative exon splicing, VEGF comprises VEGF121, VEGF165, VEGF189, and VEGF206. The most abundant isoform in tissues is VEGF165, which corresponds to VEGF164 in the mouse. VEGF165 emerges as a significant biologically relevant form, exhibiting properties intermediate between highly soluble VEGF121 and the extracellular matrix-bound VEGF189. Since then, less prevalent isoforms such as VEGF145 and VEGF183 have been identified. The capacity of various isoforms to engage heparin differently is a

fundamental property that differentiates them (Ferrara, 2010; Houck et al., 1992; Poltorak et al., 1997).

As the name implies, the vascular endothelial growth factor becomes produced by ECs (Duffy et al., 2004; Rosen, 2002; Takahashi & Shibuya, 2005; Yamazaki & Morita, 2006). However, VEGF becomes released from other cell types in response to hypoxia. These cells include malignant cells (Duffy et al., 2004; Rosen, 2002), VSMCs (Ishida et al., 2001), macrophages (Duffy et al., 2004; Rosen, 2002; Takahashi & Shibuya, 2005), primed T-cells (Duffy et al., 2004; Rosen, 2002; Takahashi & Shibuya, 2005), pericytes (Raza et al., 2010), platelets (Duffy et al., 2004), and cardiac myofibroblasts within tissue repair environments, following myocardial infarction (Duffy et al., 2004).

In 1992, (Houck et al., 1992) VEGFR1 was regarded as a tyrosine kinase VEGF receptor with a great affinity (de Vries et al., 1992). However, it became clear that VEGFR2 is a primary VEGF signalling receptor with less affinity (Terman et al., 1992). Both receptors are primarily present in ECs.

VEGF-A engages VEGFR1 and VEGFR2, whereas VEGF-B and PlGF engage VEGFR1. On the other hand, VEGF-C and VEGF-D can engage VEGFR3, though they also can attach to VEGFR2 following cleavage by proteolytic enzymes (Pajusola et al., 1992).

VEGFR2 is a significant signalling receptor that promotes vascular EC permeability with a different mechanism than angiogenesis through distinct residues. Noticeably, a sole mutation (tyrosine to phenylalanine) in position 1173 resulted in poor vasculogenic and angiogenic potentials followed by intrauterine demise in mice (Sakurai et al., 2005).

Of note, hypoxia, through the Hypoxia Inducible Factor 1 (HIF-1), is a significant modulator of VEGF expression. Other hypoxia-regulated genes, including Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF), and carcinogenic factors' mutations in the VEGF signalling pathway genes (e.g., the Von Hippel-Lindau gene (*Vhl)*, Rat Sarcoma virus gene (*Ras)*, Wingless-related integration site gene (*Wnt)*, and Kirsten rat sarcoma viral oncogene homolog (*Kras)*) synchronize VEGF production and, as a result, VEGF-driven signalling (Semenza, 2000).

The leading as well as the trailing endothelial cell populations respond to various VEGF concentrations to stimulate filopodia development (De Bock et al., 2013; Eilken & Adams, 2010a; Gerhardt, 2008; Gerhardt et al., 2003; Hellstrom, Phng, & Gerhardt, 2007; Potente et al., 2011; Ruhrberg et al., 2002). The molecular regulation of these actions features the engagement of Notch signalling and increased "Notch homolog 1, translocation-associated (Drosophila)-NOTCH1" expression in ECs. Delta-like Ligand-4 (DLL4) is a classic example (Wacker & Gerhardt, 2011). Increased Notch signalling in surrounding cells lowers VEGFR2, establishing loops to refine signalling. Such classical signalling of VEGF became essential for vascular equilibrium, yet an overactivation might ensue during angiogenesis triggered by disease settings.

6.2 SDF1 (CXCL12)/CXCR4 Pathway

C-X-C Motif Chemokine Ligand 12 (CXCL12) emerged initially as a novel discovery in 1994 before being renamed the Stromal cell-Derived Factor 1 (SDF-1) in 1996 due to the constant production in the stromal cells of the bone marrow (Bleul et al., 1996; Nagasawa et al., 1994). In humans, there are six distinct isoforms (CXCL12α to ϕ), and in mice, there are three variants (CXCL12α to γ) (Shirozu et al., 1995; Yu et al., 2006). C-X-C Chemokine Receptor Type 4 (CXCR4) is among the currently identified CXCL12 receptors (Fernandez & Lolis, 2002). CXCR4 transmits signals that affect several biological processes, including cell differentiation,

proliferation, survival, chemotaxis, and death, when activated (Togel et al., 2005). CXCL12, contrary to chemokines generated by classical inflammation, is constantly expressed in endothelial cells along with its receptor CXCR4 to signal to the VSMCs (Stratman et al., 2020), as well as in stromal cells of the bone marrow (Cheng et al., 2015).

SDF-1 loss of function resulted in significant vascular defects throughout embryogenesis (Tachibana et al., 1998).

Mainly, CXCR4 activates various signalling cascades and causes blood vessel development. CXCR4 governs cytoskeletal reorganization, stromal cell adhesion, ERK-1/2 phosphorylation, cell migration, Protein Kinase B (PKB; AKT) upregulation, cell survival and cell survival and cell death (Wu et al., 2007). It also promotes calcium transit and the stimulation of Protein Kinase C (PKC) as well as the Phosphoinositide 3-Kinase (PI3K) (Wu et al., 2007).

The involvement of chemokines and their associated receptors in the rejection response became widely researched (Nelson & Krensky, 2001). Monocyte Chemoattractant Protein 1 (MCP-1) and CXCR3 may be necessary for CAV progression (Boring et al., 1998; Zhao et al., 2002). SDF-1/CXCR4 axis was deemed vital for SMC recruitment and expansion following mechanical damage to major vessels (Olive et al., 2008; Schober et al., 2003; Zernecke et al., 2006). CXCR4 also becomes expressed on cells involved in CAV formation (ECs, T cells, VSMCs, monocytes, and neutrophils) (Mitchell & Libby, 2007). Another SDF-1 receptor has emerged (Balabanian et al., 2005; Burns et al., 2006). CXCR7, like CXCR4, is expressed on vascular ECs and helps regulate immunological processes and stem cell trafficking (Sun et al., 2010). Moreover, SDF-1 binding to CXCR7 yielded more pericyte recruitment to remodelling blood vessels in Pulmonary Arterial Hypertension (PAH), emphasizing the significance of SDF-1 (CXCL12) signalling in

controlling vascular cell proliferation and its ability to control the CXCR4- Forkhead box protein M1 (FOXM1) axis as well (Bordenave et al., 2020; Yi et al., 2021).

Notably, increased CXCL12 production, as a pro-inflammatory chemokine, can attract leukocytes to its production sites with increased Reactive Oxygen Species (ROS), proteolytic enzymes, and other mediators (Karin, 2010). This sequence of events helps interpret the promoted renal allograft fibrosis following CXCL12 activation. Thus, activation of the CXCL12/CXCR4 route can enhance the Chronic Allograft Nephropathy (CAN) evolution, although inhibition of such association can significantly slow the progression of renal allograft fibrosis, alleviating CAN (Tang et al., 2019; Xu et al., 2016). Besides, CXCR4 neutralization suppressed fibrosis expression while lowering TGFβ-1 to limit allograft fibrosis and survival (Zou et al., 2017). Focused research has also revealed that CXCL12 governs cell migration and stimulates angiogenesis in injured tissue (Du et al., 2012; Salcedo et al., 1999). CXCL12 has been shown to promote neovascularization in ischemic lesions, tumours, and wound healing (Hiasa et al., 2004; Xu et al., 2015; Xu et al., 2013). Furthermore, CXCL12 works with VEGFA to promote angiogenesis (Claes et al., 2011; Yu et al., 2009).

6.3 Apelin/Apelin Receptor (ApelinR)

The Apelin receptor (ApelinR; formerly known as APJ (Shin et al., 2017)), Angiotensin II Receptor-Like 1 (AGTRL1), or APLNR are all given as names for the apelin receptor that belongs to class A GPCRs (O'Dowd et al., 1993). The ApelinR has a similar structure (about 50% structure homology) to the Angiotensin II receptor 1 (AT1R); hence it is known as AGTRL1 (De Mota et al., 2000). However, ApelinR does not recognize Angiotensin II but rather, in pathological contexts, neutralizes its adverse effects when ApelinR is provoked (de Oliveira et al., 2022). Tatemoto et al. isolated apelin using bovine stomach tissue in 1998 (Tatemoto et al.,

1998). The human apelin gene (*APLN*) generates a 77-amino acid precursor/prepropeptide with a signalling peptide at the N-terminus and a handful of paired essential amino acids in the middle region (Tatemoto et al., 1998). Such prepropeptides can become degraded by proteases (e.g., ACE2 (Vickers et al., 2002) into active bio-peptides such as apelin-55, apelin-36, apelin-17, and apelin-13 (Kleinz & Davenport, 2005; O'Carroll et al., 2013). Distinctly, apelin-13's N-terminal glutamine residue is pyroglutamylated, resulting in the generation of the pyroglutamyl form of apelin-13 ([Pyr1]-apelin-13 ([pGlu1]-apelin-13) (Lee et al., 2000). Using mass spectrometry to discriminate these isoforms, investigators identified the pGlu1-apelin-13 as the major isoform in the human cardiovascular system and plasma (Read et al., 2019). Whereas apelin-36 and Pyr1-apelin-13 generated Nitric Oxide (NO)-dependent arterial vasodilation in humans, apelin-17 produced NO-dependent vasorelaxation in rat renal glomerular arterioles as well as in rats' aorta precontracted with Ang II or norepinephrine (Hus-Citharel et al., 2008; Iturrioz et al., 2010).

Endogenous apelin has a short plasma half-life that lasts for a few minutes (Japp et al., 2008; Japp et al., 2010). This feature appears because of apelin's susceptibility to enzymatic degradation by various metalloproteases and neutral endopeptidases (i.e., proteolysis). Examples of these metalloproteases include Neprilysin (McKinnie et al., 2016), ACE-2 (Vickers et al., 2002; Wang et al., 2016), and plasma Kallikrein (KLKB1) (Fischer et al., 2019)). Hence, newer strategies have emerged as attractive research topics to produce more stable proteolysis-resistant therapeutic apelin synthetic analogue peptides (e.g., the apelin-NMeLeu9A2-APLN-17) (Fernandez et al., 2021; Iturrioz et al., 2010; W. Wang et al., 2019).

The ApelinR expression becomes limited throughout embryogenesis, mainly to the developing vascular system's ECs. However, apelin and the ApelinR feature a comprehensive presentation in adult vessel walls (Cox et al., 2006; Kleinz & Davenport, 2004). Further, apelin expression is

primarily confined to the differentiating lead cells at the vascular sprout's tip in the mouse retina. In contrast, the APJ receptor becomes restricted to the trailing cells that create tubal structures (del Toro et al., 2010).

In vascular development, apelin loss of function yielded vascular defects throughout embryogenesis (Kasai et al., 2008).

Worth mentioning that Elabela/Apela/Toddler (ELA) is a significant natural substrate/ligand for the ApelinR (Yang et al., 2017) and emerged initially in zebrafish embryos as a heart development factor (Murza et al., 2016). Elabela's abundance changed in disease states (Yang et al., 2017). The precursor of Elabela comprises 54-amino acids. Mechanistically, Elabela internalizes the apelin receptor (Peverelli et al., 2014), inhibits the cyclic Adenosine MonoPhosphate (cAMP), moves calcium intracellularly, phosphorylates ERK1/2 MAPK, and enhances cardiac contractility (Ceraudo et al., 2014; Perjes et al., 2016).

The ApelinR conveys signals through G proteins and β-arrestins' pathways (Azzi et al., 2003; Wei et al., 2003). Biased ligands are ligands that cause biased activation of a receptor (Rajagopal et al., 2010). Elabela plays essential functions in forming cardiac tissues and controlling disease states at various times or in distinct myocardial parts (Murza et al., 2016). Pregnant Elabela knockout mice also have preeclampsia-like signs such as proteinuria and high blood pressure (Sato et al., 2017).

Researchers recently discovered that the apelin system becomes implicated in age-related sarcopenia, with apelin levels in mice and humans connecting adversely with age (Vinel et al., 2018). This correlation enables further investigation within CAV pre-clinical animal models to characterize the impact of aging on CAV outcomes.

6.4 PDGFB/PDGFR RTK

The mediators and receptors of PDGF direct the physiological mesenchymal cell activity throughout embryogenesis, angiogenesis, and wound repair (Battegay et al., 1994; Bostrom et al., 1996; Leveen et al., 1994; Ross et al., 1990; Uutela et al., 2001; Uutela et al., 2004). Additionally, PDGF regulates various pathological diseases such as arteriosclerosis, rheumatoid fibroproliferative progression and tumour formation (Lokker et al., 2002; Ponten et al., 2005; Ponten et al., 2003; Ross, 1993; Schermuly et al., 2005). The functional distinctions amongst PDGF ligands depend on their distinct receptor-binding affinity, capacity to attach to the extracellular matrix, and dependency on proteases for activation (Fredriksson et al., 2004; Raines et al., 1992). PDGF-AA and PDGF-CC are the primary ligands involved in PDGF receptor alpha (PDGFR-α)- signalling, as well as PDGF-BB plus PDGF-DD, for PDGFR-β signalling, according to studies using knockout mice in embryogenesis (Bergsten et al., 2001; Betsholtz et al., 2001; Ding et al., 2004; Li et al., 2000). PDGF-AB, unlike homodimer PDGF-AA and PDGF-BB, shows a strong affinity for both PDGFR-αα and PDGFR-αβ, but not PDGFR-ββ (Seifert et al., 1993).

Interestingly, PDGF ligands and receptors feature elevation during the first postoperative week in heart allograft endomyocardial biopsies (Sack et al., 2004). Although PDGF factors remain increased in rejection, microvascular levels of PDGFR-β are significantly reduced (Sack et al., 2004; Tuuminen et al., 2009). In chronic rejection mice with limited transplant IRI, PDGF-A, -C, and -D supplementation caused dysfunctional coronary intimal and myocardial fibroproliferation (Mancini & Evans, 2000; Tuuminen et al., 2009). There is mounting evidence that PDGF signalling, particularly PDGF-AB and -BB, contributes to cardiovascular protection by stabilizing leaky cardiac arteries and boosting endothelial cell and cardiomyocyte survival

(Edelberg et al., 2002; Fuxe et al., 2011; Hsieh, Davis, et al., 2006; Hsieh, MacGillivray, et al., 2006; Langley et al., 2004; Xaymardan et al., 2004; Zymek et al., 2006). Further, in the context of CAV, VSMCs were noted to stay dormant under physiological settings but can undergo substantial proliferative and migratory changes in response to diverse stimuli like inflammation and oxidative stress (Orr et al., 2010; Rocchiccioli et al., 2012). As an endothelial tip-cell gene, PDGF-BB is essential for vascular remodelling (G. Liu et al., 2013). Further, following vascular injury, PDGF-BB increases significantly and enhances proliferative and migratory signalling by binding to its receptor, PDGFRβ, driving VSMC growth and invasion (Park et al., 2013). An insightful study (Y. Zhao et al., 2014) looked at the role of Mitogen-Activated Protein Kinase (MAPK) in PDGF-BB-provoked human pulmonary artery Smooth Muscle Cell (SMC) proliferation. That study found that PDGF-BB upregulates the Proliferating Cell Nuclear Antigen (PCNA), Cyclin A, and Cyclin E (Y. Zhao et al., 2014). Furthermore, PDGF-BB stimulated the c-Jun N-terminal kinase (JNK) but not the extracellular signal-regulated kinase (ERK) or p38 MAPK to increase the proliferation of such SMCs (Y. Zhao et al., 2014). Thus, the PDGF-BB-JNK1/2 axis is critical for pulmonary arterial SMCs expansion in PAH remodelling.

6.5 TGFβ/TβRII

The Transforming Growth Factor-beta (TGF-β) signalling emerged initially to hinder cell migration and proliferation (Baird & Durkin, 1986; Frater-Schroder et al., 1986), but further research revealed that it stimulates cell proliferation (Iruela-Arispe & Sage, 1993; Li et al., 2001). The relative amounts of TGF-β expression explain some of these differences, with low dosages favouring angiogenesis and greater levels inhibiting EC development and vascular maturation (Hofer & Schweighofer, 2007; Li et al., 2001). TGF-β paracrine endothelial mesenchymal

transition promoted the VSMC and pericyte differentiation during vascular covering by SMCs (ten Dijke & Arthur, 2007).

During hypoxia, tissue TGF-β contributes to angiogenic activities. A classic example features the upregulated TGF-β proteins in tissues undergoing ischemia post-infarction with ongoing neovascularization (Krupinski et al., 1996).

Of note, TGF-β governs vascular proliferation and maturation by influencing the Anaplastic Lymphoma Kinase 1 (ALK1), ALK5, PDGF, basic Fibroblast Growth Factor (bFGF), TNF alpha, IL-1, and even its expression during angiogenesis (Pepper, 1997).

Notably, TGF-β facilitates angiogenic resolution through ALK5-Caenorhabditis Elegans SMA ("small" worm phenotype) and MAD family ("Mothers Against Decapentaplegic") of genes (Smad2/3)-provoked Plasminogen Activator Inhibitor-1 (PAI-1) as well as fibronectin levels under the control of ALK1 and ALK5 towards achieving vascular maturation. The latter interaction differs from Smad1/5/8 engagement by ALK1, which antagonizes angiogenic responses (Rossant & Howard, 2002; Tian et al., 2011).

Diseases correlated with pathological angiogenesis demonstrated the involvement of TGF-β mutations in their progression. Distinctly, TβR-III/endoglin signalling axis involves developing Hereditary Hemorrhagic Telangiectasia (HHT). Additionally, hepatic, pulmonary, and cerebral Arteriovenous Malformations (AVMs) are associated with developing telangiectasis and epistaxis (Marchuk et al., 2003). Subsequently, VSMC development featured disruption, resulting in unstable, leaky blood arteries (Carvalho et al., 2004). Other processes driving AVMs EC death and SMC depletion have also emerged (ten Dijke & Arthur, 2007). Furthermore,

cerebral AVM demonstrated suppressed β8-integrin levels, representing a lesser TGF-β activation in ECs (Su et al., 2010).

6.6 Dll4-NOTCH

Delta-like Ligand-4 protein (DLL4) emerged as a critical Notch ligand for angiogenesis stimulation (Benedito et al., 2009; Blanco & Gerhardt, 2013; Naito et al., 2020; Oon et al., 2017). However, Jagged 1 protein (Jag1) adversely controls angiogenesis when engaging Notch while competing with Dll4 to commence ligand-receptor binding (Taslimi & Das, 2018). In vitro research has demonstrated the robustness of the pro-angiogenic Jag1 molecule as a signalling rheostat that orchestrates Notch activation following Dll4 binding while angiogenesis is in place (Benedito et al., 2009).

Furthermore, Notch signalling may control angiogenesis via interaction with VEGF receptors (VEFGRs) (Garcia & Kandel, 2012; Luo et al., 2019). In the retinal angiogenic vascular development of mice, the potential function of Dll4 to suppress angiogenesis following VEGF activation was perfectly illustrated. To explain, the tip-EC Dll4 significantly hindered responses to angiogenesis in nearby ECs via Notch (Suchting et al., 2007). Such an effect yielded unequal responses to VEGF-sprouting in tip ECs versus adjacent ECs through variance in Dll4 expression levels (Suchting et al., 2007). Notch signalling can thus regulate the initiation and termination of angiogenesis via distinct pathways.

Such signalling is also involved in arteriogenesis (Heil et al., 2006). DLL4 selective artery blood vessel expression demonstrates that Notch is quintessential for arteriogenesis (Liu et al., 2003; Shutter et al., 2000). In addition, DLL4/Notch1 signalling appears to work downstream of VEGF throughout arteriogenesis, possibly promoting the survival and specialization of arterial ECs (Lawson et al., 2001). Mechanistically, Notch signalling might serve an essential role in

arteriogenesis by interacting with VEGF, Forkhead box protein O1 (FoxO1), and the class B scavenger receptor (CD36, also known as platelet glycoprotein 4, fatty acid translocase, scavenger receptor class B member 3, and glycoproteins 88, IIIb, or IV) (Ren, 2018; Ren et al., 2016; Ren et al., 2010; Ren et al., 2011).

Chiefly, vascular endothelial and mural cells involve Notch peptides, especially Notch1 and Notch4 receptors on vascular endothelium plus Dll1, Dll4, Jag1, and Jag2 (Leimeister et al., 2003; Liu et al., 2003; Phng & Gerhardt, 2009; Shawber et al., 2015; Uyttendaele et al., 1996; Villa et al., 2001). Murine cells featured the expression of Notch1, Notch3, and Jag1 (Joutel et al., 2000; Kofler et al., 2015; H. Liu et al., 2010; Loomes et al., 2002; Shawber et al., 2015). Notch Intracellular Domain (NICD) gets liberated to traffic and occupies the cell nucleus upon receptor engagement. Eventually, this sequence of events leads to the final Notch signalling activation of the net effector molecules. Examples of these net effector molecules include the HEY proteins (hairy and enhancer-of-split-related repressor Herp, Hesr, Hrt, CHF, gridlock) and the NOTCH Regulated Ankyrin Repeat Protein (NRARP) (Krebs et al., 2001; Phng & Gerhardt, 2009; Schweisguth, 2004).

Interestingly, Notch signalling in the donor vascular tissues and the recipient leukocyte compartment provoked tissue rejection addressed in many studies (Quillard et al., 2008). Distinctly, Riella et colleagues (Riella et al., 2011) were the first to establish a function for the Notch system in transplant rejection by using monoclonal antibodies to inhibit the Notch ligand Dll1 in a mouse model of cardiac transplantation. The combination of Anti-Dll1 antibodies and the costimulatory integral membrane protein (B7)-CD28 inhibition produced considerable, albeit moderate, rejection safeguarding cardiac tissues. This effect changes in terms of Jagged2 rejection exacerbation. Such implications relied on T-helper 2 cells (Th2) polarization following

Signal Transducer And Activator Of Transcription 6 (STAT6) mediation as well as IL-6, respectively (Riella et al., 2013).

Further, Wong and associates (Wong et al., 2003) used hematopoietic progenitors expressing Dominant-Negative MAsterMind-Like1 (DNMAML) to inhibit the total Notch activity within T lymphocytes to yield hindered rejection in allografts transplanted into mice while being exacerbated by simultaneous CD8 elimination (Wood et al., 2015). Protection was attributed to reduced T cell infiltration with higher regulatory T Lymphocytes (Tregs) in the allograft. Additionally, a 10-day period of antibody-mediated Dll1/4 blocking provided superior protection in T cells than pan-Notch inhibition, besides reduced alloantibody generation with allograft accumulation of complement. Therefore, Dll1/4 inhibition could robustly protect from allogeneic B and T cells when achieved systematically (Wood et al., 2015). Riella and associates recently showed that anti-Notch1 neutralizing antibodies had a significant protective impact when introduced to cardiac allografts at day ten post-transplant in a major histocompatibility class mismatch model (Magee et al., 2019). Evidence of enhanced Treg growth and function denotes protection. Promoted transplant survival became conceivable whenever antibodies neutralizing Notch-1 were related to the Cytotoxic T-Lymphocyte Antigen 4 (CTLA4, also known as Cluster of Differentiation 152-CD152) Immunoglobulin. Such effects inform the possibility of adopting tolerance in these settings (Magee et al., 2019).

6.7 CXCL11 (I-TAC or IP-9)/CXCR3

Chromosome 4 in humans is the location for the C-X-C motif Chemokine Ligand 11 (CXCL11). Many cells like ECs, leukocytes, Dendritic Cells (DCs), monocytes, and fibroblasts (Ohmori et al., 1997; Ohmori et al., 1993; Singh et al., 2008) can produce CXCL11.

It is usually present at constant yet reduced levels during homeostasis to be upregulated during inflammation, cancer, or other infections (Callahan et al., 2021; Cao et al., 2021; Kumar et al., 2019; Liu et al., 2021). Focused studies have found that cytokines can increase the release of chemokines from specific cells (Gao et al., 2019; Sanchez-Martin et al., 2013). Interferons have the power to enhance CXCL11 synthesis in a range of cell types, namely ECs, leukocytes, fibroblasts, and monocytes. It is generated chiefly after Interferon Gamma (IFN-γ) and IFN-β stimulation, yet IFN-α has a lesser stimulatory effect on CXCL11 production (Rani et al., 1996).

Following simultaneous IFN-γ and TNF-α treatment, monocytes, fibroblasts, ECs, and cancer cells could release CXCL11 (Tokunaga et al., 2018).

CXCL11 interacts with two distinct chemokine receptors, the C-X-C motif Chemokine Receptor 3 (CXCR3) and CXCR7.

Notably, the canonical CXCR3-bound ligands include the CXCL9 up until the CXCL11, with CXCR3 demonstrating a greater affinity for CXCL11 in comparison to CXCL9 as well -10 (Cole et al., 1998; Weng et al., 1998).

Ligands bound to CXCR3-A provoke the expansion of cells via activating the Protein Kinase B (PKB; also known as AKT) and calcium flow. On the contrary, ligand binding to CXCR3-B inhibits growth or causes cell death by dissociating the heterotrimeric Gs-protein. Subsequently, the "Gαs" becomes generated in addition to "Gβγ" while activating the Adenylyl Cyclase (AC)-cAMP-p38 MAPK signalling cascade (Lasagni et al., 2003; Petrai et al., 2008; Singh et al., 2013).

CXCL11-bound CXCR3-A suppresses CXCR3-B by triggering signalling pathways below this receptor (Lasagni et al., 2003; Wu et al., 2012). CXCR3-B's suppressive action on human microvascular EC development was attributed to CXCL11.

CXCR7 could also be activated, and CXCL12. However, CXCR7 has about a 20-fold lower affinity for CXCL11 versus CXCL12 (Burns et al., 2006).

When CXCL11 engages CXCR3-A and CXCR7, proliferative cascades become enhanced, but CXCR3-B inhibits growth activities when bound to CXCL11 (Lasagni et al., 2003). Thus, one chemokine can engage many receptors to promote various activities.

CXCL11 and CXCR3 have influenced the proliferation of several cell types, including ECs and pericytes (Lasagni et al., 2003). The new seven-transmembrane receptor, RDC1, is a putative GPCR coreceptor for human and simian immunodeficiency virus (Karin, 2010), and a CXCR7 (a CXCL11- or CXCL12-binding protein), never promote migration of cells across tissue compartments nor provoke calcium transit, unlike the traditional chemokines. It is expressed on activated ECs and promotes survival, proliferation, and enhanced adhesion qualities (Burns et al., 2006).

7. PI3-Kinases (PI3Ks)

Phosphoinositide 3-Kinase (PI3K) signalling is essential to regulate cell proliferation, migration, actin-cytoskeleton remodelling, metabolism, and vesicular trafficking (Bilanges et al., 2019a; Goncalves et al., 2018; Vanhaesebroeck et al., 2010). PI3Ks create many phosphatidylinositol derivates, driving signal transmission and membrane vesicle sorting (Bilanges et al., 2019a; Vanhaesebroeck et al., 2010). This evolutionarily conserved family of lipid enzymes entails eight catalytic isoforms classified into three major groups depending on their substrate selectivity.

7.1 Classification

The PI3K family comprises eight catalytic variants categorized into three groups depending on substrates and sequence homology in vitro. Class-I includes classes IA and IB (which received the most attention). The IA class comprises a catalytic p110 and a regulatory p85 subunit as heterodimers.

Genes that encode the p110 catalytic subunit subtypes are PIK3CA, PIK3CB, and PIK3CD.

P110γ is present in all Class IB PI3Ks, while p110α and p110β are expressed generally (Vanhaesebroeck et al., 2005). On the other hand, p110λ and p110δ fully occupy cells of the immune system (Deane & Fruman, 2004).

Chiefly, numerous cell surface receptors activate Class IA PI3Ks. The Phosphatidylinositol (4,5) bisphosphate (PIP2) gets activated through phosphorylation. Subsequently, it generates the Phosphatidylinositol (3,4,5) triphosphate (PIP3) through receptor tyrosine kinases and the G protein-coupled receptors. This phospholipid directs cytoplasmic protein mobilization into specific plasma membrane locations while serving as a messenger molecule. The Proto-oncogene tyrosine-protein kinase (Src) homology 2 (SH2) and SH3 domains are present in regulatory subunits, and equivalent binding sites occupy the target proteins. In healthy cells, growth factor stimulation momentarily induces PIP3 to be promptly processed via lipid phosphatases, ending the cascade of PI3K activation by eliminating PIP3 phosphoric acid at the 3′-position. Furthermore, inositol phosphatase that entails SH2 eliminates phosphoric acid at the 5′-location from PIP3, yielding PIP2 with a suppressed activity of its downstream effectors (Durrant & Hers, 2020).

The three members of class II PI3Ks, namely, the PI3KC2α, PI3KC2β, and PI3KC2γ, are associated with forming PIP2 via phosphatidylinositol 4 phosphate catalytic substrate.

The combination of p150 with catalytic subunits makes up the third PI3Ks class that is also known as Vps34, to govern autophagy (Jaber et al., 2012) as well as the development of cells through the mammalian (also referred to as mechanistic) Target Of Rapamycin Complex 1 (mTORC1)/Ribosomal protein S6 Kinase beta-1 (S6K1) pathway controlling for further modulation of protein formation (Mohan et al., 2016).

Despite studying all these various PI3K classes and stratifying their specific isoforms, their role(s) in vascular biology contexts has been poorly defined. This thesis will focus on Class IA PI3Ks, focusing on the PI3Kβ downstream of the apelin-ApelinR signalling pathway in ECs.

7.2 Endothelial Class IA PI3Kα Signalling

Phosphoinositide 3-Kinase Alpha (PI3Kα) is the most important class I PI3K regulating EC function during blood vessel expansion. The severe vascular abnormalities and embryonic death caused by constitutive and endothelial-specific PI3Kα deletion support the previous statement (Angulo-Urarte et al., 2018; Bi et al., 1999; Graupera et al., 2008; Lelievre et al., 2005). Such a phenotype was not evident when other class I PI3K subunits were blocked (Graupera et al., 2008). PI3Kα has been assigned several critical tasks during vascular growth, whereby it modulated EC venous identity during vasculogenesis (Herbert et al., 2009). On the other hand, Arteriogenesis necessitates inhibiting PI3K signalling (Ren et al., 2010). PI3Kα governs EC rearrangements and junctional remodelling inside the nascent tube, according to mouse and zebrafish models of sprouting angiogenesis (Angulo-Urarte et al., 2018; Nicoli et al., 2012). Homozygous PI3Kα knockout caused embryonic lethality between embryonic day (E) 9.5 to E10.5 (Bi et al., 1999).

7.3 Endothelial Class IA PI3Kβ Signaling

Knockdown of PI3Kβ in endothelial cells leads to increased VEGF-promoted PI3K/AKT signalling and angiogenesis (X. Chen et al., 2019). Thus, a reduction in myocardial ischemic injury in vivo ensued (X. Chen et al., 2019). This effect conceptualized that PI3Kβ imposes inhibitory feedback on PI3Kα in ECs (X. Chen et al., 2019).

Recent research identified VEGFR2 inhibition and the subsequent decrease in PI3Kα activity modulated angiogenesis inhibition. This effect occurred parallel with PI3K-beta inactivation (Hida et al., 2018). Furthermore, current research identified the PI3K-inhibition as a potential strategy to hinder Arteriovenous Malformation (AVM) in Hereditary Hemorrhagic Telangiectasia type 2 (HHT2) models. This strategy complements the VEGF-VEGFR2 axis inhibition, preventing excessive angiogenesis without completely reversing AVMs (Ola et al., 2016).

Notably, PI3Kβ is a distinctive peptide that receives stimulation from RTKs and GPCRs (Guillermet-Guibert et al., 2008; Kurosu et al., 1997). Rho-GTPases maintain it to guard the kinase activities and performance in cellular contexts like chemotaxis and tissue fibrosis (Fritsch et al., 2013). Furthermore, both global (Kulkarni et al., 2011) and EC-restricted PI3Kβ loss (Graupera et al., 2008) are tolerable.

Recent research studied the role of PI3Kγ isoform in vascular injury using carotid ligation animal models with specific tracking of the PI3Kγ-activation impact on Vascular Smooth Muscle Cells (VSMCs), the VSMC phenotypic transition outcomes, and vascular neointimal expansion (Yu et al., 2019). However, the exact role of the endothelial PI3Kβ isoform in vascular injury and repair necessitates further investigation.

7.4 Endothelial Class IA PI3Kγ Signaling

PI3Kγ is present at low levels in ECs and SMCs while at high levels in leukocytes. The p110γ subunit promotes chemokine-induced movement of neutrophils, eosinophils, and macrophages (Ferguson et al., 2007; Hirsch et al., 2000). It has been demonstrated that p110γ regulates neutrophil recruitment and migration and is essential for initial inflammatory reactions following sepsis challenge (Ong et al., 2005; Yum et al., 2001). Nevertheless, less knowledge is available about the role of p110γ throughout the repair phase in controlling endothelial renewal and vascular healing. The authors of this study (Huang et al., 2016) focused on the essential role of endothelial p110γ in their investigation since p110γ expression featured a significant decrease in pulmonary vascular ECs from Acute Respiratory Distress Syndrome (ARDS) patients (Huang et al., 2016). They revealed that p110γ loss seriously hampered vascular healing following Lipopolysaccharide (LPS) treatment in a mouse model of endotoxemia (Huang et al., 2016). Furthermore, restoring p110γ or FoxM1 in ECs reversed the impaired EC recovery in PI3Kγ knockout mice (Huang et al., 2016). Therefore, strategically targeting p110γ to enhance FoxM1-mediated vascular healing suggests a unique therapeutic paradigm for curing different vascular disorders like acute lung injuries (Huang et al., 2016).

Notably, in early studies of embryonic development, PI3Kγ knockout resulted in viable and fertile animals but with defective inflammation and innate immunity (Chang et al., 2007).

7.5 Vascular PTEN Signaling

Phosphatase and Tensin Homolog (PTEN) dephosphorylates the Phosphatidylinositol (3,4,5)-triphosphate (PIP3) to Phosphatidylinositol (3,4)-biphosphate (PIP2). This activity governs cell migration, survival, growth, and the cell cycle progress (Cantley, 2002; Hawkins et al., 2006; Song et al., 2011; Song et al., 2012; Vanhaesebroeck et al., 2010). By nature, PTEN features

protein and lipid phosphatase activities plus tumour suppressor capabilities, yet it utilizes its lipid phosphatase properties to hinder the PI3K pathway of signalling (Song et al., 2011).

As one of two PIP3-phosphatases, the PTEN, but not the SHIP (Src Homology 2 domain-containing Inositol polyphosphate 5-Phosphatase), regulates the PI3K activities. Research studies confirmed such distinction by using PTEN genetic knockout animal models, demonstrating embryonic lethality at an early stage of development (Di Cristofano et al., 1998), while SHIP deletion resulted in viable embryos (Helgason et al., 1998).

In physiological and pathological angiogenesis, PTEN activation hindered the vascular sprout growth and the formation of blood vessel lumen during vascular development and tumour angiogenesis (Huang & Kontos, 2002; Su et al., 2003). Therapeutic targeting of PTEN with appropriate dosing can modulate variable responses in cell growth and proliferation. This strategy can promote vascular regeneration following degenerative disorders like ischemic disease pathologies (K. Liu et al., 2010; Ruan et al., 2009). Further, it can inhibit tumorigenesis (Alimonti et al., 2010).

7.7 Therapeutics of Vascular PI3K Inhibition

As highly sought-after targets, the Phosphoinositide 3-Kinase (PI3K) inhibitors emerged to regulate vascular (Chang et al., 2007; Fougerat et al., 2008), oncological (Yap et al., 2015), pulmonary (Doukas et al., 2009; Nashed et al., 2007), inflammatory (Hawkins & Stephens, 2015), and immunological (Banham-Hall et al., 2012; Kulkarni et al., 2011; Leverrier et al., 2003; Ramadani et al., 2010) disease contexts. While research witnessed the many fruits of PI3K pan inhibition (Lonetti et al., 2015; Zhu et al., 2021), recent selective isoform-targeted interventions' benefits significantly outnumbered the initial off-targeted approaches (Bheemanaboina, 2020; Cintas et al., 2021; Janku et al., 2018; Kim et al., 2020). While PI3K

isoform-targeted inhibitors demonstrate better tolerability and higher target specificity, pan PI3K inhibitors still demonstrate potency, efficiency, and effectiveness (X. Wang et al., 2015). We can explore a relevant demonstration in cancer studies of grown tumours with PTEN deficiency, where PI3Kβ sole targeting, as an example, resulted in insufficient anti-tumour activities, unlike the used pan PI3K inhibitor in such models (Weigelt et al., 2013). On the other hand, isoform-selective PI3Kβ targeting showed a rewarding potential to modulate platelet functions in thromboembolic diseases compared to first-generation pan inhibitors (Jackson et al., 2005).

Noteworthy, PI3K-AKT pathway suppression underwent investigation in elegant recent research (Z. Wang et al., 2019) to hinder the formed atherosclerotic plaques' advancement by promoting the death of the cellular constituents of plaques (Z. Wang et al., 2019). Furthermore, in blood vessel lesions that entail calcification, like atherosclerosis, PI3K-AKT activation further worsened such pathological processes, as investigated by Liu et al. (Liu et al., 2017). This effect confirms the therapeutic potential of inhibiting the PI3K activity in vascular diseases with inflammatory (immune) injury signals and pathological remodelling such as atherosclerosis (Liu et al., 2017; Z. Wang et al., 2019). Lastly, PI3K signalling inactivation in macrophages controlled the process of autophagy. This effect, in turn, regulated the cholesterol trafficking to the exterior of the atherosclerotic plaque, as investigated by the authors of this study (Han, Kou, et al., 2019).

Interestingly, the PI3K-AKT signalling pathway emerged as a mechanistic explanation for insulin release inhibition and diabetes after immune inhibition of transplant recipients with tacrolimus, indicative of the vital role of PI3K in regulating such post-transplant complication progression (Tong et al., 2021). Besides, inactivating PI3K was deemed effective in controlling hepatic fibrosis in humans and the mouse (Gore et al., 2019). Moreover, inactivating the P110 gamma catalytic subunit of PI3K hampered transplant arteriosclerosis progression. It shifted the VSMC

phenotype towards a more responsive type during this disease pathology in vivo on cultured SMCs for gene response characterization (Yu et al., 2018). Eventually, restricting the PI3K activity through its inactivation regulated the blood vessel state of cellular quiescence. This effect is potentially significant in developing a druggable target for blood vessel hyperplasia in many vascular disease contexts (Alsina-Sanchis et al., 2018).

8. Vascular NF-κB Signaling

Following its discovery in plasma cells and B cells, the Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NFκB) emerged to control the κ-light chain levels in these cells. (That is where the name came from) (R. Sen & D. Baltimore, 1986; Ranjan Sen & David Baltimore, 1986). NF-κB emerged as a critical transcription factor involved in inflammation, immunity (Baeuerle & Henkel, 1994), and survival in almost every other cell type (Chen & Manning, 1995; Hayden et al., 2006; Kopp & Ghosh, 1995; Pahl, 1999). It is most abundant in the cytosol at an inhibited state when bound to its inhibitors. Still, it gets activated when liberated from these inhibitors and starts translocation into the nucleus. The various inflammatory, viral, bacterial, and even physical factors activate NF-κB (Harada et al., 2003). The precise understanding of the receptors that perceive the initial triggers and components altering these cellular signals required several decades and efforts by many teams (reviewed in (Hayden & Ghosh, 2012)).

8.1 NF-κB Activation Pathways and Complexes Formation

NF-κB comprises five components generated by proteasomal digestion for NF-κB activation (Liang et al., 2006; Moorthy et al., 2006; Naumann et al., 1993).

Over time, the diversity of distinct NF-κB activation signalling pathways became so perplexing that researchers advocated for a structured framework to “rationally” arrange the signalling

cascades. The NF-κB entails the canonical pro-inflammatory-mediated signalling pathway (Figure 1.1) (Hayden & Ghosh, 2008; Sun, 2017; Trask, 2004; Wulczyn et al., 1998) (initiated by Lipopolysaccharide-LPS, IL-1, or TNF alpha), an alternative pathway (stimulated with lymphotoxinβ or CD40 ligand) (Figure 1.2) (McPherson et al., 2012; Sun, 2011, 2017; Trask, 2004), and a final route of activation elicited by DNA damage signals (Trask, 2004). Notably, the activation of elements in the alternative pathway can activate signalling molecules of the canonical pathway (Seigner et al., 2018). Such transcriptional responses can be closely correlated (Seigner et al., 2018).

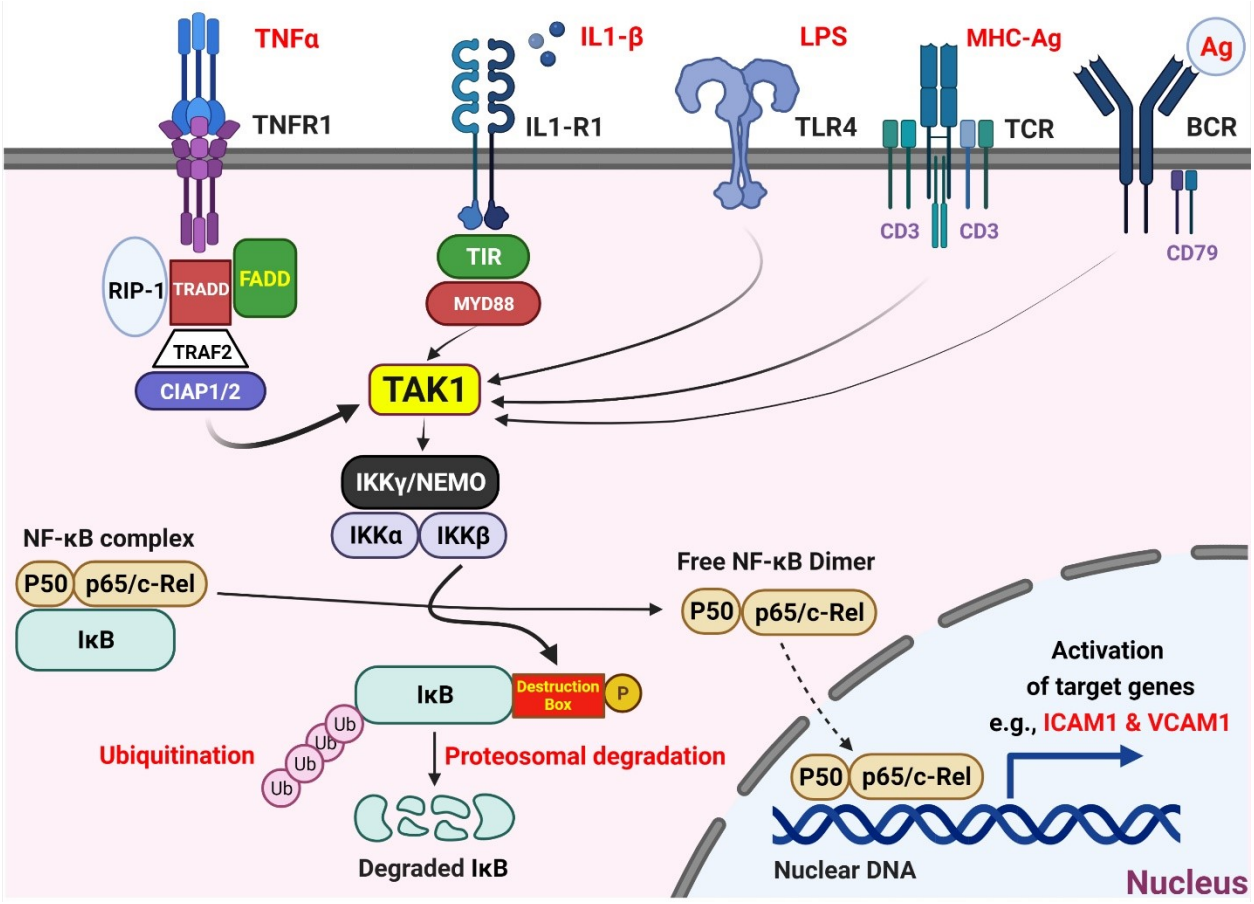

Figure 1.2: A schematic illustration of the classical NF-κB activation pathway through IκB degradation

The classical or canonical NF-κB pathway becomes activated when many inflammatory or immune factors bind to their receptors upstream of the NF-κB signalling pathway (Webster & Vucic, 2020). For example, TNF-α binds to the Tumor Necrosis Factor Receptor 1 (TNFR1, also known as CD120a), causing TNFR1 trimerization (Grell et al., 1995), to further form a receptor complex platform through recruiting various adaptor proteins that communicate via their death domains (Reddy et al., 2000) with TNFR1, such as the Receptor Interacting Protein 1 (RIP1, also known as RIPK1), TNF Receptor Associated Death Domain (TRADD) (Micheau & Tschopp, 2003), TNF Receptor Associated Factors (TRAF), E3 ligase Cellular Inhibitors of Apoptosis (c-IAP), with a series of polyubiquitination (Bertrand et al., 2008; Dynek et al., 2010; Mahoney et al., 2008; Varfolomeev et al., 2008) to construct a docking site for a signaling kinase complex that comprises Inhibitors of Kapa B Kinase 1 and 2 (IKKα and -β, respectively), NF-κB Essential Modulator (NEMO. Also known as IKKγ), Transforming growth factor beta-Activated Kinase 1 (TAK-1) in addition to the TAK-1 binding proteins 2 and 3 (TAB 2 & 3) (Haas et al., 2009; Shim et al., 2005; Webster & Vucic, 2020). The triggered TAK1, a critical activator of the canonical NF-κB pathway (Akira, 2003; Ono et al., 2001), activates the IKKβ predominantly. In turn, it phosphorylates or activates the serine residues of the destruction box (Chen, 2005; Wagener et al., 2016; Winston et al., 1999) in the IκB that is bound to the NF-κB dimer. Examples of the NF-κB dimer include the combination of P50 and P65-REL Proto-Oncogene, the NF-KB Subunit Associated protein-RELA or a mixture of P50 and cREL (Sun & Ley, 2008), and a P50-P50 variety (Hayden & Ghosh, 2008)). Such interactions allow IκB polyubiquitination and proteasomal degradation. Subsequently, this sequence of events liberates the NF-κB dimer, which shifts into the nucleus and binds to the nuclear DNA binding site. The NF-κB dimer commences further transcriptional activities via activating various target effector genes *(Brown et al., 2008)* **(Picture is created using icons available on Biorender).**

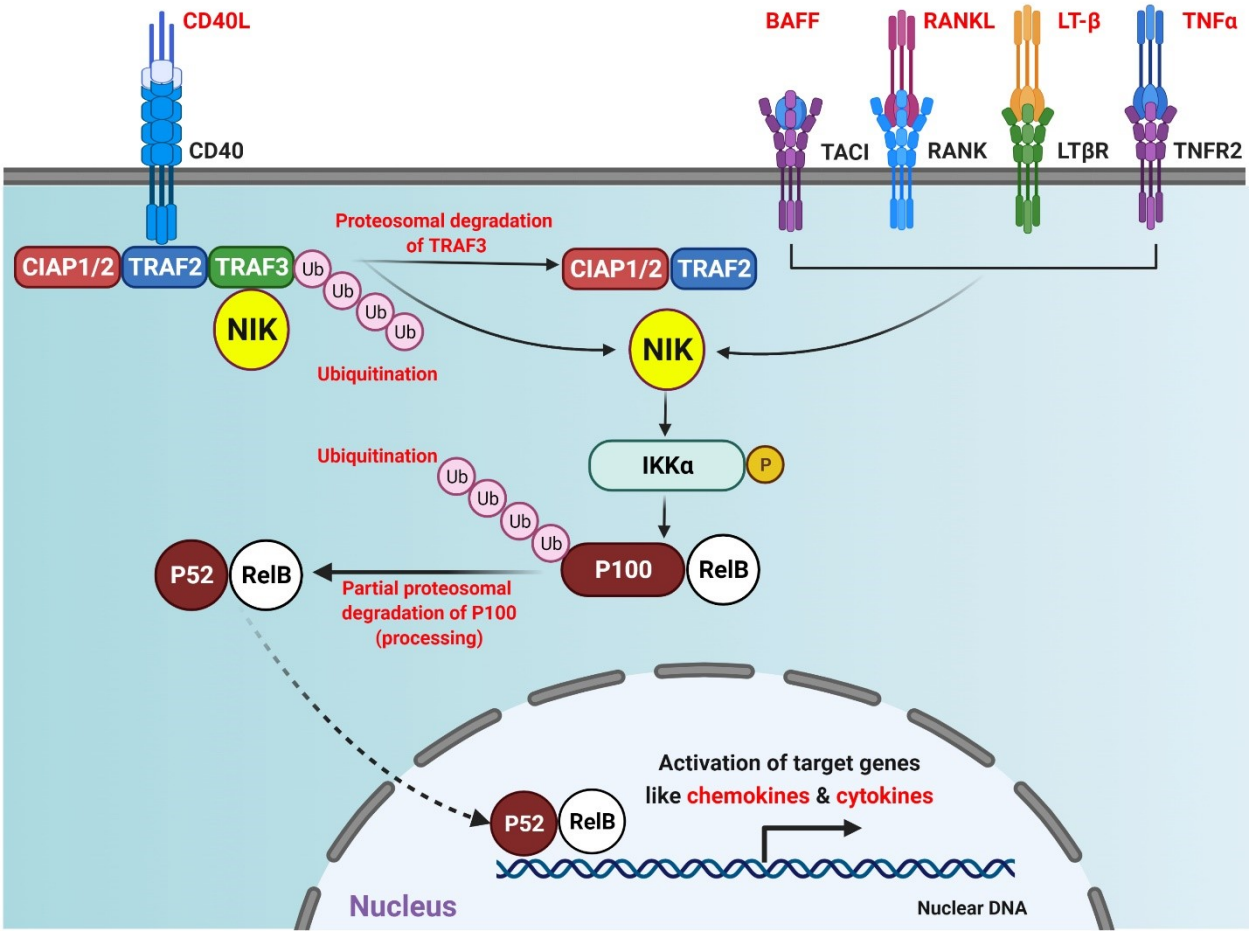

Figure 1.3: A schematic illustration of the alternative NF-κB pathway.

The critical molecular event that marks the non-canonical/alternative NF-κB route in response to ligands of the TNFR superfamily (e.g., CD40 ligand (CD40L) (Coope et al., 2002), Lymphotoxin B (LT-β), Receptor Activator of NF-κB Ligand (RANKL), and the B-cell Activating Factor-BAFF), Macrophage Colony-Stimulating Factor (MCSF), Membrane Attack Complexes (MACs), or the directed antibodies against CD3 & CD28 is the involvement of the NF-κB-Inducing Kinase (NIK) key molecule (Sun, 2011; Xiao et al., 2001) predominantly downstream the TNFR2 with its TNFR Associated Factors (TRAF) 2 & 3 in the cytoplasm, to phosphorylate

the IKKα, which in turn drives an inducible (partial) proteasomal degradation or processing via degrading the carboxy-terminus of serine residues of the Nuclear factor NF-kappa-B p100 subunit (NFKB2, also named P100, the IKKα substrate) to form P52, as a part of the NF-κB heterodimer when combined with RELB (Sun, 2012), for further transcriptional activation of the target genes assigned to the alternative pathway (Senftleben et al., 2001; Xiao et al., 2004). **(Picture is created using icons available on Biorender).**

Nuclear Factor-kappa B (NF-κB) activation is dependent on its liberation following degradation or, in certain circumstances, dissociation from IκBα (Ghosh & Hayden, 2008; Hayden & Ghosh, 2004; Imbert et al., 1996; Karin & Greten, 2005; Rahman & Fazal, 2011). A more typical mode of NF-κB activation is mediated by degrading IκBα following serine phosphorylation of IKK. As a result, IκBα breakdown by proteasomes triggers RelA nuclear translocation and net gene activation. While maintaining NF-κB dimers in the cytoplasm, IκBα stops the process of transcription (Bonizzi & Karin, 2004; Ghosh & Hayden, 2008; Karin & Greten, 2005).

Noteworthy, the IκBs do not entirely inhibit the NF-κB nuclear translocation. The reason for this effect is that NF-κB toggles from the cytoplasm to the nucleus back and forth for further detection even though IκBs are present in standard amounts (halftime is estimated to be 7 to 14 minutes) (Birbach et al., 2002; Huang et al., 2000; Johnson et al., 1999). In quiescent cells, fluorescently labelled p65 and IκB components highlighted the nuclear representation of P65 to constitute a 5% portion of cytoplasmic p65 (Birbach et al., 2002). Such bonding dynamicity justifies a modest NF-κB activation, even in quiescent cells (Carlotti et al., 2000).

8.2 NF-κB Signaling in ECs

Quiescent ECs release nitric oxide, prostacyclin (predominantly generated in major arteries) and prostaglandin-E2 (PGE2, in microvessels) to inhibit the attachment of platelets (van Hinsbergh,

2012; Yau et al., 2015). Further, negatively charged glycosaminoglycans on the endothelium membrane hinder platelet attachment. The NF-κB pathway plays essential functions in EC stress because of its effectiveness in controlling coagulation and inflammation events with their extensive interplay (Levi & van der Poll, 2005).

NF-κB components have always been available within ECs to engage in highly coagulant states and hinder barrier function (Rahman & Fazal, 2011).

Within inflammatory contexts, adhesion molecules represent essential target genes of NF-κB in ECs because they coordinate inflammatory cell attachment to vessel walls, causing efflux into tissues (Rahman et al., 1999; Sprague & Khalil, 2009; Sugama et al., 1992; Xue et al., 2009). Further, TNF-α activation of the classical NF-κB pathway was deemed transcriptionally capable of inducing the Fractalkine chemokine (also known as the (C-X3-C motif) Ligand 1-CX3CL1) (Ahn et al., 2004; Bhavsar et al., 2008), ICAM-1 as well as VCAM-1 expression (Bunting et al., 2007; Iademarco et al., 1992; van de Stolpe et al., 1994).

Notably, TNF-α and thrombin tend to be the most effective NF-κB stimulators in ECs in vitro. However, other cytokines, such as IFN-γ, stimulate EC NF-κB (Mussbacher et al., 2019). Research work noted that TNF-α and thrombin synergistically impact controlling endothelial permeability (Tiruppathi et al., 2001).

Mechanistically, thrombin interacts with the Protease-Activated Receptor 1 (PAR-1), although TNF-α engages TNFR-1 and -2 (Baud & Karin, 2001; Soh et al., 2010). Such routes coincide in the IKK complex (Delekta et al., 2010; Iwai, 2012), although it is worth noting that thrombin and TNF-α trigger redundancy, a distinct target gene expression in ECs, eventually (Luedde et al., 2005).

Noteworthy, NF-κB acted as a preservation component for ECs in sepsis by blocking endothelial apoptosis, ensuring a rapid shift from barrier damage to recovery (Liu et al., 2014). Endothelial production of a degradation-resistant version of IκB did not affect embryogenesis. Still, the EC-specific deletion of IKKβ culminated in an enhanced embryonic lethal phenotype and endothelial apoptosis, led by IKKβ kinase-independent activities (Ashida et al., 2011). Notably, endothelial NF-κB emerged as a critical modulator of atherosclerosis progression in mice models in which canonical NF-B signalling shutting down through EC NEMO knockout guarded experimental animals lacking Apolipoprotein-E against diet-provoked atherosclerotic disease progression (Gareus et al., 2008).

8.3 NF-κB Signaling in VSMCs

Decreased expression of gene factors of the smooth muscle cell phenotype of contractility like the (Calponin-related) Smooth Muscle-specific protein 22 (SM22) features many lesions of atherosclerosis (Nguyen et al., 2013). Surprisingly, in inflammatory circumstances, SM22 decreases NF-κB signalling pathways (Dai et al., 2017).

Smooth muscle cells exhibit many active elements of the NFκB, whereby under healthy conditions, VSMCs of the arteries have little basal NF-κB activation. Still, NFκB becomes boosted in VSMCs through atherosclerotic lesions. Intriguingly, inflammatory cytokines produce protracted NF-κB activity due to a continuous reduction in the inhibitory component IκBβ (Bourcier et al., 1997). TNF-α tends to play a critical role in the evolution of atherosclerotic lesions. The TNF-α/ApoE double knockout mice featured less arterial wall thickness and smaller atherosclerotic lesions (Branen et al., 2004). Of note, TNF-α binds to TNF receptors present on SMCs (Maddahi et al., 2011), activating a conventional route to promote coagulation genes (Martinez-Moreno et al., 2016) plus Matrix MetalloProteinases (MMPs) in addition to IL-1β to

inhibit the SM22 and myosin heavy chain (Ali et al., 2013). TNF-α reduces contractile gene transcription by inducing the Krüppel-like factor 4 (Klf4), a recognized modulator of SMC differentiation (Davis-Dusenbery et al., 2011), which appears to be an NF-κB target gene according to regions of attachment (Shankman et al., 2015).

8.4 PI3K/AKT and NF-κB Control

Initial research work did not identify a role for the Phosphoinositide 3-Kinase (PI3K) in modulating pro-inflammatory stimulated adhesion molecule expression in endothelial cells, as well as the inability to be regarded as a modulator of NF-κB pathways' activation (Gustin et al., 2004; Madge & Pober, 2000). Such observations emerged after testing the pan PI3K inhibitor treatments. Further research confirmed that many other NF-κB core molecular modulators downstream of inflammatory receptors like the IL-1 receptor, TNF-α superfamily of receptors, or the Toll-Like Receptors (TLRs) have been independent of PI3K (Zhang et al., 2017). Nevertheless, oncogenic PI3Kα or AKT gain-of-function mutants in cancer cells drive NF-kB activity (Bai et al., 2009; Dan et al., 2008; Hutti et al., 2012).

Interestingly, testing lung microvascular ECs from animals lacking the PI3Kγ catalytic subunit (p110γ) yielded a deficiency of Protein Kinase Cζ (PKCζ) stimulation following TNF-α within the PI3Kγ knockout cells of the endothelium. Such findings locate PI3Kγ above PKCζ during TNF-α-induced NF-κB activation (Frey et al., 2006).

Further insightful research highlighted the involvement of the PI3Kα isoform in provoking the NF-κB activation with the NF-κB65 dimer trafficked to the nucleus following the alpha isoform overexpression (Koul et al., 2001; Reddy et al., 1997; Sizemore et al., 1999).

8.5 NF-κB Effector Target Genes

Despite significant advances in recognizing target effector genes regulated by the NF-κB (https://www.bu.edu/nf-kb/gene-resources/target-genes/), the stratification and correlation of such target genes to specific physiological conditions, distinct cellular populations/phenotypes as well as stimuli have not yet been achieved (Ngo et al., 2020).

In inflammation, NF-κB target genes include *Il1b* (Hiscott et al., 1993), *Icam1* (van de Stolpe et al., 1994), *Vcam1* (Iademarco et al., 1992), *Tnfa* (Shakhov et al., 1990), *Ifng* (Sica et al., 1997), *Cx3cl1* (Fractalkine gene) (Ahn et al., 2004), the inducible nitric oxide synthase gene (*Nos2*) (Cooper et al., 1998; Xie et al., 1994), which orchestrates the production of nitric oxide following lipopolysaccharide stimulation of the NF-κB pathways, and the serine proteinase inhibitor-1 (*Serpine1)*, which codes for the Plasminogen Activator Inhibitor-1 (PAI-1) (Swiatkowska et al., 2005). In endothelial cells, inflammatory conditions can crosstalk to coagulation pathologies through NF-κB redundancy of many shared effector target genes that significantly orchestrate such disease conditions (Levi & van der Poll, 2005).

Notably, potent inhibitors of NF-κB successfully hindered the rejection of heart allografts in the heterotopic rat models that feature a genetic discrepancy in minor and major histocompatibility loci (Cooper et al., 1998). This resultant protection and prolongation of the heart allograft survival suggested a role for NF-κB in modulating allograft outcomes (Cooper et al., 1998). Further, in lung inflammation, hindering NF-κB activity via NF-κB decoy transfection introduced successfully reduced the observed acute lung injury during allograft rejection with clinical therapeutic implications on lung transplantation practice (Ohmori et al., 2005). Additionally, NF-κB decoy transfection significantly lowered the IL-8 level in donors' lungs (Ohmori et al., 2005).

Such an effect can significantly correlate to an adverse graft outcome and recipient death (Fisher et al., 2001).

9. Animal Models of CAV

Besides cost-effectiveness, mouse models in an experimental setting possess many advantages. For instance, the accessibility to a pathogen-free model with fewer inherent genetic variables, unlike those models of larger animals (Chong et al., 2013). Additionally, revealing newer subsets of lymphocytes and macrophages in response to cardiac transplantation is advantageous (Baldwin et al., 2014). Furthermore, the precise deletion or insertion of genes of interest in mice transplantation models makes it ideal to elucidate the cellular pathways of tumorigenesis and transplantation. Such advantages can drive pharmacotherapies development (Graves et al., 2018).

Some of the utilized CAV mice models exploit a mismatch in the minor histocompatibility antigens by involving different animal strains or sexes. Such a strategy allows for a gradual immune rejection response without an overwhelming rejection reaction or the need to include immunosuppression regimens post-transplantation. Immunosuppression can introduce difficulties and confounding effects on interpreting the studied transplantation mice models (Popli et al., 2014).

The first to report the most utilized technique of heterotopic heart transplantation in experimental animals was Corry and colleagues (Corry et al., 1973b). Despite encountering pericardial inflammation (which impacts epicardial vessels around the time of transplantation) and losing the cardiac pumping activity, the preclinical model of murine heart transplantation conserves the transplants' contractility, coronary perfusion, and blood flow maintenance (Libby & Pober, 2001). Essentially, the donor heart's ascending aorta, as well as the pulmonary artery, are

anastomosed to the recipient's abdominal aorta as well as the inferior vena cava, respectively (Corry et al., 1973b; Liu & Kang, 2007; Niimi, 2001; Yu et al., 2005).

Another model of interest utilizes a one-allele mutation (I-A^{Bm12}) variance of the major histocompatibility complex II- (MHCII) mismatched mice (B6.C.*H-2-Bm12*) donors into wild-type B6 recipients. In this vascularized heterotopic heart transplantation model, heart allografts can be viable for almost a hundred days to develop mononuclear cell infiltration in the fourth-week post-transplantation. In addition, these heart allografts develop significant neointimal expansion by the eighth week after transplantation (Sayegh et al., 2003; Schenk et al., 2005). This model allows for investigating chronic graft vascular disease (Bedi et al., 2010) because of the overall abundance of the regulatory T lymphocyte subsets that evolve with the lack of $CD4^+$ allogeneic T-cell indirect immune recognition (Sayegh et al., 2003). Further, this model allowed for characterizing various immunologic mediators of allograft injury, such as IFN-γ (Nagano et al., 1997), IL-17 (Yuan et al., 2008), as well as other immune functions of the chemokine receptors (Yun et al., 2004).

Interestingly, MHC I mismatched transplant models (e.g., transplanting the bm1 donor mice with mutations in the $H2K^b$ of MHC I to the wild-type B6 mice) generated a $CD8^+$-provoked alloimmune response with an allograft heart survival up to thirty days post-transplant (Mohiuddin et al., 1996; Schulz et al., 1995; Wang et al., 1990; Yang et al., 2008). Switching the used strains between the donor and recipient mice enhanced the allograft longevity by up to sixty days, yet with established heart allograft vasculopathy (Yang et al., 2008). The unifying theme among all complete MHC mismatched models is the need to employ immune inhibition regimens post-transplantation (Sho et al., 2002), with associated complexity in interpreting the transplantation outcomes in research studies (Dun et al., 2020).

Notably, the ongoing process of antibody-mediated rejection has emerged as an essential component of the chronic rejection process, especially within clinical settings with antibodies against the human leukocytic antigen 1 (Jin et al., 2005; Solez et al., 2008). Mice models of heterotopic heart transplantation that reiterated the process of antibody-mediated rejection exploited a combination of BALB/C donor mice into wild-type (Rag knockout) recipient mice with further supplementation of anti-MHC I antibodies to the recipients (Jin et al., 2005). Such a model allowed a better study of antibody-mediated rejection mechanisms (Bedi et al., 2010).

10. Female Versus Male Transplantation

In relevance to our humanized mouse H-Y-peptide mismatch CAV model, we will highlight the impact of sex difference on transplantation rejection outcomes in this section.

In various scientific reviews and studies, interpreting results about sex differences concerning transplant survival was perplexing (Horvat et al., 2009; Jindal et al., 2005). However, it has become more apparent throughout research that sex-confined factors and gender mismatching represent significant determinants when arranging for transplantation. For example, in updated studies about kidney transplantation, the research teams observed a deteriorating transplantation rejection outcome in female recipient patients who received male kidneys, with an emphasis on sensitization and the H-Y male antigen impact (Candinas et al., 1995; Gratwohl et al., 2008; Kim & Gill, 2009; Prendergast et al., 1998). Further, the most effective transplantations entailed a male donor and a male recipient, while the worst transplantation outcomes correlated with a female donor to a male recipient (Kaczmarek et al., 2013; Naderi et al., 2020; Schoening et al., 2016). Moreover, male recipients showed a poorer prognosis than female recipients due to the decreased compliance to immune inhibition and, therefore, the allograft function and survival (Rosenberger et al., 2005). Indeed, the complexity of male and female donor-recipient

combinations with the H-Y antigen mismatch represents a significant modulator of the graft outcomes (McGee et al., 2010; Pfeffer & Thorsby, 1982; Scott et al., 1997; Tan et al., 2008; Zukowski et al., 2011).

Of interest, females demonstrated a high prevalence of early transplant loss versus less delayed graft deterioration, with age as a significant co-determinant for the transplantation outcomes in sex-mismatched young versus elderly patients (Meier-Kriesche et al., 2001). We can better interpret this correlation between the female sex and the early versus the late transplantation outcomes by observing the higher prevalence of HLA antibodies formed in female hosts in response to repeated pregnancies (early transplant loss) and the effect of sex hormones in females that might protect the graft in the long run (evident by adverse longterm graft outcomes in pediatric females before adulthood) (Momper et al., 2017). Animal studies of sex hormones, such as testosterone and estradiol, confirmed the above correlation (Muller et al., 1999). Furthermore, pediatric transplant patients showed a significant effect of sex difference, which is still different from adults, whereby the female sex predicts the graft outcomes as an independent element (Smith et al., 2013). This correlation underwent testing in a research cohort of pediatric kidney transplants, where females encountered higher risks than males concerning graft survival (Bobanga et al., 2015).

Most importantly, various research studies highlighted a high CAV prevalence in female-to-male transplants (Mehra et al., 1995; Sharples et al., 1991). However, the attributable factors for such a sex-confined causality for CAV progression were perplexing (Caforio et al., 2004; Erinc et al., 2004). Sex mismatch in organ transplantation also resulted in primary graft failure among male recipients (Avtaar Singh et al., 2019; Russo et al., 2010). Still, the size of the transplanted organs contributed to the sex-mismatched graft outcomes (Young et al., 2001), and some other studies

demonstrated comparable graft outcomes in sex-mismatched transplants when perfectly matching the size or the donor-recipient body mass index (Barac et al., 2021; Correia et al., 2014). Therefore, testing these interconnected factors via pre-clinical modelling associated with sex mismatch or the contribution of distinct sex of the donor versus the recipient to CAV outcomes is vital.

Chapter 2 : Extended Materials and Methods

1. Materials and methods for chapter III

1.1 Reagents

The antibodies used in this thesis are shown in Table 2.1. The streptavidin-biotin kit (VECTASTAIN® ABC kit) and the Impact 3,3′-Diaminobenzidine (DAB) peroxidase were from Vector Labs (cat # PK4000 and VECTSK4105, respectively). HY I-Ab tetramer with the peptide sequence: NAGFNSNRANSSRSS was synthesized by the NIH tetramer core facility (Emory University, Atlanta, GA; http://tetramer.yerkes.emory.edu). QuantiTect mouse primers (*Hprt1, Apln, Esm1, Ifng, Tnfa*) were purchased from Qiagen (cat # QT00166768, QT00111762, QT00111762, QT01038821, and QT00104006, respectively). Other primer sets (Integrated DNA Technologies) are shown in Table 2.2. Diphenyleneiodonium chloride was from Sigma (cat # 300260). EasySep human monocyte isolation kit was from Stem Cell Technologies (cat # 19059). CMF019 was from Aobious (cat # AOB8242).

Human umbilical vein endothelial cells were isolated and cultured as described previously (Mullaly et al., 2002) and under approval from the Human Research Ethics Board of the University of Alberta. The M199, DMEM, FBS, HBSS and endothelial cell growth supplements were from Invitrogen. Recombinant human TNF-α, VEGF-A165 and CXCL12 were from Peprotech (cat# 300-01A, 100-20 and 300-28A). Hiperfect, AllStars scrambled control siRNA (cat # SI03650318), and apelin receptor siRNA (cat # SI00073157) were from Qiagen. NMe-Apelin (17) (APLN-17) was synthesized as previously described (McKinnie et al., 2017) by Dr. J. Vederas at the University of Alberta. Where indicated, mice were treated with 1.5 μmol/ kg /day via intraperitoneal injection.

1.2 Mice and Heart Transplantation

Animal experiments were carried out according to the Canadian Council on Animal Care guidelines and were approved by the Animal Care and Use Committee at the University of Alberta. Wild-type male and female recipient C57Bl/6J mice were purchased from Jackson Laboratories (cat # 000664). The apelin-deficient (*Apln*$^{-/y}$) male and wild-type male littermate donor mice were bred on a C57Bl/6J background at the University of Alberta, as previously described (Kuba et al., 2007b). Mouse genotype was confirmed by end-point Polymerase Chain Reaction (PCR) and gel electrophoresis.

The mice underwent surgery at eleven to fourteen weeks of age. Donors' hearts were transplanted heterotopically to the abdomen of the female wild-type recipients under isoflurane anesthesia, as previously described (Russell et al., 2011). Briefly, the inferior and superior vena cava and the pulmonary vein of the donors' hearts were ligated. The donor aorta and pulmonary artery were anastomosed to the recipient's abdominal aorta and inferior vena cava below the renal arteries. The heart grafts were harvested two or six weeks after transplantation blindly.

1.3 Left Coronary Artery Micro-dissection

At the harvest time, the heart graft was fixed with two micro pins in a silicon-coated dish in saline and visualized with a ZEISS Stemi 2000 Microscope. The proximal left coronary and left anterior descending artery was isolated and retrieved without surrounding tissue using micro-dissecting scissor and forceps. The artery was placed immediately in 1 ml RNALater, snap-frozen in liquid nitrogen, and then stored at a -80°C freezer for later RNA extraction.

1.4 Histological Processing, Staining, and Imaging Analysis

The heart was sectioned in thirds. The heart base was processed for histological morphometric evaluation in Zinc fixative. The middle third was mounted in OCT medium for frozen sections.

The apex of the heart was preserved in RNALater. The base underwent paraffin embedding, sectioning, and staining. Five μm sections were taken at 100 μm levels of the myocardium and then stained using the Van Gieson elastin stain to identify the internal elastic lamina. Unstained slides were used for immunohistochemistry (IHC) or immunofluorescence (IF) staining on zinc-fixed paraffin-embedded or acetone-fixed frozen sections for CD31, F4/80, ESM1, and EGFL7, CD8a, CD4, CD3, Ym1, Mac-2 and apelin according to the manufacturer's recommendations. The negative controls for antibody specificity were done by omitting the primary antibody and showed no staining. Conventional images were taken using an Olympus BX53 microscope with a top-mounted Infinity camera and Infinity Analyzer software. IF images were taken using Leica DM IRB Microscope and Open Lab software.

To quantitate CAV lesions, regions of interest were drawn at the internal elastic lamina and the lumen of at least three coronary arteries in cross-section/ specimen using Image-J software. The mean fractional area of intima was calculated. Endothelial gaps as a fraction of the arterial lumen circumference, microvasculature quantitation using point grids, inflammatory cell enumeration, and the corrected total fluorescence of ESM1, Apelin, and EGFL7 were each done using Image-J.

1.5 Human Heart Transplant Tissue

Human heart tissue was obtained under protocols approved by the Health Research Ethics Board of the University of Alberta. Human heart samples were obtained from patients undergoing transplantation for end-stage heart disease (n=6 transplant vasculopathy, n=6 ischemic cardiomyopathy) at the Mazankowski Alberta Heart Institute (MAHI). Transplant vasculopathy myocardium and left coronary arterial specimens were from hearts transplanted for a median of 16.5 years (range 5-24 years). Non-failing control hearts were collected from six consecutive organ donors whose hearts were not used due to medical or technical issues. All myocardium

samples from the left ventricular free wall were collected, avoiding fibrotic areas and epicardial fat. The samples were snap-frozen in liquid nitrogen and stored at -80°C. Fourteen archival formalin-fixed paraffin-embedded (FFPE) right endomyocardial ventricular biopsies from human heart transplant patients were retrieved from the pathology archive at the University of Alberta Hospital. These included one biopsy each from nine patients with at least grade 1 CAV, diagnosed by coronary angiography and intravascular ultrasound (IVUS), and one biopsy each from five patients without evidence of CAV taken at least five years post-transplantation. Only the latest available biopsy per patient was used. Six additional FFPE right ventricular endomyocardial biopsies were also obtained from unutilized non-failing human donor hearts from individuals with non-cardiac causes of death. As previously described, we exploited 20 human endomyocardial biopsies for NanoString gene expression analysis (Adam et al., 2016). Briefly, three consecutive 20 μm sections were obtained from each FFPE block, and RNA was isolated using the RNAeasy FFPE Kit (Qiagen). RNA concentration and purity were measured with a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific). Oligonucleotide probes for four endothelial tip cell-related genes (*APLN*, *EGFL7*, *ESM1*, *PDGFB*) and three housekeeping genes (*LDHA*, *HPRT1*, *GAPDH*) were manufactured (Integrated DNA Technologies). Gene expression was then quantified using the NanoString nCounter system (Nano String Technologies). Quality control assessment and data normalization were performed using nSolver Analysis Software Version 4.0 (NanoString Technologies).

1.6 qRT-PCR

The mouse heart apex, micro-dissected coronary artery, or human heart explant myocardium specimens were placed in an RNase-free tube in 1ml cold Trizol (ThermoFisher). The samples were homogenized using a TissueLyser 2 (Qiagen) for 3 minutes at a frequency of 33

cycles/second. According to the manufacturer's protocol, the RNA was extracted from the homogenates using the RNAeasy micro-Kit (Qiagen). A total of 300 ng of RNA were used to synthesize cDNA with a QuantiTect Reverse Transcription Kit (Qiagen). The primers for human and mouse genes are listed in Table 2.2. qRT-PCR was done using a 7500 ABI Thermocycler. The final components of each sample reaction were as follows: 1 µl of cDNA, one µl of 10 µM QuantiTect mouse / human primer sets and ten µl of SYBR(R) Select master mix (Applied Biosystems) in a total volume of 20 µl. Samples were normalized to the internal control (mouse Hprt1 or human GAPDH) and then to normal mouse heart samples or discarded human donor hearts. Fold changes were calculated based on the $2^{-\Delta\Delta CT}$ method.

1.7 Flow Cytometry

Splenocytes were harvested in some experiments for tetramer staining. Recipient cells were stained after incubation with an FcR block. Fluorophore-labelled antibodies against mouse TCR-β chain (TCRβ; H57–597), CD4 (GK1.5), CD8β (53–6.7), CD45R (B220; RA3–6B2) were used.

To identify alloimmune lymphocytes by tetramer staining, $1x10^6$ cells from the spleen were reconstituted in a 50 µL complete DMEM culture medium and incubated with a 50 µL Fc block cocktail for 15 minutes at room temperature. The cell suspension was then stained with 42 µg/mL PE-labelled I-Ab-HY tetramers (cat#34784; NAGFNSNRANSSRSS) or I-Ab -hCLIP tetramers (cat#34785; PVSKMRMATPLLMQA) for 3 hours at 37°C with 5% CO_2 in the incubator. Cells were subsequently stained for surface markers, i.e.CD4 (RM4-5), CD8β (53–6.7), B220 (RA3-6B2), CD45.1 (A20), and CD44 (IM7), for 30 minutes at room temperature in the dark. A Live/dead fixable yellow dead cell stain kit (cat# L34959, ThermoFisher) excluded dead cells. Tetramer and intracellular cytokine staining: spleen cells were enriched for CD4 T cells using the EasySep™ mouse $CD4^+$ T Cell Isolation kit (Stemcell Technologies, cat#19752). Enriched

populations were then reconstituted in a 50 µL complete DMEM culture medium and incubated with a 50 µL Fc block cocktail at 37°C with 5% CO_2 in the incubator with Phorbol Myristate Acetate (PMA, 20 ng/ml, to activate protein kinase C) plus ionomycin (1µg/ml, a calcium ionophore) for 5 hours in total to induce cytokine production. Brefeldin A (3µg/mL, a potent cytokine release inhibitor) and Monencin (2 µM, a cytokine release inhibitor) were added 1 hour after PMA/ionomycin. The mixture was then stained with 42 µg/mL tetramers in the incubator for the last 3 hours. Cells were subsequently stained for surface markers for 30 minutes at room temperature in the dark. Dead cells were excluded by using the live/dead fixable yellow dead cell stain kit (through interacting with free amines intracellularly and on the surface of cells, to yield fluorescent reactions with different reactivity according to the generated free amines reaction's localization on the cell surface in viable cells compared to dead cells with less fluorescent intensity. Cells were then fixed and permeabilized with eBioscience IC fixation (cat# 00-8222-49) and permeabilization buffer (cat# 00-8222) and stained with antibodies for IFN-γ (XMG1.2). Fc block cocktail was a mixture of 3 mL each of normal mouse, rat, and hamster serum, with 0.3 mg of anti-CD16/32 antibody (clone 2.4g2, BioXCell). Complete DMEM contains: Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1mM MEM Sodium Pyruvate, 100 µM non-essential amino acids, 100 units/mL penicillin, 100 units/mL streptomycin, 50 µM 2-Mercaptoethanol. All fluorophore-labelled antibodies for flow cytometry, except tetramers, were purchased from eBioscience. BD LSR II (BD Biosciences) was used for the data acquisition. Flow cytometric data analysis was performed using FlowJo (Treestar software). The gating strategy is shown in Fig 3.16.

1.8 Cell Culture

Human Umbilical Vein Endothelial Cells (HUVECs) were isolated as previously described and

used below passage 5 (Mullaly et al., 2002). According to the manufacturer's protocol, human peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood of healthy volunteers using Lymphoprep. The mononuclear cell fraction was harvested, washed, and treated with RBC lysis buffer (Invitrogen, 00-4333-57). Monocytes were isolated by negative selection (Stem Cell Technologies cat# 19059). The purity of the isolated monocyte population was > 95% by flow cytometry.

1.9 Monocyte Adhesion Assay

Confluent HUVEC monolayers were pre-treated with TNF-α (100 ng/mL) for 18 hours, then human monocytes were added in a 1:10 ratio. As indicated, the co-cultures were mock-treated or treated with an APLN-17 receptor agonist (1uM) or DPE NOS3 inhibitor (180 nM). The wells were washed twice with warm M199, and adherent monocytes in 5 random fields/ well were counted in each experiment.

1.10 Scratch (Wound-healing) Assay

HUVEC were transfected with the apelin receptor or scrambled siRNA, or mock-transfected, then plated at a high confluence. The HUVEC monolayers were wounded with a 200-µl-pipette tip, then cultured at 37°C in M199/ 2%FBS with VEGF-A165 (50 ng/mL), or APLN-17 receptor agonist alone (1 µM) or in combination as indicated. Serial images were captured at 0, 4, 8 or 10 hours in each experiment. Quantitation of the wound area was done using Image J software.

1.11 RNA Interference

HUVECs were seeded in a six well-plate at 50-60% confluence, then transfected twice over two days with either 50 nM non-specific small interfering RNA (siNS) or specific apelin receptor siRNA using Hiperfect per the manufacturer’s protocol. Cells were studied two days following the second transfection.

1.12 Western Blots

Cell monolayers were washed once with ice-cold 1x PBS then lysed using ice-cold RIPA buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 2 mM Na3VO4, 0.1% SDS, 0.5% Na deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM PMSF), and heated at 95°C for 5 minutes. Proteins were separated on SDS-PAGE, transferred to a nitrocellulose membrane (Biorad), and blocked with 5% skimmed milk in 1x TBS + 0.1% Tween 20. The membranes were immunoblotted overnight at 4°C with the desired antibodies (diluted in 5% BSA), washed three times in 1x TBST, incubated with the secondary antibodies (diluted in 5% milk), and washed three times in 1x TBST, then visualized using Odyssey infrared scanner (Licor). Protein bands were equally contrast-enhanced with Adobe Photoshop CS3 and quantified with Image Studio Lite software.

1.13 Statistical Analysis

Datasets were tested for normality by the D'Agostino & Pearson test using Prism 7 software (Graphpad). Data are presented as mean ± the standard error of the mean (SEM). Using Prism 7 software, statistical analysis was conducted using appropriate 1-way or 2-way ANOVA according to the number of variables tested, followed by the Bonferroni post hoc test. Pairwise comparisons were performed by unpaired two-sided Student t-test, or in the case of the smaller datasets, the two-sided Mann-Whitney test as indicated.

2. Materials and methods for chapter IV

2.1 Reagents

The antibodies used are shown in Table 2.3. Biotinylated secondary antibodies were from Vector Labs (cat # BA-9401), Alexa fluor-tagged highly cross-adsorbed secondary antibodies were from Invitrogen (cat # A11006, A11036, and A-11031), IRDy® 800CW donkey anti-rabbit IgG and

IRD® 680RD donkey anti-mouse IgG secondary antibodies for western blot were from Li-Cor (cat # 926-32213 & 926-68072). The ABC kit and Impact DAB peroxidase were from Vector Labs (cat # PK4000 & VECTSK4105). Class II tetramers I-A^b mouse H-Y peptide NAGFNSNRANSSRSS, I-A^b human CLIP 87-101 peptide PVSKMRMATPLLMQA, and class I tetramers H-$2D^b$ mouse H-Y (Uty) 246-254 WMHHNMDLI were synthesized by the NIH tetramer core facility (Emory University, Atlanta, GA; http://tetramer.yerkes.emory.edu). Purchased pre-designed or custom primer sets (Integrated DNA Technologies) are shown in Table 2.2. EasySep™ mouse CD45 positive selection kit (cat #18945) and EasySep™ PE-positive selection kit II (cat #17684) were from StemCell Technologies.

Recombinant human TNF-α and murine TNF-α were from Peprotech (cat # 300-01A, 315-01A). Hiperfect, AllStars scrambled control siRNA (cat # SI03650318), and *PIK3CB* siRNA (cat # SI02622214) were from Qiagen.

Selective PI3 kinase-beta inhibitor GSK2636771 (Selleckchem; cat # S8002) was dissolved in 0.5% Methylcellulose with 0.2% Tween 80 (Sigma Aldrich; cat # M7140 & P1754) and administered to mice at 30 mg/kg/day by daily gavage for four weeks starting at week two till week six post-transplantation (Costa et al., 2015). TGX221 (cat # 663619-89-4) was from Cayman Chemical.

2.2 Mice and Heart Transplantation

Animal experiments were carried out according to the Canadian Council on Animal Care guidelines and were approved by the Animal Care and Use Committee at the University of Alberta. Wild-type male and female recipient C57Bl/6 mice were purchased from Jackson Laboratories (cat # 000664). The *Pik3cb*-deficient (Tie2-Cre^{ERT2}/ $Pik3cb^{flx/flx}$) and wild-type ($Pik3cb^{flx/flx}$) male littermate donor mice were bred on a C57Bl/6 background at the University of

Alberta as previously described (Haddad, Zhabyeyev, Farhan, Zhu, Rayner, et al., 2014). Donor mice were treated with tamoxifen as described two weeks before heart harvest for transplantation. Wild-type or mutant *Pik3cb* expression was confirmed by end-point PCR of the lung (Fig 4.8).

The mice underwent surgery at 11-14 weeks of age. Donors' hearts were transplanted heterotopically to the abdomen of the female wild-type recipients under isoflurane anesthesia, as previously described (Russell et al., 2011). Briefly, the inferior and superior vena cava and the pulmonary vein of the donors' hearts were ligated. The donor aorta and pulmonary artery were anastomosed to the recipient's abdominal aorta and inferior vena cava below the renal arteries. The donor's heartbeat was assessed daily and continued to beat in these experiments until the recipient was euthanized. The heart grafts were harvested per protocol in a blinded fashion two or six weeks after transplantation.

2.3 Left Coronary Artery Micro-dissection

At the harvest time, the heart graft was fixed with two micro pins in a silicon-coated dish in saline and visualized with a Zeiss Stemi 2000 microscope. The proximal left coronary and left anterior descending arteries were isolated and retrieved using micro-dissecting scissors and forceps without surrounding tissue. The artery was placed immediately in 1 ml RNALater, frozen in liquid nitrogen, then stored at -80°C for later RNA extraction.

2.4 Histological Processing and Staining

The hearts were sectioned in thirds. The heart base was processed for histological morphometric evaluation in Zn fixative. The middle third was further divided into two halves: one half was snap-frozen in liquid nitrogen and stored at -80ºC for further western blot analysis and the second half was preserved and stored in glutaraldehyde. The apex of the heart was preserved in RNALater. The base underwent paraffin embedding, sectioning, and staining. Five μm sections

were taken at 100 μm levels and then stained using the van Gieson elastin stain to identify the internal elastic lamina. Paraffin-embedded tissue sections were used at each level for immunohistochemistry (IHC) or immunofluorescence (IF) staining for CD31, CD8a, Mac-2, aCasp3, αSMA, and CX3CL1/Fractalkine according to the manufacturer's recommendations. The negative controls for antibody specificity were done by omitting the primary antibody and showed no staining. Conventional images were taken using an Olympus BX53 microscope with a top-mounted Infinity camera and Infinity Analyzer software. IF images were taken using Leica DM IRB Microscope and Open Lab software. Confocal microscopy images were taken with a WaveFX microscope (Quorum Technologies) with Olympus IX-81 motorized base and Yokagawa CSU 10 spinning disk confocal scan-head.

To quantitate arterial CAV lesions, regions of interest were drawn at the internal elastic lamina and the lumen, the intima, of at least three coronary arteries in cross-section/ specimen using Image-J software. The mean fractional area of the intima/ the area bounded by the internal elastic lamina was calculated. As a fraction of the arterial lumen circumference, endothelial gaps were determined on CD31-stained sections; microvascular density was determined using point-grids on CD31-stained sections; inflammatory cell enumeration was done on CD8 or Mac-2-stained sections and quantified using Image-J. Manders pixel co-localization index quantification and nuclear mean fluorescent intensity of NFkB-p65 were done using the Coloc2 plugin and binary-overlay particle analysis in Fiji software. At least three images per section were analyzed for co-localization, and 30 nuclei per experimental group (n=4 biological replicates) were quantified for nuclear NFkB-p65 staining.

2.5 qRT-PCR

The mouse heart apex, micro-dissected coronary artery, or human heart explant myocardium

specimens were placed in an RNase-free tube in 1ml cold Trizol (ThermoFisher). The samples were homogenized using a TissueLyser 2 (Qiagen) for 3 minutes at a frequency of 33 cycles/second. RNA was extracted from the homogenates using the RNAeasy micro-Kit (Qiagen) according to the manufacturer's protocol. A total of 300 ng of RNA were used to synthesize cDNA with a QuantiTect Reverse Transcription Kit (Qiagen). The primers for human and mouse genes are listed in Table 2.4. qRT-PCR was done using an Eppendorf Master cycler RealPlex2 thermocycler. The final components of each sample reaction were as follows: 1 µl of cDNA, one µl of 10 µM QuantiTect mouse / human primer sets and ten µl of SYBR(R) Select master mix (Applied Biosystems) in a total volume of 20 µl. Samples were normalized to the internal control (mouse *Hprt1* or Human *HPRT*) and then to normal mouse heart samples or untreated ECs *in vitro*. Fold changes were calculated based on the 2−ΔΔCT method.

2.6 Mouse heart Allograft EC Isolation

As previously described (Kalucka et al., 2020), the heart allografts were harvested, rinsed with ice-cold PBS and dissected into small pieces using sterile scalpels and micro scissors. All pieces from one animal were transferred into a sterile Eppendorf tube containing digestion medium (KnockOut™ DMEM, Thermo Fisher Scientific, cat # 10829018), 1x penicillin/streptomycin (Thermo Fisher Scientific, cat # 15140122), 2x Antibiotic-Antimycotic (Thermo Fisher Scientific, cat # 15240062), one mM sodium pyruvate (Thermo Fisher Scientific, cat # 1360070), 1x MEM non-essential amino acids solution (Thermo Fisher Scientific, cat # 11140035) supplemented with 0.1% collagenase II (Thermo Fisher Scientific, Cat #17101015), 0.25% collagenase IV (Worthington, Cat #LS004188) and 7.5 µg/mL DNase I (Sigma-Aldrich, cat # D4527-10KU). Samples were incubated in a 37°C water bath for 30 minutes with manual mixing by inversion of the suspension every 10 minutes. Next, the cell suspension was filtered through a

100 μm cell strainer (Sigma-Aldrich, cat # CLS431752) and rinsed with a PBS-based wash buffer (0.5% BSA, Sigma-Aldrich, cat # 10735096001; 2 mM EDTA in PBS, Thermo Fisher Scientific, cat # 14190-094). The cell suspension was centrifuged at 350g for 5 minutes. The supernatant was transferred to a fresh falcon tube, and the pellet was resuspended in wash buffer and filtered through a 40 μm cell strainer (Sigma-Aldrich, cat # CLS431750). Both fractions were centrifuged again at 350g for 5 minutes. The pellets were resuspended in wash buffer, pooled, and the cell suspension was filtered again through the 40 μm cell strainer. The washing steps were repeated once more. Next, the cell suspension was treated with RBC lysis buffer (Invitrogen, cat # 00-4333-57) according to the manufacturer's instructions and enriched for ECs using the EasySep™ PE positive selection kit II (StemCell Technologies, cat # 17684) and CD31 (PECAM-1) monoclonal antibody (390) PE-conjugate (eBioscience, cat # 12-0311-83). MHEC were cultured in complete mouse EC media with VEGF kit–500 ML (Cell Biologics M1168).

2.7 Flow Cytometry

Isolated allograft cells were enriched for CD45 cells by using EasySep™ Mouse CD45 Positive Selection Kit (Stemcell Technologies, cat # 18945). Enriched populations (0.75-1.5x10^5 cells) were then reconstituted in a 50 μL complete DMEM culture medium. To identify alloreactive immune lymphocytes by tetramer staining, 1x10^6 splenocytes or 0.75-1.5x10^5 allograft infiltrating $CD45^+$ cells were reconstituted in a 50 μL complete DMEM culture medium followed by incubation with 50 μL Fc block cocktail (a mixture of 3 mL each of normal mouse, rat, and hamster serum, with the addition of 0.3 mg of anti-CD16/32 antibody (clone 2.4g2, BioXCell)) for 15 minutes at room temperature. The cell suspension was then stained with PE-labelled I-A^b-HY tetramers (NAGFNSNRANSSRSS; 42 μg/mL) or I-A^b -hCLIP tetramers (PVSKMRMATPLLMQA; 42 μg/mL), or H-2D^b-Uty (WMHHNMDLI; 6.5 μg/mL) for 3 hours

at 37°C with 5% CO_2 in the incubator. Cells were subsequently stained for surface markers, i.e. CD4 (RM4-5), CD8β (53–6.7), CD19 (6D5), CD25 (PC61), CD27 (LG.7F9), CD44 (IM7), CD223 (LAG-3, C9B7W), CD279 (PD-1, 29F.1A12) and CD366 (TIM3, RMT3-23) for 30 minutes at room temperature in the dark. A Live/dead fixable yellow dead cell stain kit (cat # L34959, ThermoFisher) excluded dead cells. For FoxP3 staining, cells were permeabilized with Permeabilization Buffer Set (cat. no. 88-8824, ThermoFisher) and fixed with Foxp3/Transcription Factor Staining Buffer Set (cat # 00-5523-00, ThermoFisher) and stained with FoxP3 antibody (FJK-16s). For intracellular cytokine staining, after FcR blockade, cells were incubated at 37°C with 5% CO_2 in the incubator with PMA (20 ng/ml) plus ionomycin (1 μg/ml) for 5 hours. Brefeldin A (3 μg/mL) and monencin (2 μM) were added 1 hour after PMA/ionomycin. The mixture was then stained with the designated tetramers for the last 3 hours in the incubator. Cells were subsequently stained for surface markers for 30 minutes at room temperature in the dark. Dead cells were excluded by using the live/dead fixable yellow dead cell stain kit. Cells were then fixed and permeabilized with eBioscience IC fixation (cat # 00-8222-49) and permeabilization buffer (cat # 00-8222) and stained with antibodies for IFN-γ (XMG1.2), TNF-α (MP6-XT22), perforin (eBioOMAK-D), or granzyme B (NGZB). A BD LSR II (BD Biosciences) was used for data acquisition. Flow cytometric data analysis was performed using FlowJo (Treestar software).

2.8 Cell Culture

Human Umbilical Vein Endothelial Cells (HUVECs) were isolated and cultured as previously described under approval from the Human Research Ethics Board of the University of Alberta (Mullaly et al., 2002). Human Aortic Endothelial Cells (HAEC) and the complete human EC medium were from Cell Biologics (cat # H6052 & H1168). M199, DMEM, FBS, HBSS and

endothelial cell growth supplement were from Invitrogen. Mouse heart endothelial cells (MHECs) were isolated using an EasySep™ PE positive selection kit II (StemCell Technologies, cat # 17684) after enrichment with the CD31 (PECAM-1) monoclonal antibody 390-PE conjugate (eBioscience, cat # 12-0311-83), then cultured in complete mouse endothelial cell medium (Cell Biologics, cat # M1168).

2.9 Endothelial Cell Adhesion Molecule Expression

Human or murine recombinant TNF-α (10 ng/ml) was used to treat primary HUVECs or MHECs, respectively, to induce ICAM-1 (12 hours) and VCAM-1 (24 hours) expression with the PI3 kinase-b selective inhibitor TGX221 (100 nM) or mock treatment, then mRNA or protein expression was analyzed.

2.10 IκBα Degradation

Murine recombinant TNF-α (10 ng/ml) was used to stimulate mock- or TGX221 (100 nM; 1 hour) pre-treated primary Mouse Heart Endothelial Cells (MHECs) as indicated for IkB alpha western blot.

2.11 NF-κB P65 Nuclear Translocation

Human or murine recombinant TNF-α (10 ng/ml) stimulated primary HAECs or MHECs respectively for 30 minutes ± TGX221 (100 nM). Endothelial cells were immunostained, and NF-kB p65 (RelA) fluorescence in the nucleus, marked by DAPI, was quantified using Image-J.

2.12 RNA Interference

Endothelial cells were seeded in a six well-plate at 50-60% confluence, then transfected serially over two days with either 50 nM non-specific small interfering RNA (siNS) or specific PI3K-β (PIK3CB) siRNA using Hiperfect per the manufacturer's protocol. Cell lysates were studied after

TNF-α stimulation for 12 or 24 hours.

2.13 Western Blots

Cell monolayers were washed once with ice-cold 1x PBS then lysed using ice-cold RIPA buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 2 mM Na3VO4, 0.1% SDS, 0.5% Na deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM PMSF), and boiled at 95°C for 5 minutes. Proteins were separated on SDS-PAGE, transferred to a nitrocellulose membrane (Biorad), and blocked with 5% skimmed milk in 1x TBS + 0.1% Tween 20. The membranes were immunoblotted overnight at 4°C with the desired antibodies (diluted in 5% BSA), washed three times in 1x TBST, incubated with the secondary antibodies (diluted in 5% milk), and washed three times in 1x TBST, then visualized using an Odyssey infrared scanner (Licor). Gels were equally contrast-enhanced with Adobe Photoshop CS3 and quantified with Image Studio Lite software.

2.14 Statistical Analysis

Datasets were tested for normality by the D'Agostino test using Prism 7 software (Graphpad). Data are presented as mean ± the standard error of the mean (SEM). Using Prism 7 software, statistical analysis was conducted using appropriate 1-way or 2-way ANOVA, followed by the Bonferroni post hoc test. Pairwise comparisons were performed by unpaired two-sided Student t-test or, in the case of the smaller datasets, the two-sided Mann-Whitney test. A P value less than 0.05 was considered significant.

2.15 Study Approval

Animal experiments were carried out according to the Canadian Council on Animal Care guidelines and were approved by the Animal Care and Use Committee at the University of

Alberta. Human tissue was obtained with the approval of the Human Research Ethics Board at the University of Alberta.

3. Materials and methods for chapter V

3.1 Reagents

Primary antibodies were as follows: Anti-CD31 (PECAM1) mouse antibody was from Dianova (DIA-310). The used anti-CD8a antibody was from eBioscience (14-0808-82). Anti-Mac-2 for type 1 macrophage detection was from Cedarlane (CL8942AP). Biotinylated goat anti-rat IgG (H+L) secondary antibody was from VectorLabs (BA-9401). The ABC kit and Impact DAB peroxidase were from Vector Labs (cat # PK4000 & VECTSK4105).

3.2 Mice and Heart Transplantation

Animal experiments were carried out according to the Canadian Council on Animal Care guidelines and were approved by the Animal Care and Use Committee at the University of Alberta. Wild-type male and female recipient C57Bl/6 mice were purchased from Jackson Laboratories (cat # 000664).

The mice underwent surgery at 11-14 weeks of age. Donors' hearts were transplanted heterotopically to the abdomen of the female wild-type recipients under isoflurane anesthesia, as previously described (Russell et al., 2011). Briefly, the inferior and superior vena cava and the pulmonary vein of the donors' hearts were ligated. The donor aorta and pulmonary artery were anastomosed to the recipient's abdominal aorta and inferior vena cava below the renal arteries. The heart grafts were harvested six weeks after transplantation in a blinded fashion.

3.3 Sacubitril Oral Gavage

Sacubitril hemicalcium salts (Selleckchem, S4426) were dissolved in 0.5% Methylcellulose with

0.2% Tween 80 (Sigma Aldrich; cat # M7140 & P1754) and administered to mice at 28.8 mg/kg/day by daily gavage for four weeks starting at week two till week six post-transplantation (Li et al., 2020).

3.4 Histological Processing and Staining

The hearts were sectioned in thirds. The heart base was processed for histological morphometric evaluation in Zn fixative. The middle third was further divided into two halves: one half was snap-frozen in liquid nitrogen and stored at -80ºC for further western blot analysis and the second half was preserved and stored in glutaraldehyde. The apex of the heart was preserved in RNALater. The base underwent paraffin embedding, sectioning, and staining. Five μm sections were taken at 100 μm levels and then stained using the van Gieson elastin stain to identify the internal elastic lamina. According to the manufacturer's recommendations, paraffin-embedded tissue sections were used at each level for immunohistochemistry (IHC) staining for CD31, CD8a, and Mac-2. The negative controls for antibody specificity were done by omitting the primary antibody and showed no staining. Conventional images were taken using an Olympus BX53 microscope with a top-mounted Infinity camera and Infinity Analyzer software.

To quantitate arterial CAV lesions, regions of interest were drawn at the internal elastic lamina and the lumen, the intima, of at least three coronary arteries in cross-section/ specimen using Image-J software. The mean fractional area of the intima/ the area bounded by the internal elastic lamina was calculated. On CD31-stained sections, microvascular density was determined using point-grids; inflammatory cell enumeration was done on CD8 or Mac-2-stained sections and quantified using Image-J. At least three images per section have been analyzed for intimal thickening, microvascular density, s well as inflammatory immune-cell infiltrate quantification.

3.5 Statistical Analysis

Datasets were tested for normality by the D'Agostino & Pearson test using Prism 7 software (Graphpad). Data are presented as mean ± the standard error of the mean (SEM). Using Prism 7 software, pairwise comparisons were performed by unpaired two-sided Student t-test, or in the case of the smaller datasets, the two-sided Mann-Whitney test as indicated.

3.6 Study Approval

Animal experiments were carried out according to the Canadian Council on Animal Care guidelines and were approved by the Animal Care and Use Committee at the University of Alberta. Human tissue was obtained with the approval of the Human Research Ethics Board at the University of Alberta.

Table 2.1: Names and sources for antibodies in Chapter III

Primary Antibodies (Supplier Catalogue #)	Secondary Antibodies (Supplier Catalogue #)
CD31-Mouse (Dianova-DIA310)	Biotinylated Goat anti Rat / Rabbit & Mouse IgG (H+L) (Vector Labs BA-9401, BA-1000, and BA-9200, respectively)
CD31-Human (Dako GA610)	Goat anti Rat (H+L) Cross-Adsorbed Alexa Fluor 488 (ThermoFisher: A-11036)
CD4 (BD Pharmingen 550280)	Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody Alexa Fluor 568 (ThermoFisher: A-11057)

CD8a (eBioscience 14-0808-82)	Donkey anti-Rat IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (ThermoFisher: A-21208)
CD3 & CD45 (Biolegend 100201 & 103101)	
Apelin (Santa Cruz sc33469)	
Mac-2 (Cedarlane CL8942AP)	
Ym1 (StemCell: 60130)	
ApelinR (APJ) (Phoenix H-001-79)	
Esm-1 (MyBiosource MBS2006250)	
EGFL7 (BIOMATIK CAU21428)	
F4/80 (Biolegend 123102)	
CD14 & CD16 (StemCell 60004AZ & 60041PE)	
T-AKT, P-AKT^{473}, T-eNOS, P-eNOS^{1177}, and βActin (Cell signalling 4691, 4060, 5880, 9571, and 4970/3700)	
Rabbit Anti-ESM1 polyclonal antibody (Causabi: CSB-PA007825LA01HU)	
Anti-VE-Cadherin Antibody (Sinobiological 50192-T56)	
Anti-CD34 Monoclonal Antibody (ThermoFisher: QBEND/10, MAI-10202)	

Anti-human Apelin polyclonal antibody (Abcam: ab59469)	
Anti-Fibrinogen antibody (Dako: A008002-2)	

Table 2.2: PCR primers for Chapter III

Mouse primers

Esm1	F: 5' AGCGAGGAGGATGATTTTGGT 3' R: 5' TGCATTCCATCCCGAAGGT 3'
Apln	F: 5' TAGCCCCTGACACTGGTTGTC 3' R: 5' TTCTCCATCCCCCAAAAGC 3'
Pdgfb	F: 5' CCCTCGGCCTGTGACTAGAA 3' R:5'AATGGTCACCCGAGCTTGAG3'
Pecam1	F: 5' AGGACGATGCGATGGTGTATAA 3' R: 5' AAGACCCGAGCCTGAGGAA 3'
Tnfa	F: 5' ATGATCCGCGACGTGGAA 3' R: 5' TAGGCACCGCCTGGAGTTC 3'
Vegfa	F: 5' GCAGGCTGCTGTAACGATGA 3' R: 5' TCCGCATGATCTGCATGGT 3'
Cxcl11	F: 5' GGGCCGATGCAAAGACA 3' R: 5'GAGATGAACAGGAAGGTCACAG 3'

Human primers

APLN	F: 5' CCCATGCCCACATATTGCA 3' R: 5' TCAGTTTGAGGCCACTTGACCTA 3'
PECAM	F: 5' AGTGGAGTCCAGCCGCATAT 3' R: 5' CAGTTCGGGCTTGGAAAATAGT 3'
PDGFB	F: 5' AGATCGAGATTGTGCGGAAGA 3' R: 5' GCTGCCACTGTCTCACACTTG 3'
GAPDH	F: 5' GATTCCACCCATGGCAAATT 3' R: 5 TGATGGGATTTGCATTGATGAC 3'
ESM1	F: 5' GGTGGACTGCCCTCAACACT 3' R: 5' GTCGTCGAGCACTGTCCTCTT 3'

Table 2.3: Names and sources for primary antibodies in Chapter IV

Target	**Cat #**	**Company**	**Application**	**Dilution**
CD31	DIA-310	Dianova	IHC & IF	1/20
αSMA	A5228	SigmaAldrich	IF	1/200
aCasp3	NB100-56113	Novus Biologicals	IF	1/100
P-AKT (Ser473)	9271	Cell Signaling	WB	1/1000
T-AKT	2920	Cell Signaling	WB	1/1000
β Actin	4970 & 3700	Cell Signaling	WB	1/1000
VCAM-1	13662	Cell Signaling	WB	1/1000
ICAM-1	67836	Cell Signaling	WB	1/1000

PE-labeled CD31	12-0311-83	Invitrogen	EC isolation	1/100
Granzyme B	25-8898-82 & MHGB05	eBioscience & Invitrogen	Flowcytometry	1/100
Perforin	11-9392-82	eBioscience	Flowcytometry	1/100
TNF alpha	17-7321-82	eBioscience	Flowcytometry	1/100
IFN-γ	564336	BD Biosciences	Flowcytometry	1/100
CD279 (PD-1)	135231	Biolegend	Flowcytometry	1/100
CD19	115538	Biolegend	Flowcytometry	1/100
CD27	11-0271-82	eBioscience	Flowcytometry	1/100
CD366 (TIM3)	25-5870-82	eBioscience	Flowcytometry	1/100
CD223 (LAG-3)	17-2231-82	eBioscience	Flowcytometry	1/100
CD44	56-0441-82	eBioscience	Flowcytometry	1/100
CD8b	47-0083-82	eBioscience	Flowcytometry	1/100
CD4	46-0041-82	eBioscience	Flowcytometry	1/100
CD19	115538	Biolegend	Flowcytometry	1/100
ICAM-1 Recombinant Rabbit Monoclonal Antibody (9H21L19)	701254	ThermoFisher	IF	1/100
Human CX3CL1/Fractalkine Chemokine Domain Antibody	MAB3651-100	R & D Systems	IF and WB	1/200 & 1/1000

VCAM-1 (D2T4N) Rabbit mAb (Mouse Specific)	#32653	Cell Signaling	WB	1/1000
Monoclonal Anti-β-Actin antibody produced in mouse	A2228	Sigma Aldrich	WB	1/5000
NF-κB p65 (Ser536) (93H1) Rabbit mAb	#3033	Cell Signaling	ICC	1/300
Total IκBα Antibody	#9242	Cell Signaling	WB	1/1000

Table 2.4: PCR primers for Chapter IV

Mouse primers

Pik3cb ***Exon 16-24***	5'-CACTCCTGCTGTGTCCGTACA-3' 5'-TCAGTGCTTCCTCCTCGCTCT-3'
Hprt	5'-CCCCAAAATGGTTAAGGTTGC-3' 5'-AACAAAGTCTGGCCTGTATCC-3'
Esm1	5'-TGGTGACGAGTTTGGTATCTG-3'

	5’-GTCACCCTGTCACATATGCC-3’
Pdgfb	5’-TGAGGAACTGTATGAAATGCTGA-3’ 5’-GTCATGTTCAAGTCCAGCTCCA-3’
Vegfa	5’-CCGAAACCATGAACTTTCTGC-3’ 5’-GACTTCTGCTCTCCTTCTGTC-3’
Pecam1	5’-TCATTGGAGTGGTCATCGC-3’ 5’-TGTTGGAGTTCAGAAGTGGAG-3’
Ifng	5’-CTGAGACAATGAACGCTACACA-3’ 5’-TCCACATCTATGCCACTTGAG-3’
Tnfa	5’-AGACCCTCACACTCAGATCA-3’ 5’-TCTTTGAGATCCATGCCGTTG-3’
Apln	5’- AGGCATAGCGTCCTCACCTCTT-3’ 5’-GGTGCAGAAACGACAAAGACGG-3’
Cxcl11	5’- CCGAGTAACGGCTGCGACAAAG-3’ 5’- CCTGCATTATGAGGCGAGCTTG-3’
Il6	5’-TACCACTTCACAAGTCGGAGGC-3’ 5’- CTGCAAGTGCATCATCGTTGTTC-3’

Human primers

HPRT1	5’-TTGTTGTAGGATATGCCCTTGA-3’ 5’-GCGATGTCAATAGGACTCCAG-3’
VCAM1	5’-TGACTCCGTCTCATTGACTTG-3’

	5’-ACTTGACTGTGATCGGCTT-3’
CX3CL1	5’CTTCTGCCATCTGACTGTCC-3’ 5’-TGCCTGGTTCTGTTGATAGTG-3’
CXCL10	5′-GTGAGAATGAGGGCCATAGG-3′ 5′-GGCTAAACGCTTTCATTAAATTC-3′
CD74	5’-CTGAGACACCTTAAGAACACCA-3’ 5’-TGGCACTTGGTCAGTACTTTC-3’
ICAM1	5’-CCCGAGCTCAAGTGTCTAAAGG-3’ 5’-CAAGATCTCGAGTGACAGTCACTG-3’

Chapter 3 : Apelin Directs Endothelial Cell Differentiation and Vascular Repair Following Immune-mediated Injury

Abstract

Sustained, indolent immune injury of the vasculature of a heart transplant limits long-term graft and recipient survival. This injury is mitigated by a poorly characterized, maladaptive repair response. Vascular endothelial cells respond to pro-angiogenic cues in the embryo by differentiation to specialized phenotypes associated with the expression of apelin. In the adult, the role of developmental pro-angiogenic cues in the repair of the established vasculature is largely unknown. We found human and minor histocompatibility mismatched donor mouse heart allografts with alloimmune-mediated vasculopathy upregulated expression of apelin in arteries and myocardial microvessels. In vivo, loss of donor heart expression of apelin facilitated graft immune-cell infiltration, blunted vascular repair, and worsened occlusive vasculopathy in mice. In vitro, an apelin receptor agonist analogue elicited endothelial nitric oxide synthase activation to promote endothelial monolayer wound repair and reduce immune cell adhesion. Thus, apelin acted as an autocrine growth cue to sustain vascular repair and mitigate the effects of the immune injury. Treatment with an apelin receptor agonist after vasculopathy was established markedly reduced the progression of arterial occlusion in mice. Together, these initial data identify pro-angiogenic apelin as a key mediator of coronary vascular repair and a pharmacotherapeutic target for immune-mediated injury of the coronary vasculature.

Introduction

Heart transplantation is the most effective treatment of end-stage heart failure to prolong life. Modern immune-suppression regimens blunt the directed alloreactive immune responses against the transplant but are nevertheless associated with appreciable rates of early acute cellular

rejection and late chronic cell- and antibody-dependent graft injury (Lund et al., 2015b). Long-term heart allograft survival is primarily limited by chronic allograft rejection (Lund, Khush, Cherikh, Goldfarb, Kucheryavaya, Levvey, Meiser, Rossano, Chambers, Yusen, Stehlik, et al., 2017). Currently, no treatment exists for this occlusive chronic allograft vasculopathy (CAV) which is the lead cause of recipient death.

The transplant coronary artery endothelium is the principal target of the indolent immune response (Pober et al., 2014). Recent examination of failing heart grafts explanted for re-transplantation of the recipient has identified that the entire vascular tree, from the coronary artery to the microvasculature of the allograft, is damaged (Loupy et al., 2016). Cell-mediated injury is required to elicit vasculopathy (Choy et al., 2004; Lin et al., 2016). Immune responses dominated by interferon-γ production and mononuclear cell infiltration of the graft arteries have been demonstrated in mouse models (Kitchens et al., 2007; Nagano et al., 1997; Uehara et al., 2005) and among human heart transplants with vasculopathy (van Loosdregt et al., 2006), and mechanistically linked to the development of vasculopathy in humanized mouse models (Nadig et al., 2010; Tellides et al., 2000; Wang et al., 2007b).

Injury to the arterial or microvascular endothelium elicits a repair response, but little is known regarding the mediators involved in the repair of the damaged, mature vasculature. The pro-angiogenic mediators are better defined in development and tumour neo-angiogenesis (Claesson-Welsh & Welsh, 2013b). Among these, vascular endothelial growth factor (VEGF) is a dominant growth cue, but angiogenesis is modified by a variety of ligands for G-protein-coupled receptors. These cues elicit differentiation of “tip” endothelial cells (EC), leading an angiogenic sprout to acquire motility and elaborate soluble and matrix-associated molecules to cross-talk with

adjacent trailing EC and neighbouring smooth muscle cells (del Toro et al., 2010; Eilken & Adams, 2010b).

Apelin is an endothelial cell-derived peptide agonist for the apelin G-protein-coupled receptor (Cox et al., 2006; del Toro et al., 2010). Apelin features many effects as a potent inotropic agent for cardiac myocytes (Charo et al., 2009; Scimia et al., 2012). However, apelin is induced in the tip endothelial cell directly by tissue hypoxia and indirectly by VEGF and signals to trailing stalk ECs that express the apelin receptor (Eyries et al., 2008; Pi et al., 2017). Apelin loss in the developing embryo is associated with subtle defects in vascularization (Charo et al., 2009; Kasai et al., 2008). In the adult, apelin is required for tip EC sprouting during regenerative angiogenesis after tailfin amputation in zebrafish (Eyries et al., 2008) and plays a role in myocardial remodelling after infarction (Wang et al., 2013a).

Here we tested the hypothesis that the developmental angiogenesis program contributes to the vascular endothelial repair of the established adult coronary circulation under chronic immune injury. We define the expression of characteristic EC-enriched tip cell transcripts to mark vascular repair in murine and human arteries and microvessels after heart transplantation. Of these, we focused on apelin since apelin signalling to vascular ECs might cue repair and be amenable to therapeutic intervention. We find apelin expression by the graft vasculature is critical to mediate vascular repair and defend the graft against immune-cell invasion. An apelin receptor agonist mitigates both immune-cell infiltration and maladaptive vascular repair.

Results

Endothelial tip-cell genes are expressed during immune injury

First, we sought to determine if the injury of the established mammalian vasculature is accompanied by reparative endothelial differentiation in vivo. Initially, heart transplantation is associated with reperfusion injury to the graft vasculature. Later, the vascular endothelium of the male heart graft coronary artery and microvessels are known to be targeted by the minor HY-histocompatibility antigen-directed alloimmune response in female mice (Bagai et al., 2005; Marelli-Berg et al., 2004). We characterized microvascular endothelial density in the myocardium two weeks after heterotopic heart transplantation of a male donor heart to a female major histocompatibility complex-matched recipient mouse to confirm the injury. We observed ~ 45% loss of microvascular profiles in sections immunostained for endothelial CD31 or cadherin 5 among hearts transplanted to female or male recipients versus normal heart tissue, consistent with ischemia-reperfusion injury-induced loss (Figure 3.1A). We evaluated gaps in the continuity of the endothelium in coronary artery cross-section profiles of grafts after transplantation. Focal endothelial cell (EC) loss was seen in grafts of female, but not male, heart recipients (Figure 3.1B (left panel), Figure 3.7A).

Similarly, we observed an increase in cleaved caspase 3 co-staining $CD31^{+}$ endothelial cells among hearts transplanted to female recipients, consistent with apoptotic stress of the endothelium (Figure 3.1B (right panel), Figure 3.7B). Fibrin immunostaining was associated with the injured arterial and microvascular endothelium of hearts transplanted to female recipients (Figure 3.7C). These data indicate that the loss of microvessel density at two weeks post-transplant is mainly attributable to earlier reperfusion injury and suggests additional active vascular injury in the allograft but not the syngeneic graft.

Angiogenesis in the developing vasculature, cancer neo-angiogenesis, and sprouting from endothelial spheroids cultured in vitro is associated with characteristic genes expressed by the lead 'tip' EC (del Toro et al., 2010; Gerhardt et al., 2003; Roudnicky et al., 2013; Saint-Geniez et al., 2002; Villa et al., 2016). We hypothesized that tip cell genes are similarly expressed during the repair of the endothelium of the established vasculature. Among genes known to be upregulated by the tip cell during angiogenesis, we selected *Apln, Egfl7, Esm1, and Pdgfb*, as readouts with endothelial-selective expression. We examined tip gene expression in epicardial coronary arteries micro-dissected from the myocardium of the heart transplant (Figure 3.1C). Since only small amounts of RNA were isolated from the epicardial left coronary arteries, specimens were pooled in pairs, then analyzed for expression of the selected genes. We observed that each tip cell gene was markedly upregulated at two weeks after transplantation in the allogeneic male wild-type hearts (Figure 3.1C and Figure 3.8), whereas little change in expression was seen with the constitutively expressed, endothelial-specific gene *Pecam1* (CD31).

Moreover, high tip cell gene expression persisted at six weeks after transplantation. In contrast, tip cell gene expression among male-to-male transplanted hearts at 2- and 6-weeks post-transplant was like freshly isolated native heart tissue, consistent with resolution of reperfusion injury-associated repair that occurred at the time of transplant. Remarkably, we observed a parallel pattern of expression of the tip genes among the coronary artery and the heart microvascular endothelial cells in the myocardium (Figure 3.1C, D).

We examined the deposition of the tip cell matrix protein ESM1 in the heart by immunohistochemistry to confirm protein expression. As shown in (Figure 3.1E, F), focal deposits of ESM1 were found in the myocardium associated with CD31-positive microvessels,

and in the wall of the expanded arterial intima, in the allogeneic, but not syngeneic, heart transplants. Similarly, allografts upregulated the expression of EGFL7 and apelin in the arterial endothelium (Figure 3.2, Figure 3.3A, B). These findings are consistent with the resolution of an early wave of repair in the male-to-male heart transplants and indicate vascular repair in response to active injury from the alloimmune response in the male-to-female heart transplants. Notably, the repair genes were expressed in the isolated artery, indicating arterial repair is associated with a similar repair program as the heart microvasculature.

Apelin loss decreases endothelial repair gene expression

Loss of apelin receptor signalling is associated with defects in vascular development (Charo et al., 2009; Kasai et al., 2008); hence we hypothesized that apelin cues vascular repair in the allograft. To test this, we transplanted hearts from apelin-deficient male donors to major histocompatibility complex-matched, apelin wild-type female recipient mice.

We examined the effect of apelin loss on endothelial reparative gene expression. Apelin expression in the apelin-deficient hearts was undetectable by qRT-PCR, indicating apelin is endogenous to the graft vasculature and is not supplied by circulating donor progenitor cells or infiltrating leukocytes (Figure 3.1C, D). We found that apelin loss abolished the induction of endothelial tip cell gene expression in the isolated artery and myocardium (Figure 3.1C, D). Similarly, we found that deposition of ESM1 and EGFL7 protein markedly decreased in the perivascular matrix of the apelin-deficient hearts (Figure 3.1E, F, Figure 3.8). In contrast, expression of *Vegfa*, produced by parenchymal cells and leukocytes infiltrating the graft coronary artery and myocardium, remained elevated, consistent with a pro-angiogenic tissue microenvironment (Figure 3.10).

Apelin loss exacerbates vasculopathy

We found that intimal expansion and occlusion of the arterial lumen were more pronounced among apelin-deficient versus wild-type littermate heart allografts (Figure 3.2A, B). Apelin loss in the allograft further decreased the microvessel density versus transplanted male control hearts (Figure 3.1A, 2C, Figure 3.11). In contrast, no effect of apelin loss was seen on the normal arterial histology after reperfusion injury among the syngeneic grafts (Figure 3.2B).

Apelin receptor agonist promotes endothelial repair in vitro

We studied the effect of apelin receptor stimulation on endothelial repair in vitro. Functionally, knockdown of apelin by siRNA treatment of EC decreased monolayer repair of a scratch wound in response to VEGF (Figure 3.2D). Further, apelin receptor agonist treatment using a proteinase-resistant APLN-17 analogue (Figure 3.12A) (McKinnie et al., 2017) augmented VEGF-stimulated scratch wound repair (Figure 3.2D, Figure 3.12B). Scratch wound repair in vitro was associated with induction of ESM1, which was abolished by RNAi-mediated knockdown of APLN (Figure 3.2E, F). Moreover, sprouting and tip cell gene expression is induced by the apelin receptor agonist in 3D endothelial spheroid cultures in vitro (Figure 3.12C, D). These findings indicate vascular endothelial reparative differentiation, reflected by induced expression of apelin and ESM1, directly mitigates vascular injury. Further, traditional pro-angiogenic cues such as VEGF are insufficient to mediate repair optimally.

Endothelial repair genes are expressed in human heart transplants

Next, we examined reparative endothelial gene expression in human heart transplants. We studied coronary artery and endomyocardial samples of end-stage human hearts with advanced transplant vasculopathy, explanted during re-transplantation of the recipient (Figure 3.3A). Non-utilized donor hearts were used as a reference sample. Features of atherosclerosis were evident in

both sets of human coronary arteries, but the transplanted vessels had additional features of CAV (van Loosdregt et al., 2006) and, in some cases, prominent calcification of the expanded intima. A small intramyocardial artery in the reference sample shown in Figure 3.3A has a patent lumen and a single-cell intimal layer.

In contrast, the intramyocardial artery in the explanted heart graft features marked luminal narrowing by intimal myofibroblast proliferation, matrix accumulation, and chronic inflammation. Epicardial arteries from the explant show similar prominent intimal myofibroblast proliferation. In contrast, the reference heart artery shows established intimal fibrosis with cholesterol deposits. The intimal expansion among the explant arteries was marked (Figure 3.3B), consistent with advanced disease. The explant arteries heterogeneously expressed the repair genes (Figure 3.3C). We found *APLN* expression was increased among the explant coronaries and a trend for *ESM1* induction compared to the reference arteries. Apelin expression was detected by immunohistochemistry co-localized with endothelial CD34 in the explant arteries (Figure 3.9C). The allograft samples with the most extensive intimal calcification had the lowest expression of the EC repair genes.

We also examined tip EC gene expression among the explant myocardium samples. We found upregulation of *APLN*, *ESM1*, and *PDGFB* compared to the reference myocardium (Figure 3.3D). The magnitude of the reparative gene expression was like the expression in the peri-infarct zone after myocardial infarction (data not shown).

Further, we characterized *APLN*, *ESM1*, and *PDGFB* expression in endomyocardial biopsies of the interventricular septum of functioning heart transplants, obtained at a mean of 3 (range 0.3-7) years post-transplant. We identified nine recipients with transplant vasculopathy defined clinically by intravascular ultrasound and coronary angiography and five recipients without

vasculopathy. We compared gene expression among heart transplants with CAV, no CAV, and the non-transplanted donor hearts as the reference (Figure 3.3E). We observed elevated expression of endothelial repair genes among the post-transplant samples with vasculopathy. Together, these data indicate that the endothelial repair gene program in the heart is conserved among mice and humans.

Apelin loss promotes inflammation

We examined the alloimmune response to the apelin-deficient graft. As reported, the male heart allograft is infiltrated by lymphocytes and monocytes at day 14 post-transplant, i.e. cellular rejection without an alloantibody response (Kaul et al., 2015). We observed an increase in the number of lymphocytes and monocyte/macrophages at both 2- and 6-weeks post-transplant in the apelin-deficient grafts (Figure 3.4A, B, and Figure 3.13). There was little qualitative difference in the relative fractions of CD4, CD8 lymphocytes, F4/80 macrophages, or M1 versus M2 monocytes, determined by marker immunohistochemical staining, in the graft among the apelin-deficient versus wild-type hearts. However, M1 monocyte infiltration of the arterial intima was more prominent among apelin-deficient hearts at two weeks (Figure 3.4B). Further, we found that interferon-γ-dependent *Cxcl11* transcript expression was increased early in the rejection response among apelin-deficient hearts in both the artery and myocardium compartments (Figure 3.4C). Similarly, interferon-γ was markedly increased in the 2-week apelin-deficient myocardium samples (Figure 3.4D). Interferon-γ was comparable among apelin-deficient and wild-type grafts at the later time point, but increased *Cxcl11* expression persisted among the apelin-deficient hearts. The pro-inflammatory cytokine, TNF-α, was modestly higher among apelin-deficient hearts (Figure 3.4D).

Since lymphocyte-generated interferon-γ has been linked to CAV in human clinical material, we further characterized the frequency of alloreactive lymphocytes between recipients of apelin-deficient and wild-type grafts. The spleen is the primary site of alloantigen presentation after murine heart transplantation (Liu et al., 2016). Therefore, we harvested splenic lymphocytes two weeks post-transplant and quantitated the frequency of allogeneic responsive lymphocytes among the recipient mice using H-Y antigen I-Ab tetramers and flow cytometry. We observed no increase in the frequency of total $CD44^{hi}$ memory or interferon-γ-positive, alloreactive lymphocytes among the apelin-deficient versus wild-type recipients (Figure 3.4E). This strongly argues that apelin loss does not render the graft more immunogenic or promote the expansion of the alloreactive lymphocyte population.

We reasoned that apelin loss might alter leukocyte trafficking to the graft through autocrine signals among ECs. First, we reduced apelin receptor expression among ECs in vitro using RNA interference (Figure 3.5A). Apelin is known to signal to vascular EC to elicit Akt and eNOS activity. We confirmed that an APLN-17 analogue acts as a selective apelin receptor agonist (McKinnie et al., 2017). APLN-17-stimulated Akt and eNOS phosphorylation required endothelial apelin receptor expression (Figure 3.5B, C).

To test the hypothesis that apelin receptor stimulation may repel leukocyte entry to the heart, we stimulated EC monolayers with TNF-α for 18 hours to simulate inflamed endothelium, then evaluated the adhesion of primary human monocytes to the monolayer. We found that monocyte adhesion to an EC monolayer in vitro was decreased with apelin receptor stimulation of the monocyte/EC co-culture in an eNOS-dependent fashion (Figure 3.5D, Figure 3.14).

APLN-17 treatment blocks the progression of transplant vasculopathy

To determine if an apelin-receptor agonist treatment could modify graft coronary arterial injury, we treated recipient mice daily with the synthetic apelin receptor agonist APLN-17 peptide that is resistant to degradation by endogenous plasma proteinases (McKinnie et al., 2017). Treatment was begun two weeks after transplantation to model the clinical scenario of treatment of early, established injury. Hearts were harvested at six weeks post-transplant, and the extent of graft vascular injury was evaluated. We found the graft coronary artery intimal expansion was markedly decreased in the apelin receptor agonist- versus saline-treated control grafts (Figure 3.6A, B), and we observed a marked reduction in arterial gaps and cleaved caspase three staining of the endothelium among the treated hearts (Figure 3.15). Further, allograft microvessel density was increased among the APLN-17-treated animals (Figure 3.6A, C). After four weeks of treatment, native *Apln* expression in the graft was suppressed in the isolated coronary artery- (Figure 3.6F) and myocardium compartments (Figure 3.6H). Similarly, EC repair biomarkers (*Esm1, Pdgfb*) and *Vegfa* expression levels were normalized versus untreated grafts in both isolated coronary arteries and the myocardium.

We examined the effect of the apelin agonist treatment on leukocyte infiltration in the allograft. We found a markedly decreased number of T cells and monocytes infiltrating the APLN-17-treated allografts (Figure 3.6A, D). In line with this observation, the allograft expression of interferon-γ and TNF-α were both decreased versus the saline-treated hearts in both coronary artery and myocardium compartments (Figure 3.6G, I, respectively). This data indicates that apelin receptor-agonist treatment decreases graft immune-cell infiltration with an accompanying decrease in proinflammatory cytokine generation.

We tested the hypothesis that APLN-17-stimulated nitric oxide was required to protect the graft from immune cell infiltration. L-NAME was administered with APLN-17 (Kazakov et al., 2013), and graft survival was evaluated. Inhibition of nitric oxide synthases reversed the effect of apelin (17) to reduce inflammation (Figure 3.6D) and promoted graft loss (Figure 3.6E). This was associated with coronary vasculopathy (Figure 3.6B, C). Taken together, apelin receptor agonist treatment both decreases the immune injury burden on the graft vasculature and promotes vascular repair resulting in the normalization of endothelial repair gene expression. Conversely, inhibition of nitric oxide generation facilitates rejection and vascular injury despite APLN-17 treatment.

Discussion

In this study, we sought to determine if the embryonic vascular development program expressed by vascular endothelial cells contributes to the repair of the established vasculature in the adult. We focused on three biomarkers, apelin, ESM1, and PDGFB, selectively expressed in ECs, and well-characterized in mouse developmental angiogenesis, to report on angiogenic "tip" cell differentiation in the heart. We found transient upregulation of the angiogenic biomarker panel in the myocardium after ischemia-reperfusion injury associated with isogeneic heart transplantation. However, transplanted hearts exposed to chronic alloimmune injury showed persistent expression of biomarker RNA and protein. Thus, despite the similar density of endothelial cells, the pro-angiogenic biomarkers reveal underlying endothelial repair among allogeneic heart transplants. We found that this upregulated expression was conserved among samples of two independent groups of human transplanted hearts: those undergoing explant during recipient re-transplantation for end-stage allograft vasculopathy and those with developing vasculopathy. Moreover, the tip cell genes were induced in the peri-infarct zone of

human hearts after myocardial infarction. Thus, the endothelial repair gene expression in response to immune injury is conserved in mice and humans.

Further, we examined the expression of the biomarkers in micro-dissected epicardial coronary arteries in mouse heart transplants. This segment of the coronary vasculature is recognized to be prone to developing occlusive vasculopathy lesions in humans. Notably, the biomarker panel expression was similarly regulated among paired coronary artery and myocardium samples in the mouse. This is consistent with the observation that the transplant vasculature in humans undergoes alloimmune injury along the length of the arterial tree, from the artery to the microvasculature (Loupy et al., 2016). Recent work identifies reparative endothelial progenitor cells embedded in the mouse aortic endothelium, but repair from mechanical injury involved EC de-differentiation and proliferation (McDonald et al., 2018). We confirmed that apelin expression in human heart explant coronary artery endothelium was induced versus non-utilized donor arteries, but these specimens had advanced disease with a heterogeneous expression of the biomarkers and may not all reflect evolving lesions. Taken together, the findings suggest biomarker interrogation of clinical endomyocardial samples reflects events in the arteries, but the features of the response may depend on the nature or duration of the injury.

Apln, *Pdgfb*, and *Esm1* are each associated with vascular defects in knockout mice (Kasai et al., 2008; Leveen et al., 1994; Rocha et al., 2014), consistent with the notion that the expression of these genes by the injured vascular endothelium participates in vascular repair. For example, loss of the *Esm1* gene reduces matrix-bound VEGF presentation to EC and perturbs capillary outgrowth (Rocha et al., 2014). In vitro, loss of apelin signalling reduces monolayer wound repair and tip cell differentiation, whereas apelin itself can cue differentiation in cultured human EC. To directly address the hypothesis that apelin signalling within the injured tissue

compartment, i.e., the heart allograft, cues vascular repair, we found that heart allografts from donor knockout mice lacking expression of apelin exhibited more areas of denuded endothelium and developed more aggressive arterial occlusion than hearts from wild-type littermate donors. Apelin-deficient allografts failed to induce the expression of reparative tip cell markers in both the arterial and microvascular compartments. Microvascular density was decreased among the apelin-deficient allografts. VEGF appears insufficient to mediate vascular repair and induce expression of EC tip cell genes in the absence of apelin signals since we find abundant expression of VEGFA among both apelin-sufficient and -knockout heart allografts. Optimal induction of reparative endothelial cells in the adult requires co-incident signalling by VEGF and apelin and perhaps a series of supporting cues (Hara et al., 2013; Kasai et al., 2008; Oladipupo et al., 2014).

Apelin loss increased, and apelin receptor agonist treatment suppressed neointima expansion and vascular smooth muscle cell (VSMC) accumulation under chronic immune injury. This finding contrasts with the neointimal formation in arterial ligation and atherosclerosis-prone mouse models, where apelin signalling directly to vascular smooth muscle cells is reported to drive myofibroblast migration and expansion (Hashimoto et al., 2007; Kojima et al., 2010). Our observation that apelin loss is associated with reduced expression of PDGFB indicates that paracrine cues intrinsic to the vascular wall that drive VSMC movement and myofibroblast differentiation are blunted in the setting of apelin deficiency (Hosaka et al., 2016; Malabanan et al., 2012). Conversely, EC-derived EGFL7 production, which was reported to inhibit VSMC migration (Soncin et al., 2003), is also blocked in apelin-deficient hearts. However, the VSMC response to the intima microenvironment is affected by leukocyte-derived mediators that are also influenced by apelin.

We find that the loss of apelin expression by the heart vasculature promotes inflammation. Infiltrating immune cells supply VSMC with growth factors and cytokines, such as interferon-γ, known to drive transplant vasculopathy (Nagano et al., 1997; Tellides et al., 2000; Wang et al., 2007b). Our investigation of alloimmune T cells demonstrates that apelin loss does not modify HY-alloantigen peptide-specific T lymphocyte expansion or differentiation to produce interferon-γ. These findings exclude the effect of apelin loss to increase the alloantigen burden or enhance alloantigen presentation in mice carrying apelin knockout grafts. However, trafficking of T cells to the graft and intragraft interferon-γ expression are increased among apelin-deficient hearts.

Apelin receptor stimulation of the EC elicits PI3 kinase and downstream eNOS activation. Apelin increases NO availability in the angiotensin-stimulated aortic aneurysm model, which is attributed to a direct antagonistic interaction between the angiotensin receptor and apelin receptor (Chun et al., 2008). In in vitro co-culture, we find the net effect of apelin receptor agonist stimulation decreases monocyte recruitment to an activated EC monolayer. This is consistent with findings that endothelial NO generation reduces trans-endothelial migration of dendritic cells (Weis et al., 2002). Co-treatment of allograft recipients with L-NAME dominated the effect of APLN-17 and elicited early graft rejection. Thus, vascular endothelial apelin normally functions to indirectly guide inflammatory cells away from a repairing endothelium, but this defence response is overwhelmed under sustained alloimmune attack.

We find that treatment of the recipient mice with an apelin receptor agonist blunts the development of the occlusive vasculopathy lesion in the allogeneic heart. In our experiment, we began treatment on day 14 post-transplant when the alloimmune response was established, intragraft expression of interferon-γ was increased, and the angiogenic repair biomarkers were

elevated. We reasoned that these features establish an early vascular injury. Further, in a human heart transplant recipient, established vascular injury would prompt intervention if effective treatment was available. The apelin receptor agonist acts in part to protect the graft by reducing established local graft inflammation and interferon-γ since these features are decreased versus the saline-treated recipients. However, earlier work identified that rapid re-endothelialization reduces maladaptive repair after mechanical angioplasty injury of the endothelium (Asahara et al., 1995) and in allogeneic tracheal transplants (Jiang et al., 2011). Interestingly, the angiogenic repair biomarkers, including VEGF expression, are normalized in both artery and microvessels among heart allografts treated with the apelin receptor agonist, suggesting that reparative re-population of the injured endothelium was achieved.

Remarkably the apelin analogue treatment effectively promotes vascular repair microvascular density and decreases maladaptive intimal expansion of the coronary arteries despite upregulation of endogenous apelin production in the heart allograft. This is consistent with an earlier finding that tumour neovascularization is hyper-induced by cancer cell overexpression of apelin (Sorli et al., 2007). The finding suggests that the induced native apelin production or availability in the setting of chronic immune injury remains rate-limiting for vascular repair. The apelin analogue used in our experiments is resistant to endogenous proteinases, specifically angiotensin-converting enzyme two and neprilysin, that degrade and may limit angiogenic responses elicited by the native apelin peptide (McKinnie et al., 2017). Optimization of the stability and delivery of the analogue, or development of small molecule apelin receptor agonists, are needed for the treatment of humans.

It will be important in future studies to examine whether our findings in this H-Y minor histocompatibility model also occur in the setting of an MHC mismatched transplant. The H-Y

model does not reliably generate an alloantibody capable of binding the surface of donor cells (Simpson et al., 1997). Notably, however, the H-Y model does include the direct and indirect T cell response against the donor (Y. Chen et al., 2003; Valujskikh et al., 2002), responses thought to play a major role in chronic rejection (Chan et al., 2008; Y. Chen et al., 2004; Kaul et al., 2015).

Our experiments reveal that apelin receptor signalling mediates complex interactions between vessel wall cells and the immune system. Apelin induction in reparative vascular EC acts in a paracrine fashion to support arterial re-endothelialization. In parallel, apelin cues the endothelium to repel mononuclear cell adhesion and invasion of the injured tissue. These functions are rate-limiting for a successful repair since pharmacologic treatment with the apelin receptor agonist markedly suppresses inflammation, leukocyte-dependent interferon-γ effects, and maladaptive intimal scarring. Apelin agonists may be an important treatment for an otherwise untreatable, fatal disease in heart transplant recipients. Further, the approach is relevant to autoimmune and mechanical arterial injuries since repair mechanisms are likely to overlap.

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Figures

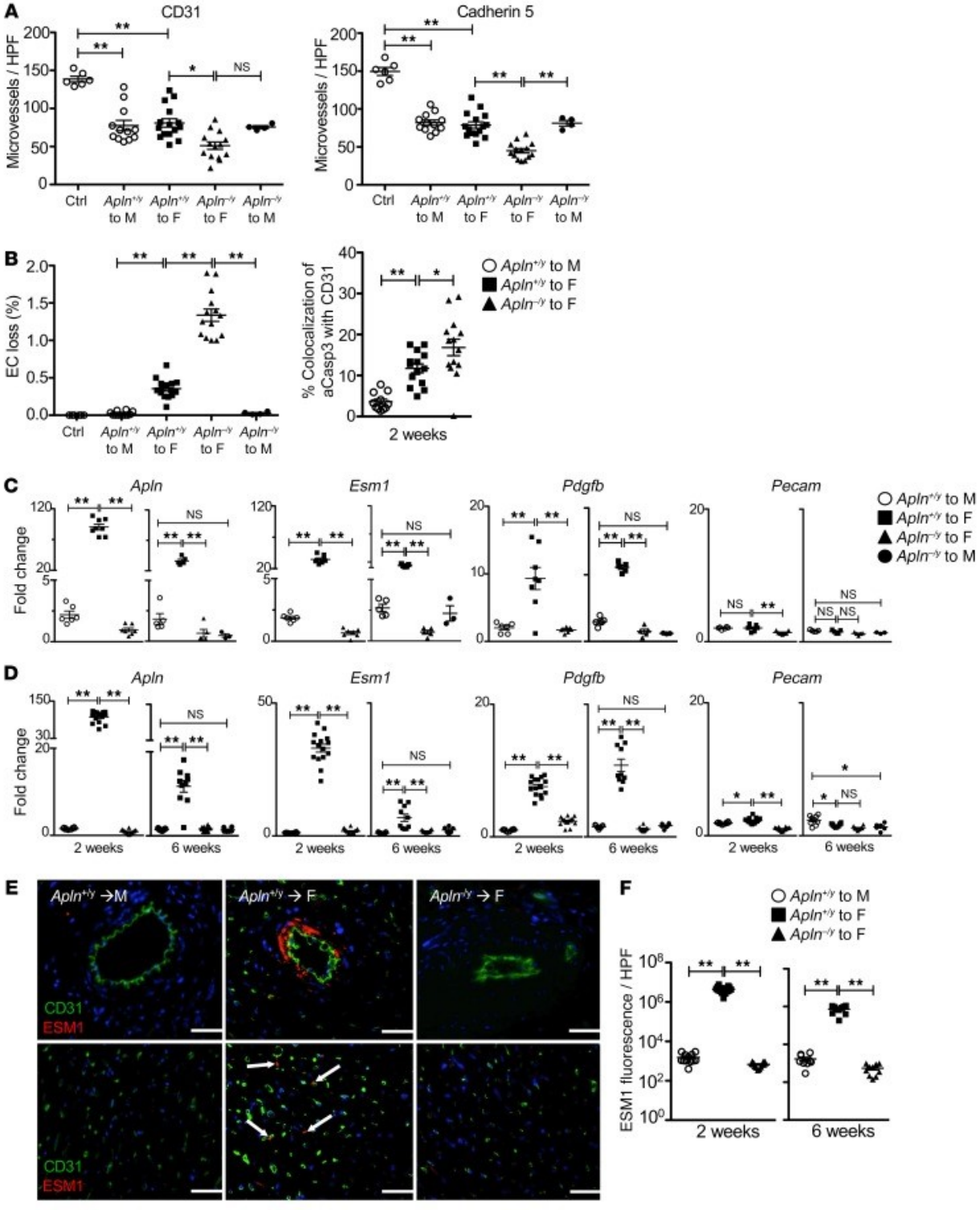

A
CD31
Cadherin 5
Microvessels / HPF
Ctrl
Apln+/y to M
Apln+/y to F
Apln−/y to F
Apln−/y to M
B
EC loss (%)
% Colocalization of aCasp3 with CD31
2 weeks
Apln+/y to M
Apln+/y to F
Apln−/y to F
C
Apln
Esm1
Pdgfb
Pecam
Fold change
Apln+/y to M
Apln+/y to F
Apln−/y to F
Apln−/y to M
D
Apln
Esm1
Pdgfb
Pecam
Fold change
2 weeks
6 weeks
E
Apln+/y →M
Apln+/y → F
Apln−/y → F
CD31
ESM1
F
Apln+/y to M
Apln+/y to F
Apln−/y to F
ESM1 fluorescence / HPF
2 weeks
6 weeks

Figure 3.1: Post-transplant vascular injury is associated with endothelial repair gene expression.

A), Hearts recovered two weeks post-transplantation were immunostained for endothelial cell markers CD31 or cadherin 5. Myocardial microvessel density was quantitated ($CD31^+$ (PECAM1), left panel) and (cadherin 5^+, right panel). $Apln^{+/y}$ to male recipients (n=12 biological replicates) experienced reperfusion injury alone, $Apln^{+/y}$ (n=15) and $Apln^{-/y}$ (n=14) to $Apln^{+/+}$ female recipients experienced reperfusion and chronic alloimmune injury. **B**, Gaps in the arterial endothelium in cross-section (left panel) and the fraction of cleaved caspase 3^+ immunostaining among the $CD31^+$ arterial endothelium (right panel) were quantitated among the samples from (A). Endothelial repair gene expression among transplanted hearts was determined by qRT-PCR and expressed relative to non-transplanted control hearts. **C**, Gene expression among micro-dissected coronary arteries at 2- or 6-weeks post-transplant. Consecutive artery samples were pooled in pairs for analysis. ($Apln^{+/y}$ to male recipients (n=6 pairs), $Apln^{+/y}$ (n=8), $Apln^{-/y}$ (n=7) to $Apln^{+/+}$ female recipients) and 6 weeks ($Apln^{+/y}$ to male recipients (n=5 pairs), $Apln^{+/y}$ (n=5), $Apln^{-/y}$ (n=5) to $Apln^{+/+}$ female recipients). **D**, Gene expression among individual myocardium samples at 2- or 6-weeks post-transplant ($Apln^{+/y}$ to male recipients (n=12 biological replicates), $Apln^{+/y}$ (n=15), $Apln^{-/y}$ (n=14) to $Apln^{+/+}$ female recipients), and 6 weeks ($Apln^{+/y}$ to male recipients (n=9 biological replicates), $Apln^{+/y}$ (n=10), $Apln^{-/y}$ (n=10) to $Apln^{+/+}$ female recipients). **E**, Hearts recovered two weeks post-transplantation were immuno-stained for endothelial CD31 (green) and ESM1 (red, arrows). Medium-sized to larger arterial cross-sections are represented in the top panels, whereas myocardial microvessels are in the lower panels. **F**, ESM1 immunofluorescent quantitation among heart transplants in (E); $Apln^{+/y}$ to male recipients

(n=12 biological replicates), *Apln* $^{+/y}$ (n=15) and *Apln* $^{-/y}$ (n=14) to *Apln*$^{+/+}$ female recipients. Mean ± SEM; * $p<0.05$, ** $p< 0.01$ by one-way ANOVA with Bonferroni's post-hoc test.

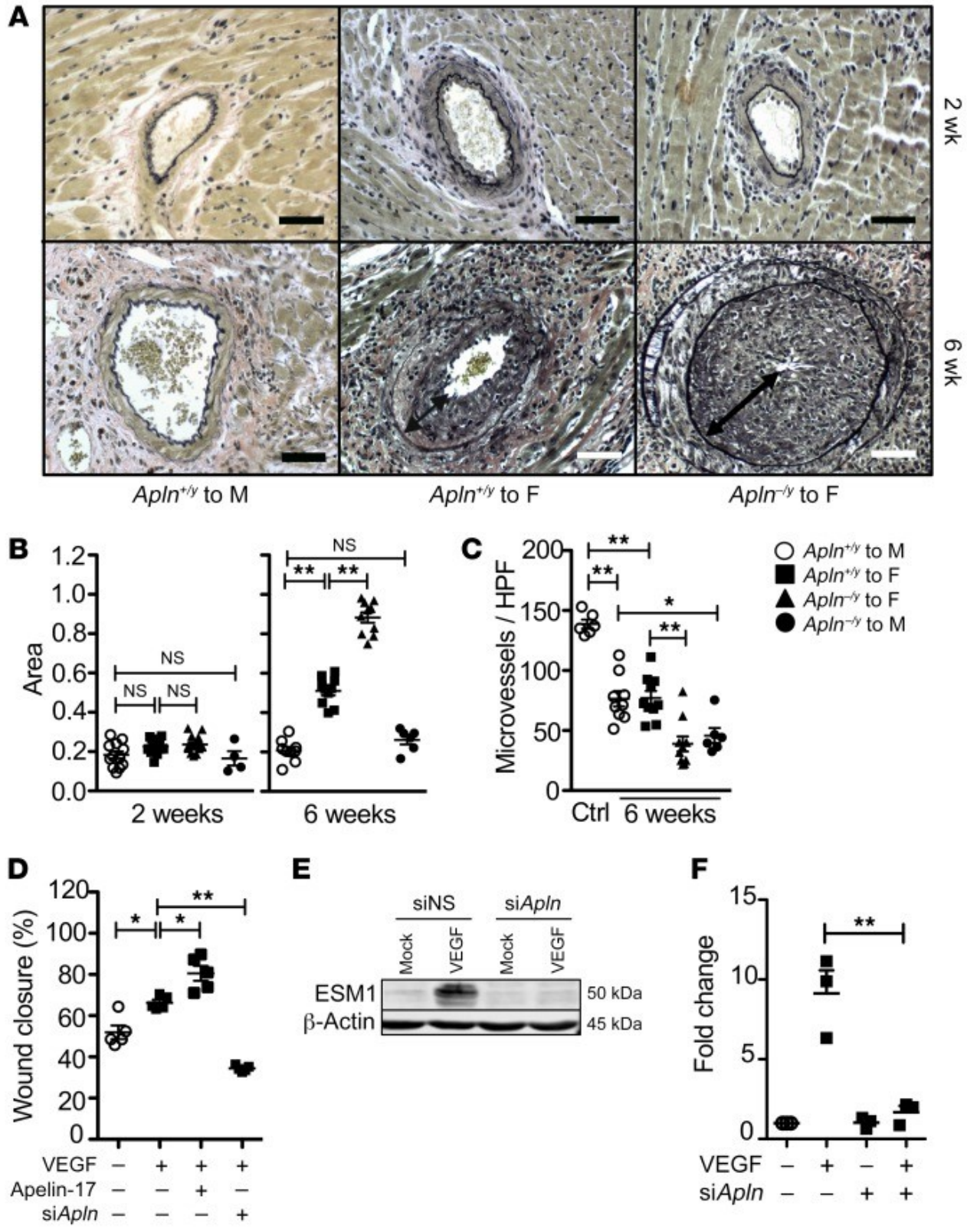


Figure 3.2: Apelin loss exacerbates arterial vasculopathy and blunts endothelial repair.

(A) Photomicrographs of coronary arteries from mouse heart transplants, with van Gieson stain to highlight the internal elastic lamina in black. Syngeneic male-to-male transplants have a single-cell layer of the intima. The progressive expansion of the intima is evident from 2 weeks (top panels) to 6 weeks (bottom panels) after transplantation in allogeneic male to apelin WT female transplants, which is more marked among apelin-deficient male donor hearts. Scale bars: 50 μm. **(B)** Quantitation of the intima area as a fraction of the area within the internal elastic lamina at two weeks and six weeks after transplantation among grafts, as in Figure 3.1. **(C)** Microvessel density among freshly harvested donor reference hearts versus WT or apelin-deficient hearts from B recovered six weeks after transplantation. Mean ± SEM; *P < 0.05, **P < 0.01 by 1-way ANOVA with Bonferroni's post hoc test. **(D)** ECs were transfected with scrambled or apelin siRNA, then plated at a higher confluence. The monolayers were injured with a scratch wound, then stimulated with VEGF (50 ng/mL) or APLN-17 analogue (1 μM) as indicated (n = 5 biological replicates in independent experiments). Mean ± SEM; *P < 0.05, **P < 0.01 by 1-way ANOVA with Bonferroni's post hoc test. **(E)** ECs were transfected with scrambled or apelin siRNA, then plated at a higher confluence. The monolayers were injured with multiple scratch wounds, then stimulated with VEGF (50 ng/mL) or mock-treated. Western immunoblot of EC lysates for ESM1. Representative of 3 biological replicates. **(F)** Quantitation of data in E (n = 3 biological replicates). Mean ± SEM; **P < 0.01 by 1-way ANOVA with Bonferroni's post hoc test.

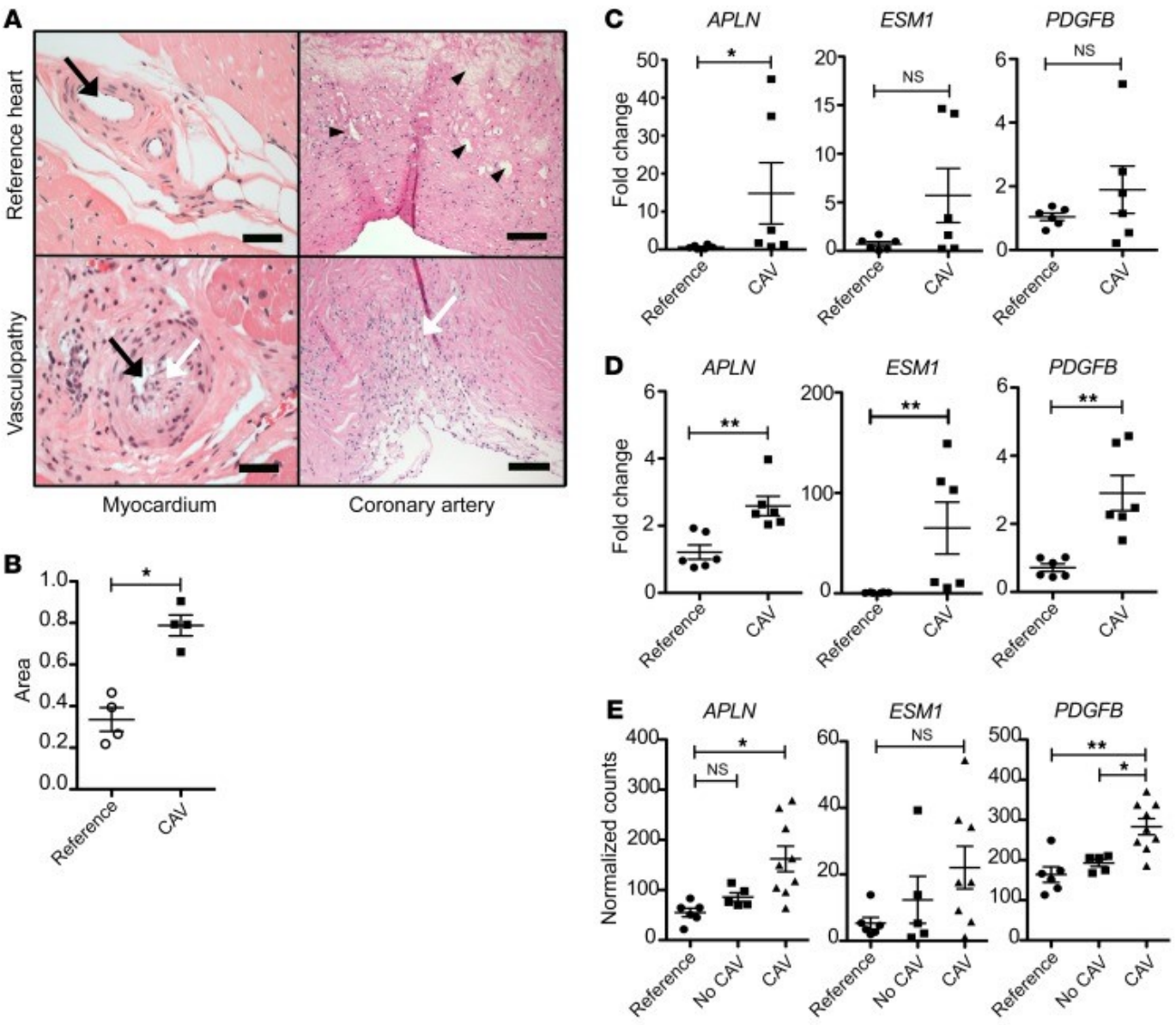


Figure 3.3: Endothelial repair genes are expressed in injured human hearts.

(A) Histology of coronary artery and myocardium of human hearts, H&E stain. The top panels show an unutilized donor reference heart. The bottom panels show a transplant heart explant with arterial CAV, recovered at recipient re-transplantation. The arterial lumen (black arrows) is occluded in the explant, with prominent myofibroblasts (white arrows) and a matrix in the explant intima. The reference arteries show atherosclerotic cholesterol accumulation

(arrowheads). Scale bars: 50 μm (left panels), 200 μm (right panels). **(B)** Quantitation of the intima area as a fraction of the area within the internal elastic lamina of epicardial coronary arteries among unutilized donor and explant hearts (n = 4 biological replicates). **(C)** Expression of endothelial repair genes in the coronary artery of reference or explant hearts (n = 6 biological replicates). **(D)** Expression of endothelial repair genes in myocardium among reference (n = 6 biological replicates) or explant allograft hearts with vasculopathy (n = 6). **(E)** Expression of endothelial repair genes in myocardium among endomyocardial biopsy specimens of reference (n = 6 biological replicates) or transplant hearts with (n = 9) or without (n = 5) arterial CAV. Mean ± SEM; *P < 0.05, **P < 0.01 by 1-way ANOVA with Bonferroni's post hoc test.

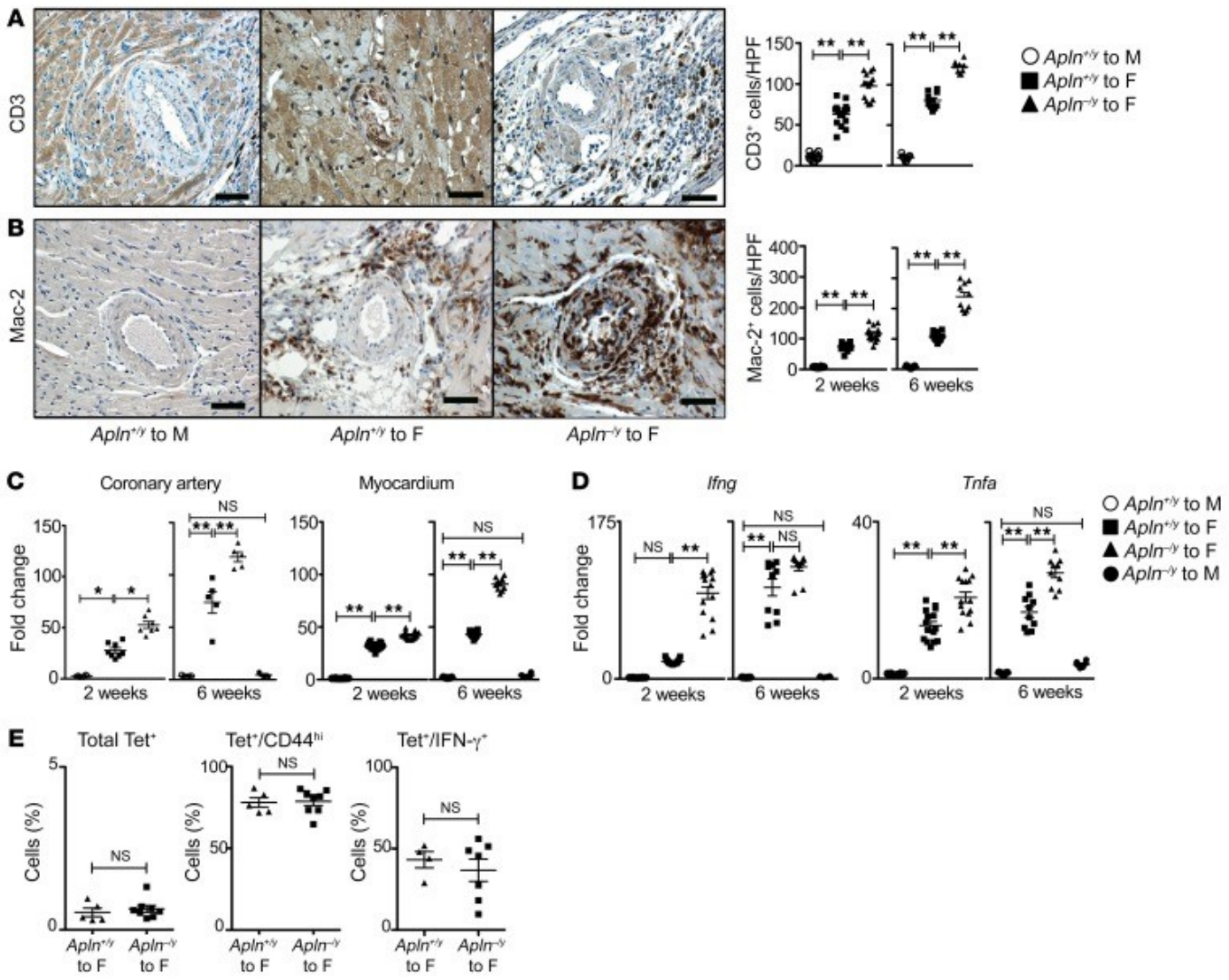


Figure 3.4: Apelin loss increases heart inflammation after transplantation.

Hearts were recovered after transplantation. **(A** and **B)** Immunostains for CD3, a lymphocyte marker (left panel), with quantitation at two weeks and six weeks after transplantation among grafts as in Figure 3.1 **(A)**; and for Mac-2, a pro-inflammatory (M1) monocyte/macrophage marker (left panel), with quantitation at two weeks and six weeks after transplantation among grafts as in Figure 3.1 **(B)**. Mean ± SEM; **P< 0.01 by 1-way ANOVA with Bonferroni's post hoc test. Scale bars: 50 μm. (C and D) Expression of proinflammatory cytokines among heart

transplants. **(C)** CXCL11 expression among micro-dissected coronary arteries and myocardium at 2 and 6 weeks after transplantation among grafts, as in Figure 3.1. Mean ± SEM; *P < 0.05, **P < 0.01 by 1-way ANOVA with Bonferroni's post hoc test. **(D)** Myocardial cytokine expression at 2 and 6 weeks after transplantation among grafts, as in Figure 3.1. Mean ± SEM; **P < 0.01 by 1-way ANOVA with Bonferroni's post hoc test. **(E)** Recipient anti-male H-Y–alloreactive lymphocytes were isolated from the spleen two weeks after transplantation, identified by staining with H-Y I-Ab tetramers, and characterized by dual staining with CD44 and intracellular IFN-γ. The data from individual mice are displayed. Mean ± SEM; P = NS by Mann-Whitney test.

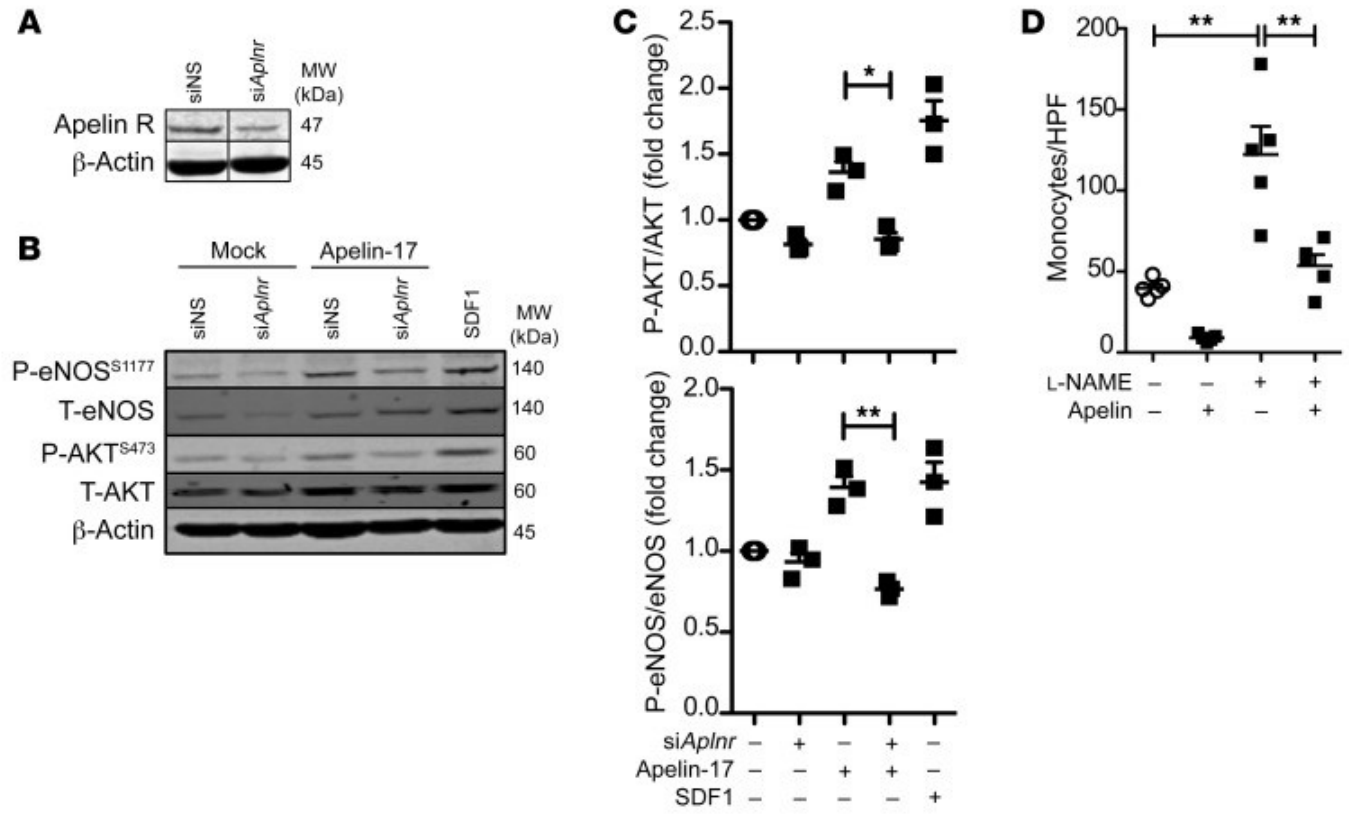


Figure 3.5: Apelin stimulates EC eNOS activation and inhibits monocyte adhesion.

(A) Primary human ECs were transfected with siRNA against the apelin receptor, or scrambled siRNA, then lysed, and the apelin receptor was immunostained on Western blot. Representative of 4 biological replicates. **(B)** Apelin receptor stimulation of human ECs elicits phosphorylation of AKT and eNOS. **(C)** Quantitation of the data in B (n = 3 biological replicates). Mean ± SEM; *P < 0.05, **P < 0.01 by ANOVA. **(D)** Human ECs were stimulated with TNF-α overnight, then cocultured with human monocytes. As indicated, the cocultures were mock-treated or treated with proteinase-resistant APLN-17 (1 μM), DMSO, or the eNOS inhibitor L-NAME (100 μM). The number of adherent monocytes was quantitated (n = 5 biological replicates). Mean ± SEM; **P < 0.01 by 1-way ANOVA with Bonferroni's post hoc test.

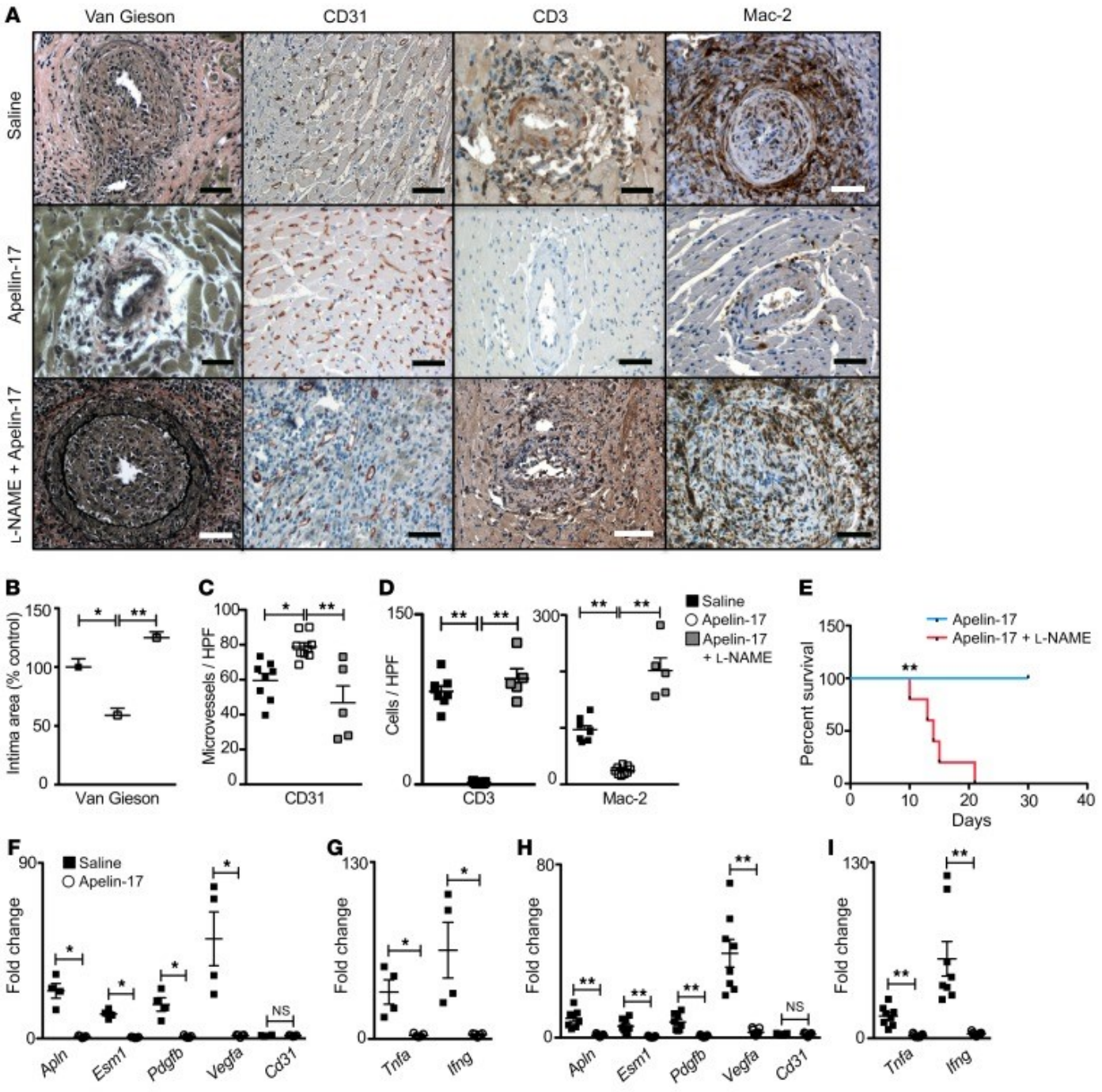


Figure 3.6: APLN-17 analogue treatment suppresses arterial vasculopathy and immune cell infiltration of heart transplants.

Apln$^{+/y}$ male hearts were transplanted into WT female recipients. Two weeks after transplantation, the recipient mice were treated daily with saline or APLN-17 analogue or with APLN-17 analogue plus L-NAME; then, the heart allografts were recovered when the graft heartbeat stopped or after six weeks post-transplantation. **(A)** Photomicrographs of transplanted hearts stained with Van Gieson or immunostained for CD3, Mac-2, or EC CD31. Scale bars: 50 μm. **(B)** Quantitation as in Figure 2 of the intima area of graft arteries in heart recipients treated with saline (n = 8 biological replicates), APLN-17 analogue (n = 9), or APLN-17 analogue plus L-NAME (n = 5). **(C)** Quantitation of $CD31^+$ microvessels in grafts of heart recipients treated with saline (n = 8 biological replicates), APLN-17 analogue (n = 9), or APLN-17 analogue plus L-NAME (n = 5). **(D)** Quantitation of immune cell infiltration in the myocardium of grafts from heart recipients as in C. Mean ± SEM; *P < 0.05, **P < 0.01 by 1-way ANOVA with Bonferroni's post hoc test. **(E)** Survival of heart allografts in mice treated with APLN-17 analogue without (n = 9) or with L-NAME (n = 5 biological replicates) starting on day 14 after transplantation. **P < 0.01 by log-rank. (F and G) Expression of endothelial repair **(F)** and proinflammatory **(G)** genes in micro-dissected coronary arteries from recipient mice treated with saline (n = 4 pairs) or APLN-17 analogue (n = 5 pairs). Coronary artery data were analyzed by the Mann-Whitney test. Mean ± SEM; *P < 0.05, **P < 0.02. (H and I) Expression of endothelial repair **(H)** and proinflammatory **(I)** genes in the myocardium of heart grafts from recipient mice treated with saline (n = 8) or APLN-17 analogue (n = 9). Mean ± SEM; **P < 0.01 by Student's t-test.

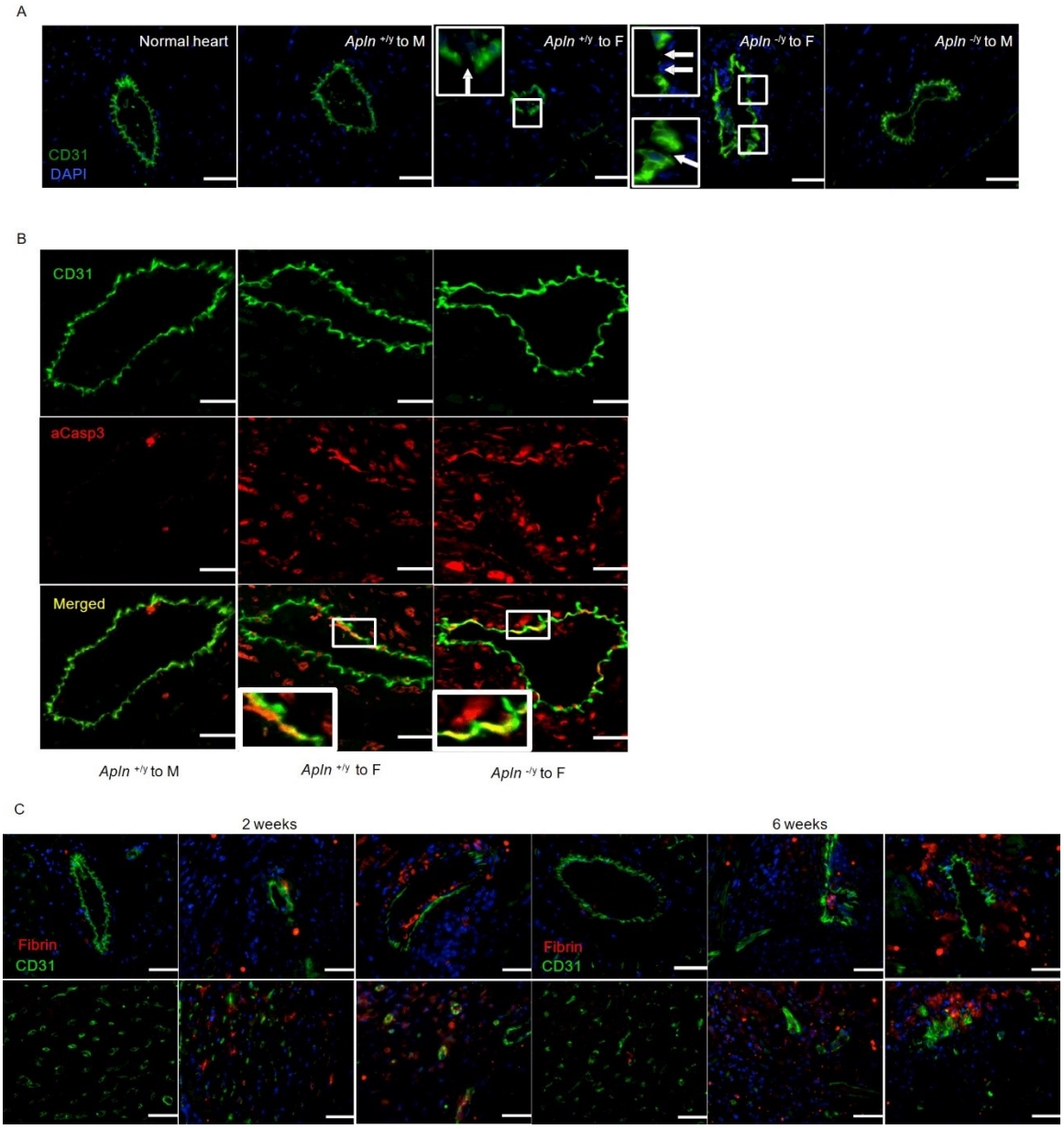


Figure 3.7: Graft coronary arterial injury post-transplantation.

A) Loss of continuity of the arterial endothelium in hearts at two weeks post-transplantation. Photomicrographs show immunofluorescent staining of the endothelial marker PECAM1 (CD31; green) with DAPI nuclear staining (blue). Arrows indicate areas of endothelial loss/gaps in the endothelium (quantitation is in figure 3.1B). **B)** Arterial endothelial apoptosis in hearts at two weeks post-transplantation. Confocal photomicrographs show double immunofluorescent

staining of the endothelial marker PECAM1 (CD31; green) with cleaved caspase 3 (red). Insets show caspase 3^+ endothelial cells. Colocalization is quantitated and is shown in Figure 3.1B (right panel). **C)** Fibrin is associated with the graft arterial (upper panels) and microvascular (lower panels) endothelium post-transplantation. Photomicrographs show double immunofluorescent staining of the endothelial-cell marker PECAM1 (CD31; green) and fibrin (red). n=4-15. Scale bar = 50μm.

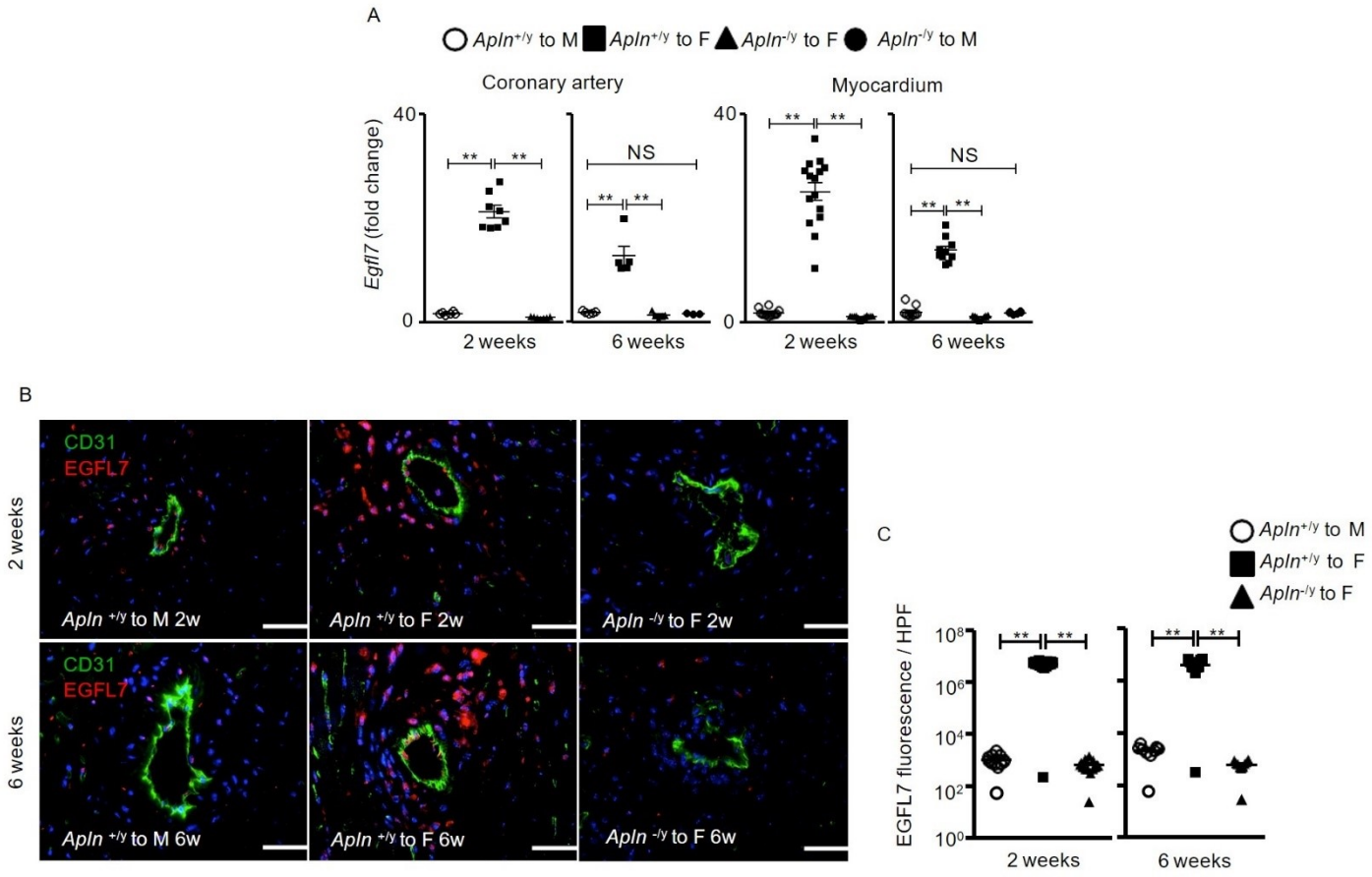


Figure 3.8: *Egfl7* gene expression in graft coronary artery or myocardium post-transplantation.

A) Consecutive pairs of coronary artery samples were pooled for qRT-PCR analysis (n=3-8 pairs); individual myocardium samples are shown (n=6-15 biological replicates) relative to normal hearts. As indicated, samples of heart grafts were obtained at two weeks and six weeks post-transplant. Mean ± SEM; **P<0.01 and NS=nonsignificant by one-way ANOVA with Bonferroni's post-hoc test. **B)** Double immunofluorescent staining of CD31 (green) and EGFL7 (red) in the heart grafts. **C)** Quantitation of the total corrected EGFL7 fluorescence/ HPF from (C). n=9-15 biological replicate hearts/ group. Scale bar = 50 μm. Mean ± SEM; **P<0.01 by one-way ANOVA with Bonferroni's post-hoc test.

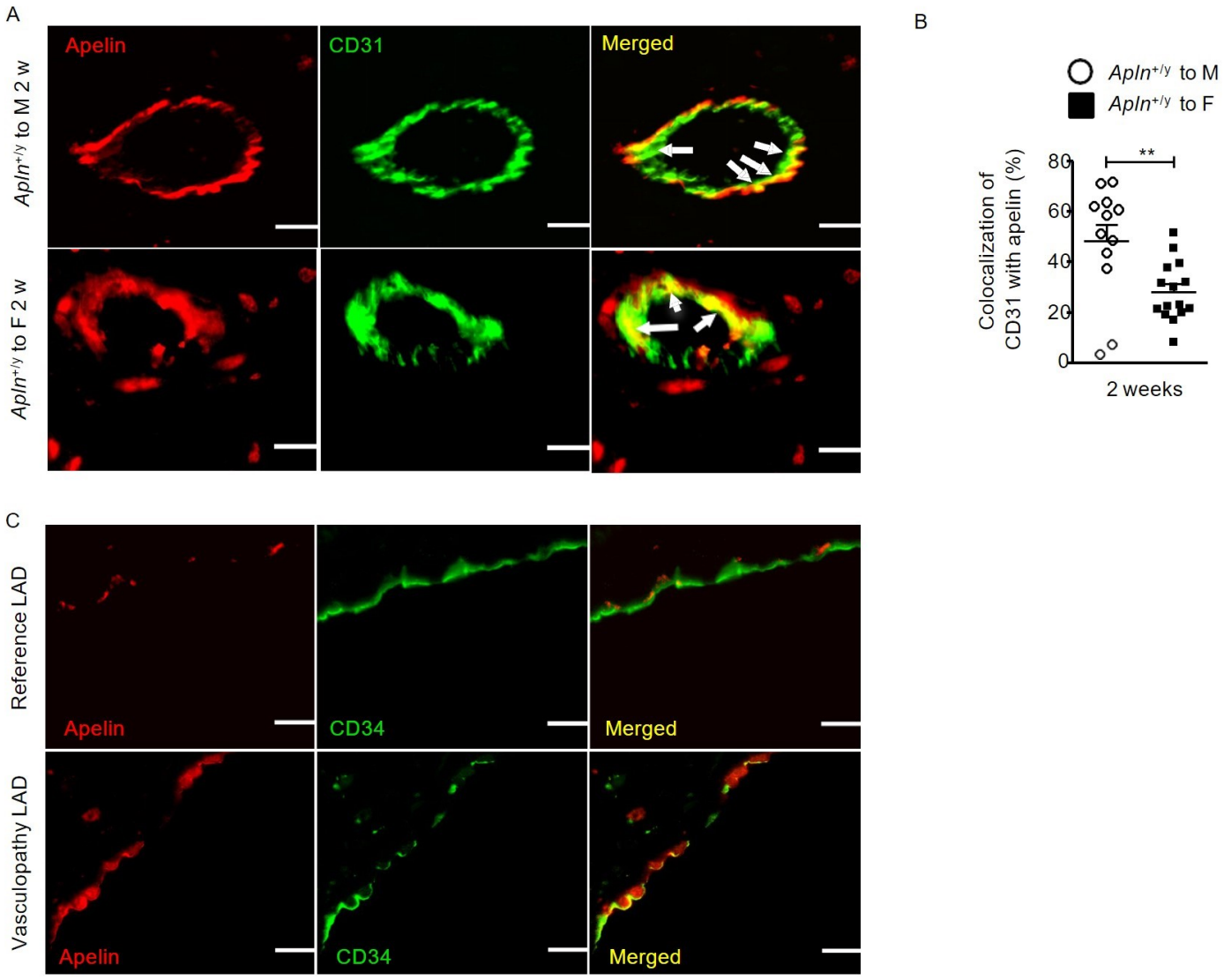


Figure 3.9: Apelin expression in heart grafts.

A) Confocal photomicrographs show double immunofluorescent staining of apelin (red) and endothelial CD31 (green) in heart grafts at two weeks post-transplant (isograft hearts with reperfusion injury are represented in the top panel *vs* heart allografts with immune and reperfusion injury are in the lower panel). Apelin knockout ($Apln^{-/y}$) heart grafts show no apelin staining. Scale bar = 50 µm. **B)** Quantitation of endothelial cell co-localization with apelin (n=12-15 biological replicate hearts/ group). Mean ± SEM; **P<0.01 by Student's t-test. **C)**

Photomicrographs of reference human left anterior descending artery (LAD samples; upper panel) and human LADs with vasculopathy (n=4, lower panel) double immunofluorescence-stained for the human endothelial marker CD34 (green) and apelin (red). Scale bar = 50 μm.

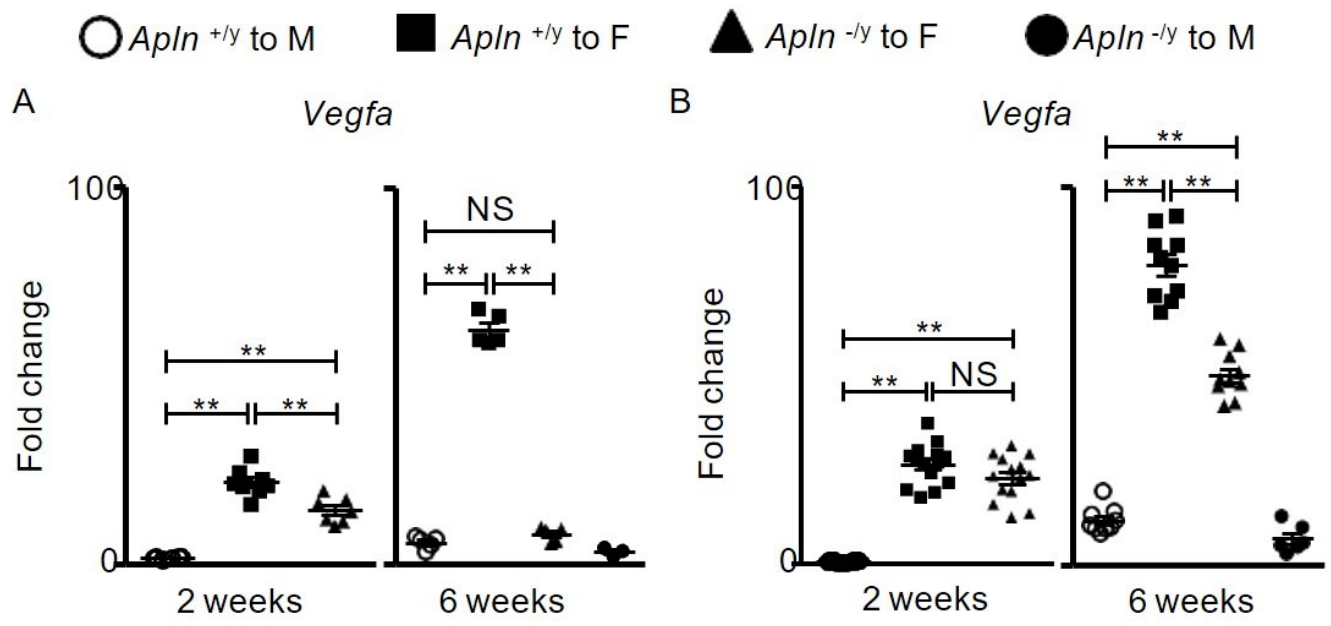


Figure 3.10: *Vegfa* expression in mouse heart allografts.

A) Isolated coronary arteries (consecutive samples pooled in pairs for analysis (n=3-8 pairs)), and **B)** myocardium (n=6-15 biological replicates) at 2- and 6-weeks post-transplant, relative to normal hearts. Mean ± SEM; **P<0.01 and NS= non-significant by one-way ANOVA with Bonferroni's post-hoc test.

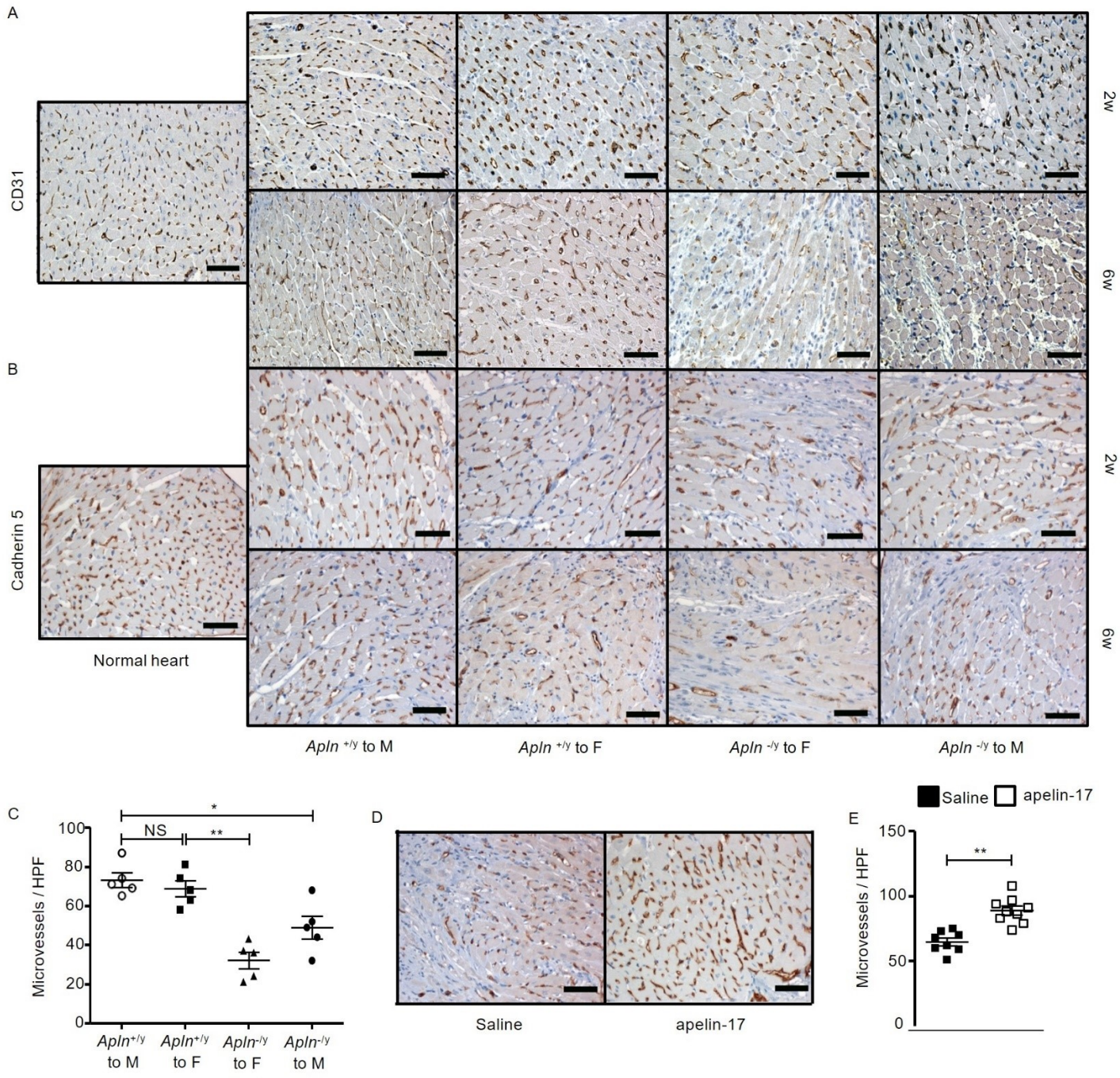
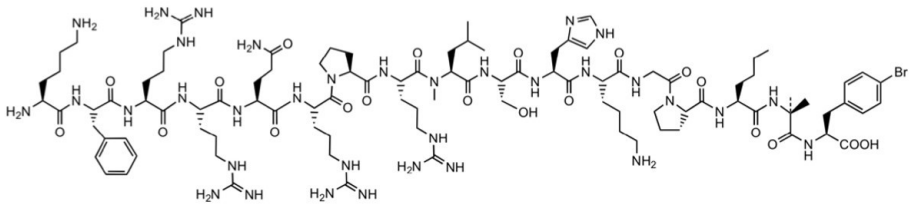


Figure 3.11: Microvessel density in mouse heart grafts.

Immunohistochemical staining of endothelial marker **A)** CD31 (brown) or **B)** VE-Cadherin (brown) among mouse heart grafts at 2- and 6-weeks post-transplant. Quantitation is shown in Figures 3.1A and 3.3C. **C)** Quantitation of VE- Cadherin^{+} microvessels at six weeks post-

transplantation. n=6-10 biological replicate grafts/ group. Scale bar= 50μm. Mean ± SEM; NS= nonsignificant, **P<0.01 by one-way ANOVA with Bonferroni's post-hoc test. **D)** Photomicrographs show VE-Cadherin microvessel staining of hearts of allograft recipient mice treated with saline (n=8) or APLN-17-analogue (n=9) from week 2 through week six post-transplantation. Quantitation of microvessel density is shown in **E)**. **P<0.01 by Mann Whitney.

A



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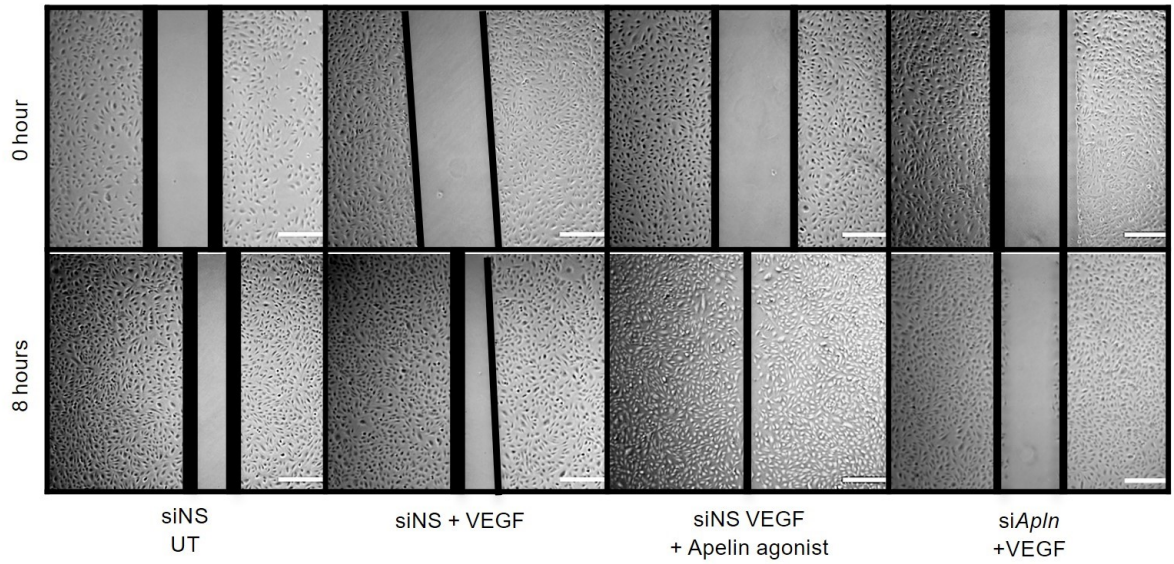
0 hour
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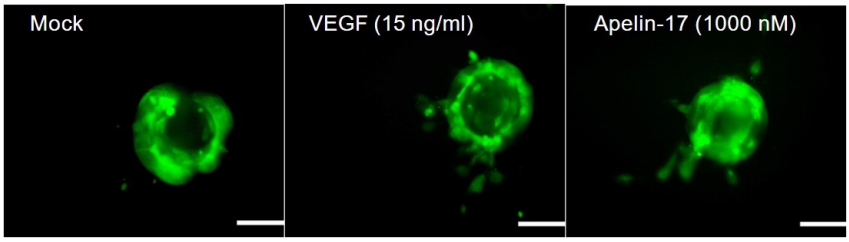
siNS
UT
siNS + VEGF
siNS VEGF
+ Apelin agonist
si*Apln*
+VEGF

C


Mock
VEGF (15 ng/ml)
Apelin-17 (1000 nM)

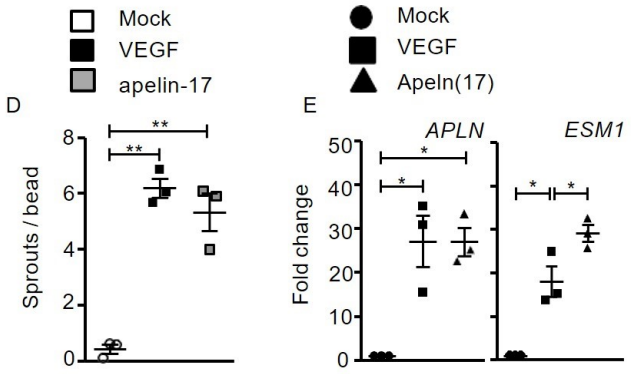

Mock
VEGF
apelin-17
D
Sprouts / bead
Mock
VEGF
Apeln(17)
E
APLN
ESM1
Fold change

Figure 3.12: An APLN-17 analogue promotes closure and tip-cell differentiation in wounded endothelial cell monolayers.

A) The synthetic N-MeLeu9-apelin-17 (APLN-17) agonist peptide, compound 11 (McKinnie et al., 2017). **B)** HUVECs were transfected with non-specific (siNS) or *APLN* siRNA, then plated at a higher confluence.

The monolayers were wounded and then treated with VEGF (50 ng/mL) or APLN-17 (1uM). Quantitation of the experiments is shown in Figure 3.3D. n=5. Scale bar = 50 μm. **C)** Synthetic APLN-17 agonist peptide induces angiogenic sprouting in 3D HUVEC cultures (n=3). Scale bar = 95 μm. **D)** Quantitation of angiogenic sprouting in mock-, VEGF- (15 ng/ml), or APLN-17- (1 μM) stimulated cultures. **E)** Tip cell gene expression in cultures from (D). n=3 biological replicates. Mean ± SEM; * $p<0.05$, ** $p< 0.01$ by one-way ANOVA with Bonferroni's post-hoc test

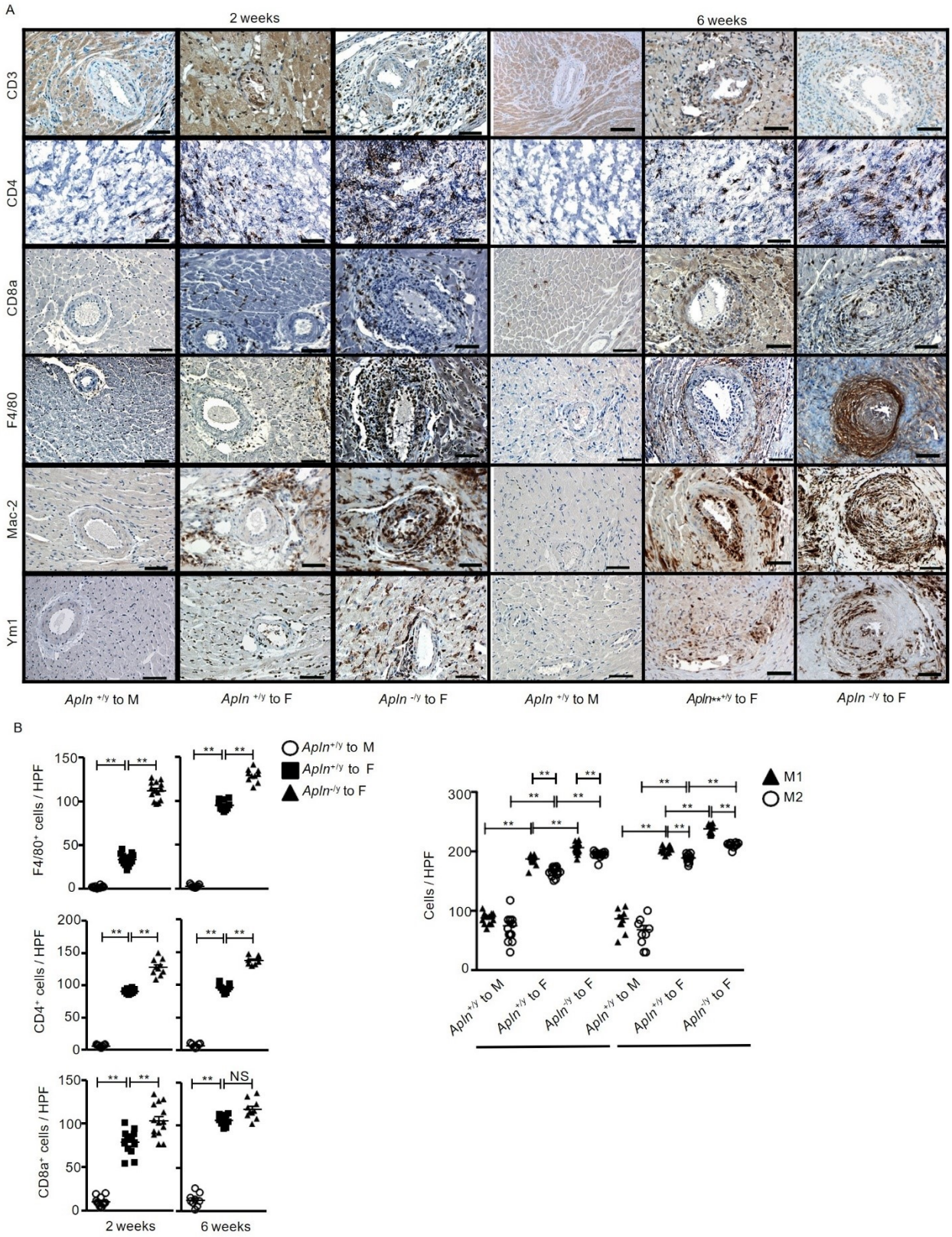


Figure 3.13: Characterization of leukocytes infiltrating mouse heart allografts.

A) Photomicrographs showing immunohistochemical staining of $CD3^+$ total, $CD4^+$, or $CD8a^+$ subsets of T lymphocytes; F4/80 macrophages, Mac-2^+ M1, or $YM1^+$ M2 macrophage subsets at 2- and 6-weeks post-transplant. CD3 and Mac-2 immunostains of week-2 grafts have been shown in Fig 3.4A. **B)** Quantitation of the inflammatory cellular infiltrate among groups. Scale bar = 50μm. n=9-15 biological replicate hearts. Mean ± SEM; **P<0.01 and NS= nonsignificant by one-way ANOVA with post hoc Bonferroni's post-hoc test.

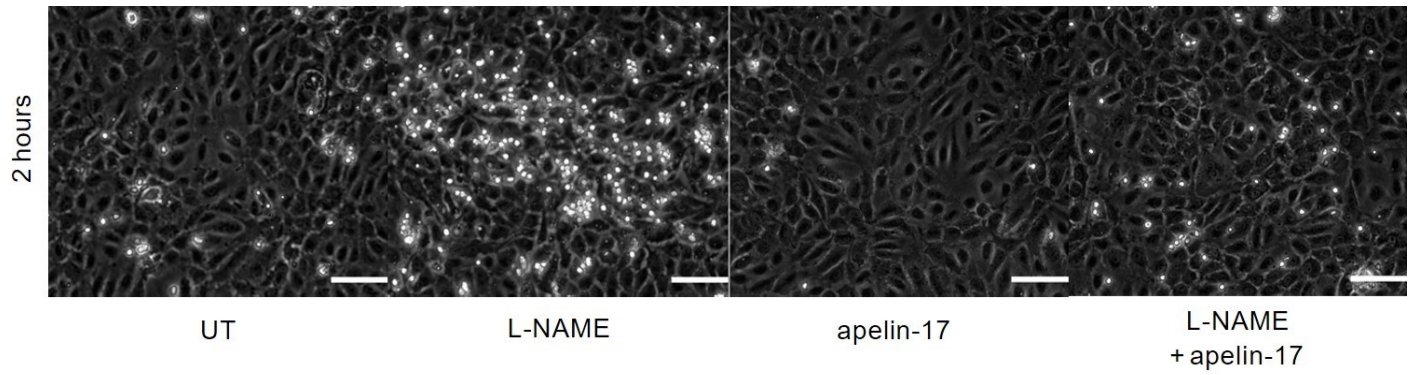


Figure 3.14: APLN-17 inhibits monocyte adhesion to endothelial cell monolayers.

Human umbilical vein endothelial cells were pretreated with TNFα (100 ng/mL) for 18 hours, then primary human monocytes were added to the co-culture for 2 hours in the presence of APLN-17 with or without the nitric oxide synthase inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), as indicated. The (phase-bright) monocytes adherent after gentle washes were photographed. The quantitation is shown in Figure 3.5D. Representative of n=5 biological replicates. Scale bar = 50 μm.

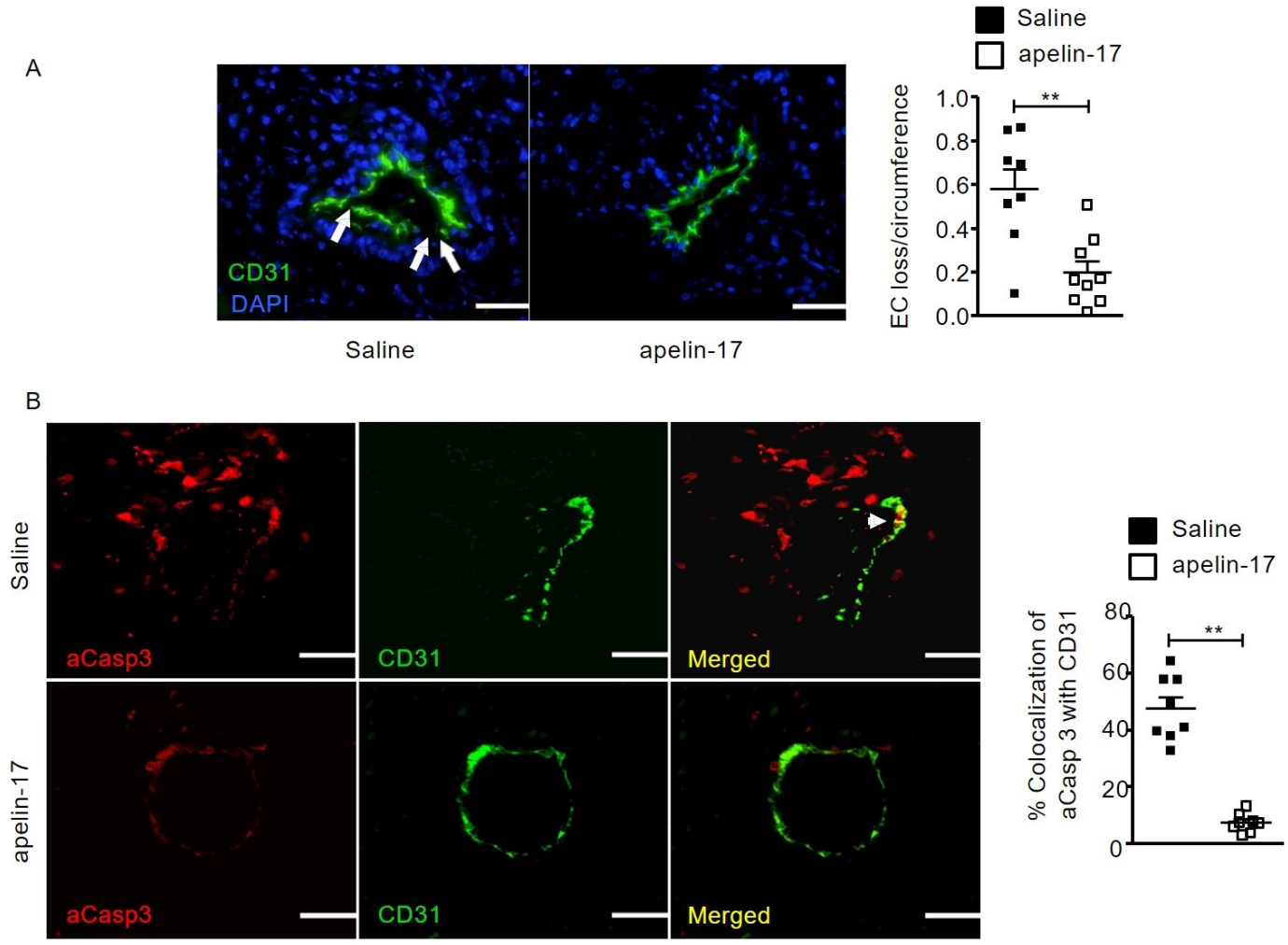


Figure 3.15: APLN-17 reduces endothelial cell loss and apoptosis.

A) Photomicrographs show immunofluorescent staining of endothelial cell CD31 (green) in heart allografts from mice treated with either saline (n=8) or APLN-17 (n=9) from week 2 through week six post-transplantation. Quantitation of arterial endothelial gaps (right panel). **B)** Confocal photomicrographs show immunofluorescent staining for cleaved caspase 3 (red) and endothelial cell CD31 (green; left panel). Quantitation of the cleaved caspase 3^+ co-localization with $CD31^+$ ECs by Mander's coefficient analysis (right panel). n=8-9 mice. Scale bar = 50 µm. Mean ± SEM; **P<0.01 Mann Whitney.

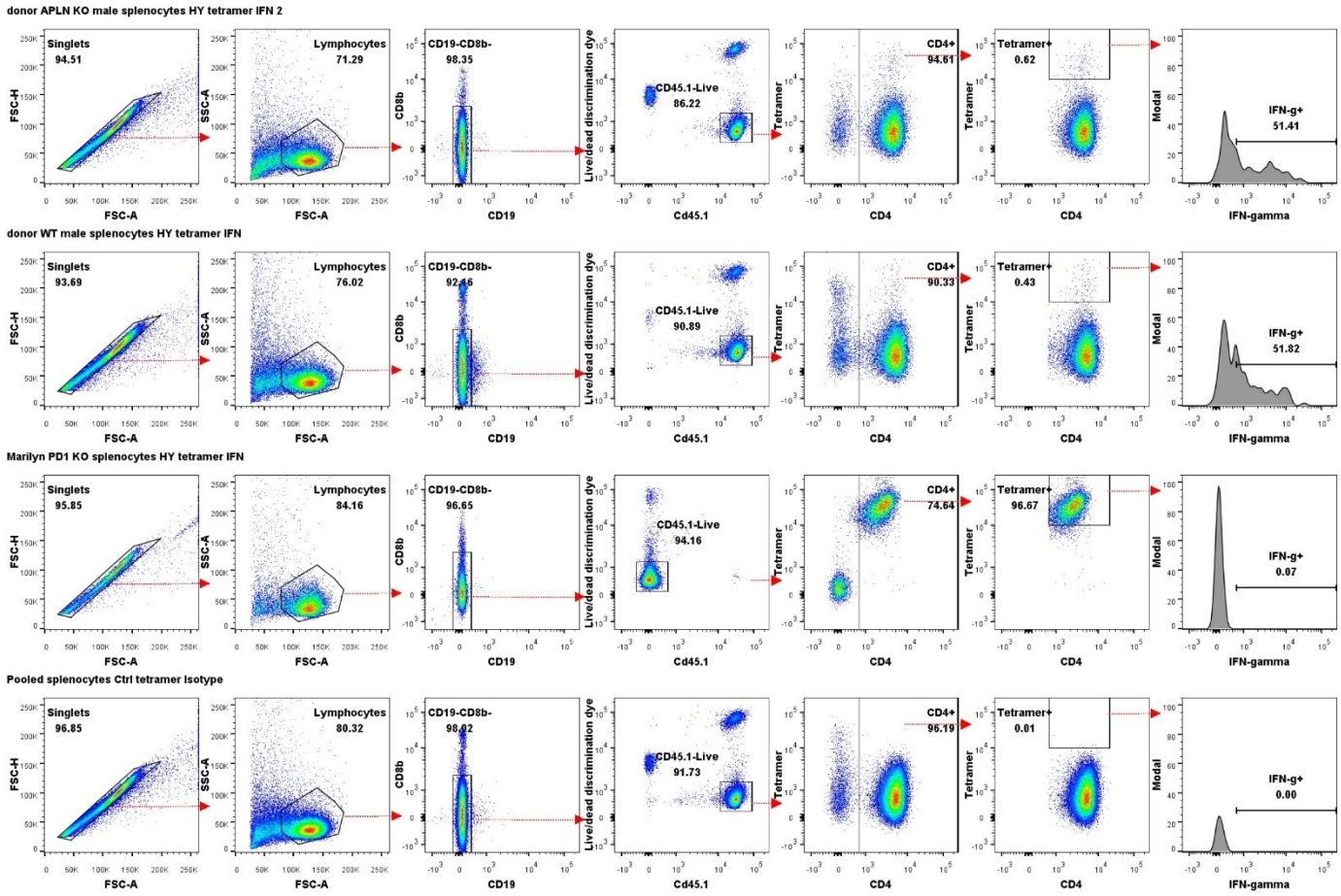


Figure 3.16: Gating strategy for male-antigen-specific CD4 T cells.

A depiction that shows the gating strategy used to identify live CD19-CD8β-CD45.1^{+} (host) IFN-γ^{+} HY-I-A^{b+} CD4^{+} cells from the spleens of the *Apln*$^{-/y}$ (1st row) *Apln*$^{+/y}$ (2nd row) heart grafted mice at two weeks post-transplantation. Splenocytes from female Marilyn-*Rag2*$^{-/-}$-*pd1*$^{-/-}$ mice were used as positive control cells (3rd row). Isotype control of anti-mouse IFN-γ antibodies (4th row) excluded non-specific binding from the staining (n=4-7 biological replicates). The same gating strategy is used for determining the frequencies of CD44^{hi} cells.

Chapter 4 : Endothelial Phosphoinositide 3-Kinase-beta Inactivation Confers Protection from Immune-mediated Vascular Injury

Abstract

Heart transplant and recipient survival are limited by immune cell-mediated injury of the graft vasculature. We examined the role of the phosphoinositide 3-kinase-beta (PI3Kbeta) isoform in endothelial cells (EC) during coronary vascular immune injury and repair in mice. In minor HY-antigen mismatched allogeneic heart grafts, a robust immune response was mounted to each wild-type, PI3Kbeta inhibitor-treated, or endothelial-selective PI3Kbeta knockout (ECbetaKO) grafts. However, microvascular EC loss and progressive occlusive vasculopathy only developed in control, but not PI3Kbeta -inactivated hearts. We observed a delay in inflammatory cell infiltration of the ECbetaKO grafts, particularly in the coronary arteries. Surprisingly, this was accompanied by an impaired display of pro-inflammatory chemokine and adhesion molecules by the ECbetaKO ECs. In vitro, TNF-alpha-stimulated endothelial ICAM1 & VCAM1 expression was blocked by PI3Kbeta inhibition or RNAi. Selective PI3Kbeta inhibition also blocked TNFalpha-stimulated degradation of IKB-alpha and nuclear translocation of NF-kB p65 in EC. These data identify PI3Kbeta as a therapeutic target to reduce vascular inflammation and injury.

Introduction

Heart failure is associated with high morbidity and mortality, and despite advances in heart failure management, heart transplantation (HTx) remains the best therapy for individuals with end-stage disease (Toyoda et al., 2013). However, solid-organ transplant and heart transplant recipient survival is limited by chronic immune responses directed against the graft vasculature (Costello et al., 2013b; Khush et al., 2021; Loupy et al., 2020). In heart transplants, immune-mediated vascular injury affects both coronary arteries and the heart microvasculature (Chih et

al., 2016; Loupy et al., 2016). Dynamic vascular repair is mitigated by loss of the microvasculature and arterial injury (A. G. T. Masoud et al., 2020; Raisky et al., 2007), but the maladaptive repair response elicited in coronary arteries results in progressive occlusion of the arterial lumen and occurs despite modern immune suppression regimes. These therapies target mononuclear leukocyte proliferation and function; hence, there is a need to develop complementary clinical strategies that address the vascular response to inflammation and injury.

Previously we demonstrated that the expression of local vascular pro-reparative genes, such as the endothelial-derived peptide apelin, are induced by alloimmune vascular injury and mitigate structural damage to the coronary arteries (A. G. T. Masoud et al., 2020). The apelin receptor is expressed by vascular smooth muscle and endothelial cells (EC). In EC, the apelin receptor is linked, among others, to the pro-reparative phosphoinositide 3-kinase (PI3K) signal transduction pathway by the p110β catalytic isoform (Abul K. Azad et al., 2020; Guillermet-Guibert et al., 2008). However, it is not clear how apelinergic signals are translated by vascular cells to mitigate injury.

Endothelial PI3K-β activity couples G-protein coupled cell surface receptors to mTOR and other effectors such as Akt (Ackah et al., 2005; Phung et al., 2006). PI3K activity in EC is both required and rate-limiting for angiogenesis (Graupera et al., 2008; Hamada et al., 2005; Huang & Kontos, 2002). The class I PI3Ks comprise four catalytic subunits (p110α, β, γ and δ) that are bound to regulatory subunits (Vanhaesebroeck et al., 2016). Whereas p110α and p110β show a broad tissue distribution, p110γ and p110δ are mainly found in leukocytes (Bilanges et al., 2019b). The p110α isoform is the dominant form coupled to receptor tyrosine kinases such as the VEGF receptor-2 (VEGFR2) in EC (Graupera et al., 2008), whereas p110β and p110γ are coupled to pro-angiogenic endothelial GPCRs (Graupera et al., 2008). Selective perturbation of PI3K-b isoform activity in

mice has been found to have subtle effects on glycemic control, platelet adhesion, and spermatogenesis (Arcucci et al., 2021). Defective angiogenesis is noted in pericyte-deficient PI3K-β mutant mice (Figueiredo et al., 2020) and EC-deficient mice if PI3K-α is inhibited (Abul K. Azad et al., 2020).

In this report, we studied the effect of vascular PI3K-β inactivation on immune cell-mediated vascular injury. The experiments reveal a previously unknown requirement for endothelial PI3K-β activity to regulate EC expression of pro-inflammatory chemokines and adhesion molecules.

Results

Vascular injury and maladaptive repair limit the survival of the heart and other vascularized solid organ allografts. The disease is characterized by progressive expansion of the arterial intima with consequent obliteration of the vascular lumen and microvessel loss. Earlier work identified a role for the G-protein coupled apelin receptor to promote vascular repair; however, key downstream signalling events that mediate the effect are not well defined. We investigated the Phosphoinositide 3-kinase (PI3K) pathway, coupled to the apelin receptor by the catalytic p110β isoform, to determine if beta isoform activity was required.

We exploited male-to-female heart transplantation in mice, known to elicit an indolent immune response and vascular disease akin to transplant vasculopathy in humans. In the first series of experiments, recipient mice were treated with the PI3K-β isoform-selective oral inhibitor, GSK2636771 (GSK), starting two weeks after transplantation. The hearts were retrieved at six weeks post-transplantation. We found that hearts harvested from recipients treated with the carrier developed marked expansion of the coronary arterial intima, largely occluding the arterial lumen (Fig 4.1 A, B). In striking contrast, arterial intimal expansion in hearts from GSK-treated mice showed little change compared to non-transplanted donor hearts.

Further, we observed that the microvessel density of the myocardial compartment was reduced by more than 50% in the vehicle-treated mice, reflecting alloimmune injury directed against vascular endothelium. GSK-treatment protected the microvasculature from loss. This surprising result suggests that PI3K-β inhibition of immune cells or parenchymal cells of the allograft served to mitigate CAV vascular disease.

To determine if PI3K-β inactivation of the heart coronary endothelium accounted for vascular protection from the effects of immune injury, we transplanted hearts from inducible Tie2-Cre$^{ERT2+/-}$/ *Pik3cb*$^{f/f}$ (ECβKO) donor mice, pretreated with tamoxifen two weeks before the donor's heart was harvested for transplant. The effect of endothelial cell PI3K-β inactivation was compared with tamoxifen-treated CRE$^{ERT2-/-}$/ *Pik3cb*$^{f/f}$ littermates. The effect of recombination was monitored by RT-PCR of donor lung endothelium acquired at the time of donor heart transplantation (Fig 4.8). Hearts were recovered at two weeks and six weeks post-transplant. We observed a progressive increase in the extent of the intima expansion in the littermate control hearts and a reduction in the microvessel density in the myocardium (Fig 4.1C, D). Endothelial *Pik3cb* knockout protected the graft artery and microvasculature. This finding establishes that endothelial PI3K-β activity, independent of PI3K-β expression in the immune system, is required to protect against vasculopathy.

The vascular endothelium is a target for alloimmune injury; hence we directly evaluated the effect of PI3K-β inactivation on injury to the graft endothelium. As shown in Fig 4.2A and Fig 4.9, discontinuities or gaps in the arterial endothelium were quantified from tissue sections immune-stained for CD31. The Isogeneic male-to-male heart transplant group has no endothelial gaps detected at two weeks post-transplant. However, a progressive increase in the focal loss of endothelial cell coverage was observed in the littermate control hearts at 2- and 6-weeks post-

transplant. In contrast, in ECβKO hearts, the area of focal gaps in the endothelium was markedly reduced at two weeks post-transplant and did not measurably increase at the later timepoint. This data is supported by evaluating activated caspase three immunostaining of the CD31-positive coronary arterial endothelium in 1 μm confocal tissue sections. Cleaved caspase 3 (aCasp3) staining patterns of the endothelium, the cells in the expanded intima, and medial vascular smooth muscle cells were evident in littermate control hearts (Fig 4.2B, C; Fig 4.10). A substantial reduction in the $CD31^+$ EC co-stained with aCasp3 was evident in the ECβKO coronary arteries. Notably, the aCasp3 staining of VSMC was also markedly reduced in ECβKO hearts (Fig 4.10). Taken together, this data indicates that the ECβKO graft coronary arteries displayed less immune-cell-mediated injury compared to the wild-type littermate controls.

Next, we evaluated vascular repair in the micro-dissected coronary artery and myocardial compartments. Previous work identified the upregulation of endothelial tip cell genes in mouse and human transplant coronary vessels and suggested that apelin-dependent repair is rate-limiting in progressive vasculopathy (A. G. T. Masoud et al., 2020). Although the EC tip cell gene *Apln* was upregulated *vs* expression in the baseline donor heart, we did not find increased expression in the 2-week ECβKO *vs* wild-type hearts in either artery (e.g., *Apln* 8 ± 1.4 vs 32 ± 9-fold change; p= 0.01) or microvascular myocardial (e.g., *Apln* 14.9 ± 6.3 vs 15.1 ± 4.8; p=NS) compartments (Fig 4.13). Pro-angiogenic *Vegfa* expression in the myocardium trended lower in the 6-week ECβKO heart transplants, consistent with better-maintained perfusion. These data indicate that enhanced repair is not a dominant feature of the protection afforded by EC PI3K-β inactivation.

Immune cell infiltration and subsequent injury of the graft vasculature initiate vasculopathy. We, therefore, examined the effect of endothelial PI3K-β loss on $CD8^+$ lymphocytes and Mac-2^+ (M1) macrophage infiltration of the artery and myocardium. At two weeks post-transplant, when an

early vascular injury is evident, we observed a modest ~25% reduction in the density of lymphocytes and macrophages in the ECβKO myocardium, which at six weeks post-transplant was like the wild-type littermate hearts (Fig 4.3A, B). In addition, M2 macrophage (labelled with $YM1^{+}$) infiltration was decreased in ECβKO hearts (Fig 4.14). In contrast, the reduction in leukocyte infiltration of the artery was more pronounced (Fig 4.3C). At six weeks post-transplant, the leukocytes in the expanded arterial intima of the control hearts were too numerous to count, whereas the infiltrate was little changed from 2 weeks post-transplant in the ECβKO (Fig 4.3A).

We further examined the gene expression of *Ifng* and *Tnfa*, characteristic cytokines of alloimmune responses implicated in the development of vasculopathy. We found reduced *Ifng* in the ECβKO artery at the early 2-week timepoint (Fig 4.3D) but could resolve little change in *Ifng* or *Tnfa* between the ECβKO and littermate coronary arteries at six weeks post-transplant. In the myocardial compartment, reduced *Ifng* and *Tnfa* were evident at the later time point (Fig 4.3E). Moreover, interferon-γ-dependent expression of *Cxcl11* and the tumour necrosis factor alpha-dependant expression of *Il6* in the artery and the myocardial compartment were unchanged by endothelial PI3K-β loss (Fig 4.11). These data suggested that leukocyte infiltration of the artery was delayed in the ECβKO *vs* wild-type hearts, but the vascular cells were nevertheless exposed to complex pro-inflammatory stimuli.

Perturbation of immune cell trafficking to the allograft has been associated with altered populations of lymphocytes, reported to be biased to regulatory T cell enrichment, which might mitigate direct alloreactive T cell activities against graft cells (Plenter et al., 2018). We, therefore, extracted lymphocytes infiltrating the myocardium for more detailed phenotyping by flow cytometry at two weeks post-transplantation. We exploited H-2^{b}-HY and I-A^{b}-HY tetramers to identify alloreactive lymphocytes within cell populations in the spleen and myocardial infiltrate.

We confirmed that an increased frequency of alloreactive $CD8^+$ and $CD4^+$ T cells was detected in the spleen of grafted mice, consistent with an established alloimmune response at two weeks post-transplant (Fig 4.4A). Heart-infiltrating lymphocytes were enriched in the fraction stained with each of class I and class II tetramers, but no difference in total or $CD44^{hi}$ memory alloreactive lymphocytes was observed between ECβKO and wild-type hearts (Fig 4.4B, C).

Further, a similar fraction of $CD8^+$ T cells expressed cytolytic markers granzyme B and perforin between the groups (Fig 4.4D). We observed a low fraction of $FoxP3^+$ CD4 T cells but no enrichment in the ECβKO hearts (Fig 4.4E). Further, we did not find a difference in the expression of exhaustion markers, TIM3 or LAG3, among lymphocytes from ECβKO *vs* wild-type hearts (Fig 4.4F, G). Taken together, these data indicate that the lymphocytes egress from blood to the artery and the myocardium was reduced; however, the alloreactive lymphocytes maintained similar functional capacity in ECβKO *vs* wild-type hearts.

To investigate the defective recruitment of mononuclear inflammatory cells to the arterial intima, we examined the expression of CX3CL1, a chemokine that participates in graft rejection and is selectively expressed by arterial endothelial cells (Ahn et al., 2004; Haskell et al., 2001). CX3CL1 was absent from the coronary endothelium of the naïve donor heart. CX3CL1 was highly expressed by arterial but few microvascular ECs two weeks after transplantation of wild-type hearts (Fig 4.5A, B). In striking contrast, CX3CL1 expression was absent from ECβKO endothelium post-transplant. Next, we studied *CX3CL1* expression in human aortic EC (HAEC) *in vitro*. As previously reported, CX3CL1 was optimally induced with dual interferon-γ plus TNFα stimulation. However, treatment of HAEC with TGX221 to selectively inhibit PI3K-β activity completely blocked CX3CL1 expression by western blot (Fig 4.5C, D). TGX221 treatment completely blocked HAEC IFN-γ plus TNFα-induced CX3CL1 expression by qRT-

PCR and expression of EC adhesion molecules VCAM1 and ICAM1, but not the expression of the interferon-γ-induced genes, CD74 or CXCL10 (Fig 4.5E).

We studied the effect of endothelial PI3K-β inactivation on the EC expression of pro-inflammatory adhesion molecules. ICAM-1 immunostaining of the heart allografts two weeks post-transplant demonstrated widespread ICAM-1 expression by microvascular EC in wild type, but not ECβKO hearts (Fig 4.5F, G). ICAM-1 staining of infiltrating leukocytes was visible in the myocardium of both groups (Fig 4.5F).

In vitro, HUVEC were treated with TGX221 to inhibit PI3K-β activity, then stimulated with TNFα and examined by western blot to evaluate adhesion molecule expression (Fig 4.6A, B, E, F). We observed TNFα treatment-induced phosphorylation of Akt, and the Akt substrate eNOS, indicating upstream PI3K activity, in a TGX221-sensitive manner (Fig 4.6A). Thus, TNFα stimulation elicits PI3K activity via the p110β isoform in EC. Further, TGX221 blocked TNFα-stimulated ICAM-1 induction (Fig 4.6A, B). To confirm the observation, HUVEC were treated with siRNA to knock down *PIK3CB* expression (Fig 4.6C, G). TNFα-stimulated expression of ICAM-1 was abolished in PIK3CB-deficient EC (Fig 4.6C, D). Moreover, the effect of EC PI3K-β inactivation on EC expression of VCAM-1 was examined. TNF-α-stimulated induction of VCAM-1 was blocked by either TGX221 or PIK3CB knockdown, like the effect on ICAM-1 (Fig 4.6E-H). Together, we observe endothelial cell inactivation of PI3K-β *in vivo* in the mouse heart and *in vitro* in human ECs prevents the expression of pro-inflammatory chemokine and adhesion molecules used to recruit mononuclear cells to sites of inflammation.

The transcription factor NF-kB integrates a variety of pro-inflammatory cues to elicit chemokine and adhesion molecule expression by vascular endothelial cells (e.g., CX3CL1, ICAM-1, and VCAM-1). We tested the effect of endothelial cell PI3K-β inhibition on TNF-α-stimulated NF-

kB activation. Western blot of TNF-α-stimulated mouse coronary endothelial cells showed a time-dependent degradation of IκBα, the final regulatory component that prevents the release of sequestered NF-kB from the cytosol (Fig 4.7A, B). Treatment with TGX221 blocked TNF-α-stimulated IκBα degradation. Employing a second line of evidence, we examined the translocation of NF-kB p65 from the cytosol to the nucleus. TNF-α stimulation of EC monolayers for 30 minutes resulted in nuclear accumulation of NF-kB p65, visualized by immunofluorescent confocal microscopy of the mouse (Fig 4.7C, D) and human (Fig 4.12) EC. Treatment with TGX221 blocked p65 translocation. These data indicate that TNF-α recruitment of PI3K-β-dependent signalling is required for TNF-α-stimulated NF-kB activity and provides a mechanistic explanation for the dependence of TNF-α-stimulated proinflammatory CX3CL1, ICAM-1, and VCAM-1 induction on PI3K-β activity.

Discussion

Immune injury and maladaptive repair of the arterial vascular endothelium limit graft and recipient survival after heart transplantation despite modern immune suppression treatment (Loupy et al., 2020). Here we identify PI3 kinase-β blockade, and a repurposed pharmacologic compound currently in clinical trials for salvage cancer chemotherapy (Sarker et al., 2021), as an approach to reduce vascular inflammation and limit injury.

The principal finding of this study is that TNF-α-stimulation of inflammatory responses in vascular endothelial cells requires PI3K-β activity. We show that TNF-α uses the PI3K-β isoform to elicit mTORC2 and downstream Akt activation in cultured EC. Further, induction of the endothelial chemokine, CX3CL1, stimulated by IFN-γ plus TNF-α, or adhesion molecules ICAM-1 and VCAM-1, elicited by TNF-α stimulation alone, was blocked by selective PI3K-β inhibition or genetic inactivation. The induction of genes dependent only on IFN-γ was not

affected by selective PI3K-β inactivation. Blockade of adhesion molecule and CX3CL1 expression was evident in both human and mouse EC, indicating that the requirement for PI3K-β activity is conserved across species.

In vivo murine heart transplantation, directed against the minor HY histocompatibility antigen expressed by the male coronary arterial and microvascular endothelium, elicits chronic vascular inflammation without confounding immune suppression. We observed that the inhibition of PI3K-β by oral administration of GSK2636771 after the alloimmune response arrested the progression of obliterative arterial vasculopathy and preserved the coronary microvasculature against immune injury. Selective knockout of the PI3K-β catalytic isoform in the graft endothelium phenocopied the effect of the inhibitor to protect the graft vasculature from injury. A study of the graft-infiltrating lymphocytes confirmed the presence of $CD4^+$ and $CD8^+$ lymphocytes in the graft myocardium, albeit with delayed kinetics. EC PI3K-β loss was not associated with the expression of markers of T cell exhaustion or enrichment of regulatory T cells, as has been reported in chronic tumour inflammation (Popovic et al., 2018). $CD8^+$ and $CD4^+$ T cells were functional, reflected by a transcript for TNF-α, interferon-γ, and interferon-γ-dependent chemokine CXCL11 expression in the graft myocardium and arterial compartments. Nevertheless, immune stains showed markedly reduced *in vivo* expression of CX3CL1 and ICAM-1 on EC, consistent with the *in vitro* findings.

The dramatic protection against arterial disease likely reflects more stringent requirements for chemokine and adhesion molecule presentation by the vascular endothelium to recruit leukocytes under high shear stress conditions. Conversely, the low shear condition of the capillary and post-capillary coronary vessels allowed lymphocyte and monocyte trans-endothelial migration. Similar parenchymal lymphocyte infiltration has been demonstrated in transplanted ICAM1-1

deficient donor hearts, consistent with redundant adhesion ligand use by leukocytes undergoing trans-endothelial migration (Raisanen-Sokolowski et al., 1999). Moreover, CX3CL1-deficiency alone does not change heart transplant survival unless immune suppression is added (Haskell et al., 2001). The perturbed expression of numerous pro-inflammatory molecules by the ECs cumulatively reduces inflammation (Ley, 2001).

The effect of endothelial PI3K-β loss of function on the vascular response to inflammation is complex since PI3K-β activity contributes to pro-angiogenic repair responses and defends the repairing vasculature from immune cell infiltration. For example, apelin-stimulated nitric oxide synthase activity in the endothelium attenuates leukocyte recruitment and is required to protect the vasculature against aggressive immune injury (A. G. T. Masoud et al., 2020). Here we find that TNF-α-stimulated eNOS activation is similarly dependent on PI3K-β. Thus, PI3K-β blockade-mediated inhibition of TNF-α-stimulated chemokine and adhesion molecule display dominates blunted TNF-α-stimulated NO production by the inflamed vasculature.

Selective perturbations of the graft microenvironment have been shown to confer immune tolerance to an allograft. For example, disruption of co-stimulation molecules (Miller et al., 2019) or macrophage activation (Braza et al., 2018) may induce long-term allograft survival associated with impaired lymphocyte function and enrichment of regulatory T lymphocytes. Similarly, in antitumor immunity, suppression of T cell activation with the Programmed death-ligand 1 (PD-L1) or exhaustion of the infiltrating lymphocytes from chronic stimulation may occur (Popovic et al., 2018). Disruption of selective adhesion molecules has generally not afforded long-term protection of graft rejection (Bergese et al., 1995; Lacha et al., 2002; Raisanen-Sokolowski et al., 1999; Stepkowski et al., 1994), but systemic ICAM-1/ LFA-1 blockade has been shown to limit recipient alloreactive lymphocyte expansion (Kwun et al., 2015; Setoguchi et al., 2011). Our

experiments found robust enrichment of the graft with $CD44^{hi}$ allospecific lymphocytes showing cytotoxic perforin and granzyme molecules and interferon-γ expression, indicating competent effector function. The lymphocytes were not enriched in $FoxP3^{+}$ Treg cells or cells bearing exhaustion markers. We speculate that in the face of preserved vascular repair, T cell-mediated cytotoxicity against the microvascular EC may be blunted by defective interaction with EC displaying low amounts of adhesion molecule ligands, enabling net preservation of the microvasculature.

The finding that selective PI3K-β inactivation blocks the effect of TNF-α-dependent pro-inflammatory responses in EC was unexpected. NF-kB-dependent gene transcription has been shown to drive the expression of CX3CL1 and endothelial adhesion molecules to promote leukocyte recruitment in response to inflammatory cues and disturbed flow at sites of atherosclerosis (Mohan et al., 1997; Nagel et al., 1999). However, an earlier study of the effect of PI3 kinase inhibition using pan-isoform first-generation compounds did not identify a role of the PI3K in EC NF-kB activation nor the pro-inflammatory adhesion molecule expression in response to TNF-α (Gustin et al., 2004; Madge & Pober, 2000). In agreement with this finding, subsequent work has defined the molecular detail of NF-kB pathway activation downstream of TNF-α superfamily receptors in various cell types (Zhang et al., 2017). However, oncogenic PI3Kα gain-of-function mutations have been demonstrated to drive NF-kB activity in cancer cells (Hutti et al., 2012).

Recent interest in clinical PI3 kinase-β inhibition, primarily for the treatment of PTEN-deficient cancers that upregulate PI3 kinase activity using the p110β isoform, has prompted a detailed investigation of the roles of individual PI3K isoforms and revealed complexity in the regulation of the parallel pathways. In cancer cells, for example, resistance to PI3K-α appears dependent on

PI3K-β activity to recruit the mediators downstream of the PI3 kinases (Costa et al., 2015; Schwartz et al., 2015). These studies identify complex cellular responses to isolated PI3 kinase isoform inhibition, including the use of alternate PI3K isoforms and the release of constitutive inhibitory pathways that normally constrain mTOR and Akt activities (Costa et al., 2015; Rodrik-Outmezguine et al., 2011; Schwartz et al., 2015). Taken together, these findings suggest that PI3K-β inactivation is likely to have an indirect effect on TNF-α-induced NF-kB pathway activation in EC.

Recognition that endothelial cells depend on PI3K-β to elicit NF-kB-regulated pro-inflammatory gene expression is relevant to a variety of clinical syndromes of vascular inflammation, including, for example, atherosclerosis. Earlier work identifies that genetic inactivation of EC NF-kB signalling decreases atherosclerotic plaque formation (Gareus et al., 2008). Similarly, EC loss of RGC-32 is reported to impair NF-kB activation and suppress adhesion molecule expression and lesion development in an atherosclerosis model (Cui et al., 2018). Re-purposed oral PI3K-β inhibitors may be broadly useful in these diseases.

Acknowledgments

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Figures

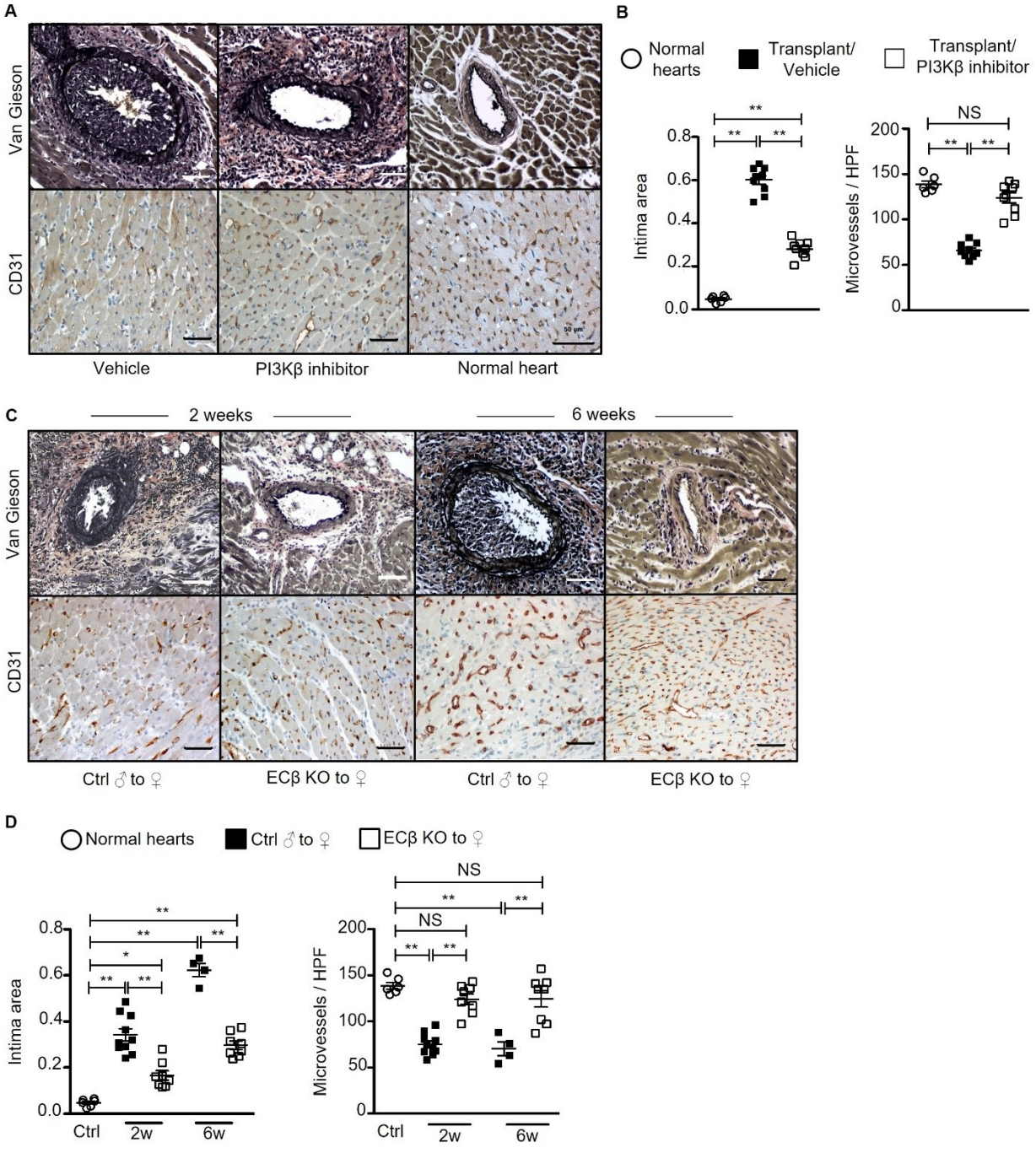

Figure 4.1: PI3 kinase-β inactivation mitigates vasculopathy in transplanted hearts.

(A) Transplanted hearts from vehicle (n = 9) or GSK2636771(GSK)-treated mice (n = 9) were recovered six weeks after transplantation, stained with the Van Gieson elastin stain (top panels)

or immuno-stained for endothelial CD31 (PECAM; bottom panels). Fibro-cellular expansion of the intima, bounded by the internal elastic lamina and the lumen, is marked in a coronary artery from a vehicle-treated heart (upper panels). Little neointima expansion is evident in coronary arteries from a normal heart or a transplanted heart from a GSK-treated mouse. Rarefaction of myocardial microvessels is evident in a vehicle-treated vs normal or GSK-treated heart (lower panels). **(B)** Quantification of the neointima area (left) and myocardial $CD31^+$ microvessel density (right) of the groups in (A) as described in Methods. **(C)** Transplanted ECβKO or wild-type hearts (n=4-10) were recovered at 2- or 6-weeks post-transplant and stained as in (A). **(D)** Quantification of the neointima area (left) and myocardial microvessel density (right) of the groups in (C). HPF = high-power field. Scale bars: 50 μm. Mean ± SEM; *P < 0.05, **P < 0.01, NS= non-significant by 1-way ANOVA with Bonferroni's post hoc test.

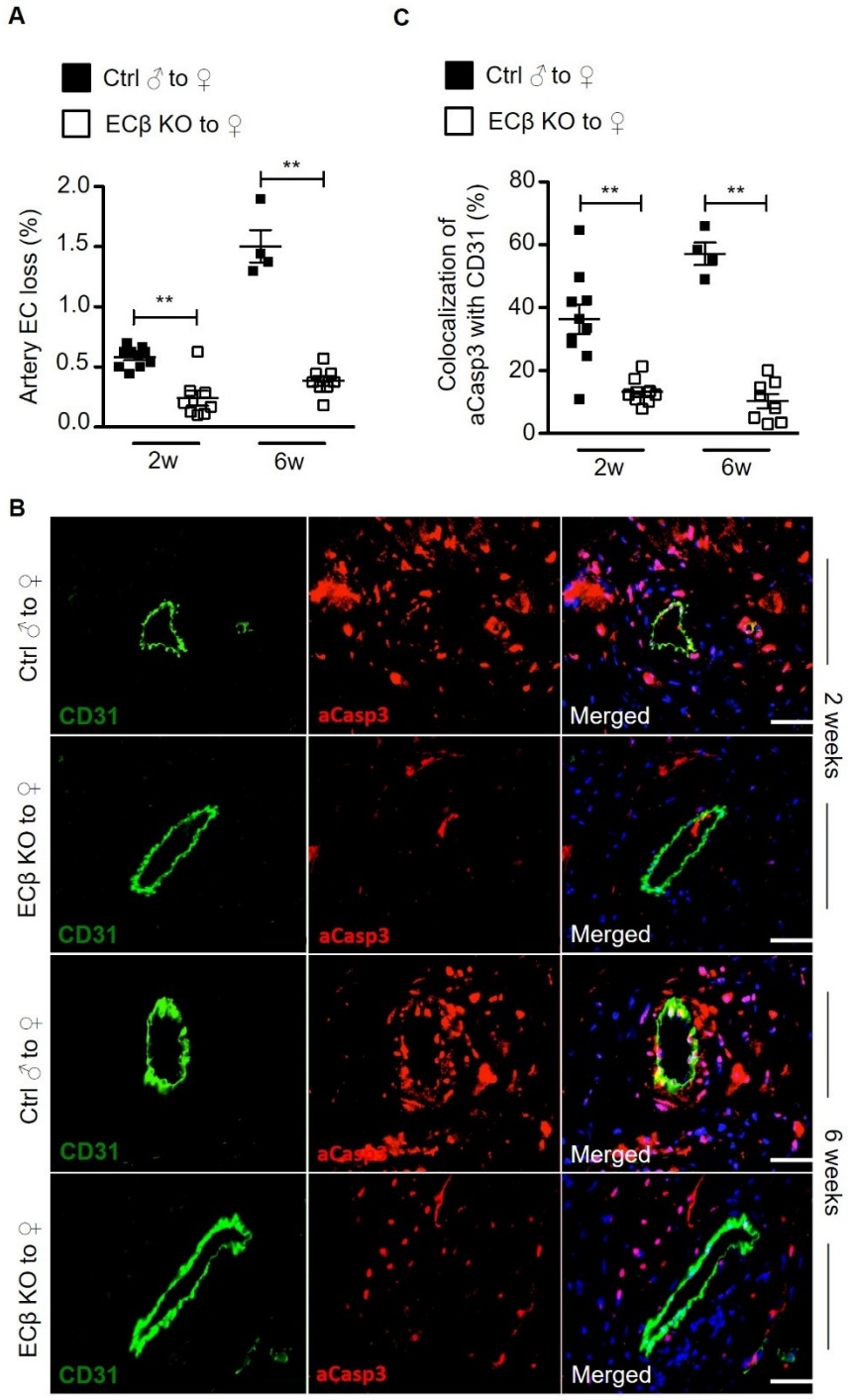

Figure 4.2: PI3 kinase-β inactivation protects heart allografts from endothelial injury.

(A) Quantification of gaps in the arterial endothelium in cross-section as described in Methods, from ECβKO or wild-type hearts recovered post-transplant. **(B)** Confocal photomicrographs (1

μm optical section) of intramyocardial coronary arteries cross-sections from the transplanted hearts. $CD31^+$ EC (left panels), activated caspase 3 (aCasp3, red; center panels), and the merged channels (right panels) are shown. **(C)** Quantification of the fraction of $CD31^+$ EC co-stained with aCasp3 in the groups (n=4-10 mice/ group) in (B). Scale bars: 50 μm. Mean ± SEM; **P < 0.01, by 1-way ANOVA with Bonferroni's post hoc test.

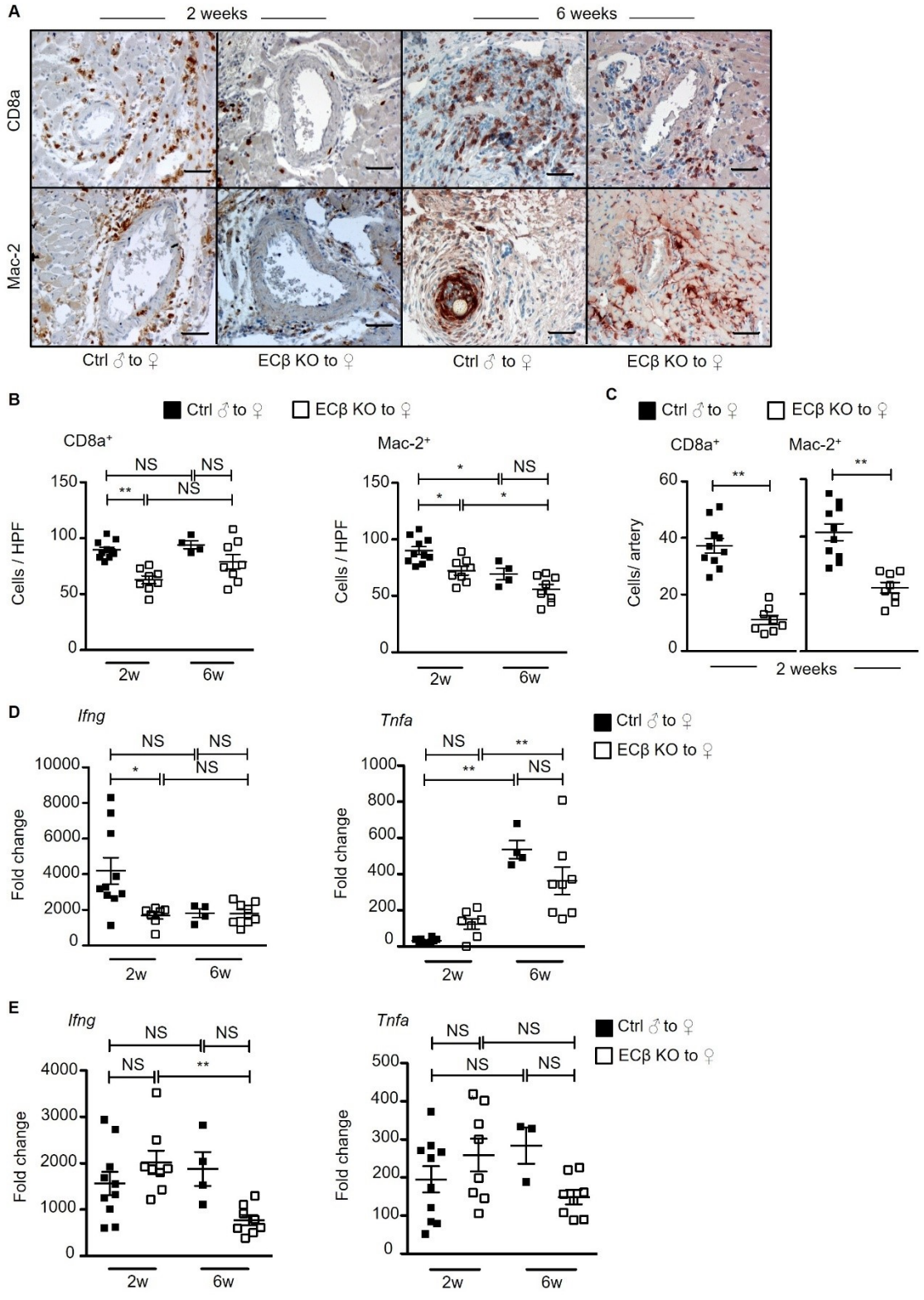

Figure 4.3: Immune cell infiltration of heart allografts.

(A) Photomicrographs of the $CD8^+$ lymphocytes (top panels), and Mac-2^+ monocyte/ macrophages (bottom panels) among the heart from ECβKO or wild- type donors (n=4-10 mice/ group) recovered at 2- or 6-weeks post-transplant. **(B)** Quantification of the number of myocardial-infiltrating $CD8^+$ (left) and Mac-2^+ cells (right) in (A). **(C)** Quantification of the $CD8^+$ (left) and Mac-2^+ (right) cells infiltrating the arterial compartment in hearts recovered 2 weeks post- transplant (n=8-10 mice/ group). Mean ± SEM; **P < 0.01 by Student's t-test. Pro-inflammatory *Infg* or *Tnfa* cytokine gene expression determined by qRT-PCR among micro-dissected coronary arteries **(D)** or myocardium **(E)** samples from ECβKO or wild-type donor transplanted hearts (n=4-10 mice/ group). HPF = high-power field. Scale bars: 50 μm. Mean ± SEM; *P < 0.05, **P < 0.01, NS, non-significant by 1-way ANOVA with Bonferroni's post hoc test.

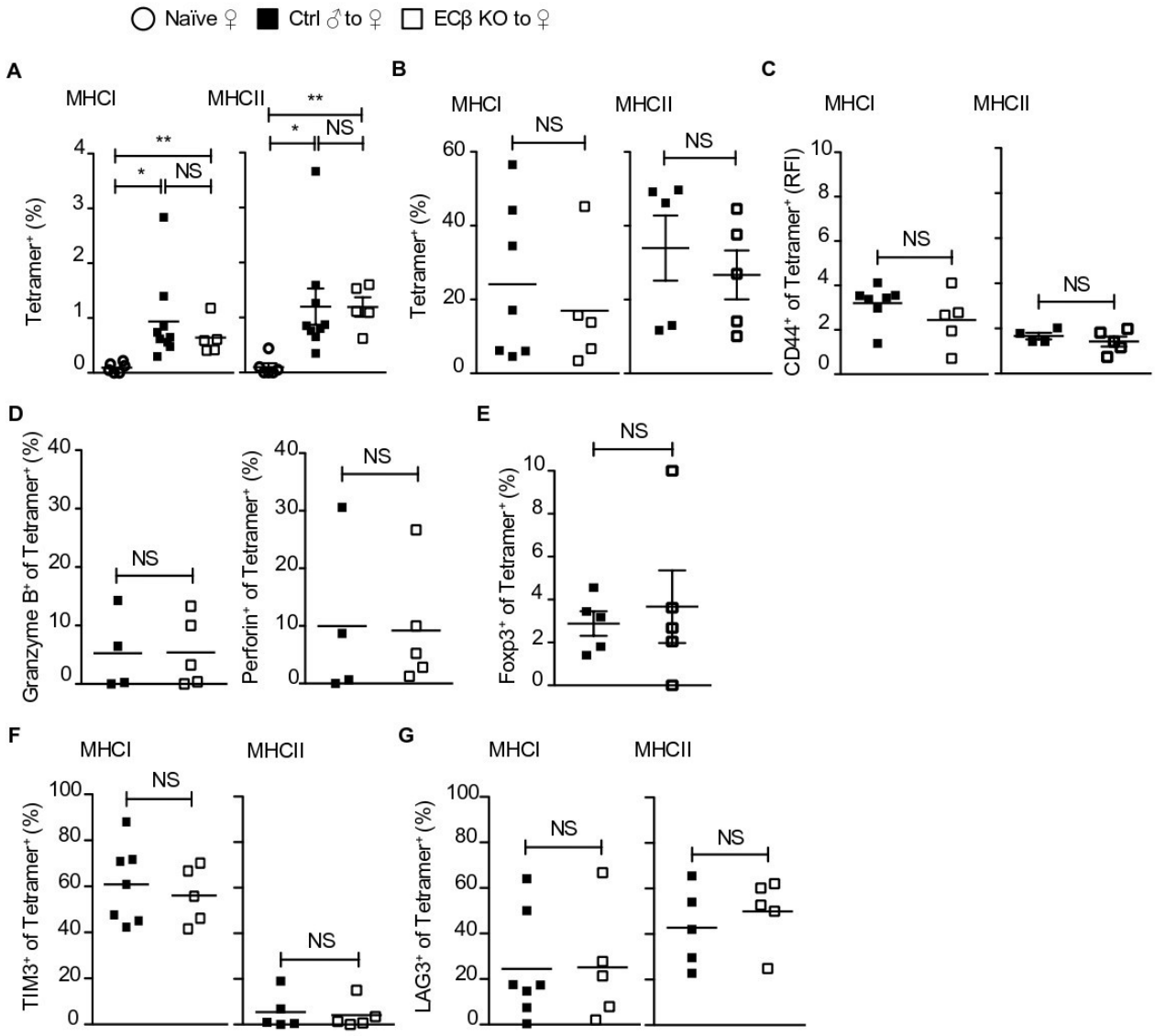

Figure 4.4: Characterization of male HY-alloreactive lymphocytes in heart allografts.

Splenic or graft-infiltrating lymphocytes were isolated from non-transplanted female, or ECβKO or wild-type donor heart recipient mice (n=4-7 mice/ group) recovered two weeks post-transplant, then stained with HY-peptide/ Major Histocompatibility Class (MHC) I or II tetramers to identify alloreactive cells, counter-immuno-stained as indicated, then detected by flow

cytometry as described in Chapter 2 of the methods. **(A)** Quantification of the abundance of MHC I (left) or MHC II (right) tetramer-stained lymphocytes to detect $CD8^+$ and $CD4^+$ alloreactive T lymphocytes, respectively, in the spleen of the heart allograft-harbouring recipient mice. **(B)** Quantification of the abundance of tetramer-stained lymphocytes recovered from the hearts two weeks post-transplant. **(C)** Quantification of the $CD44^+$ memory T lymphocytes among alloreactive lymphocytes in heart allografts. **(D)** Quantification of the cytotoxic granzyme B^+ (left) or $perforin^+$ (right) fraction of the HY-MHC class I $tetramer^+$ cells. **(E)** Quantification of the $FoxP3^+$ Treg fraction of the HY-MHC class II $tetramer^+$ cells. Quantification of the lymphocyte exhaustion markers **(F)** $Tim3^+$ and **(G)** $LAG3^+$ populations among alloreactive lymphocytes in heart allografts. Mean ± SEM; NS = non-significant by unpaired Student's t-test.

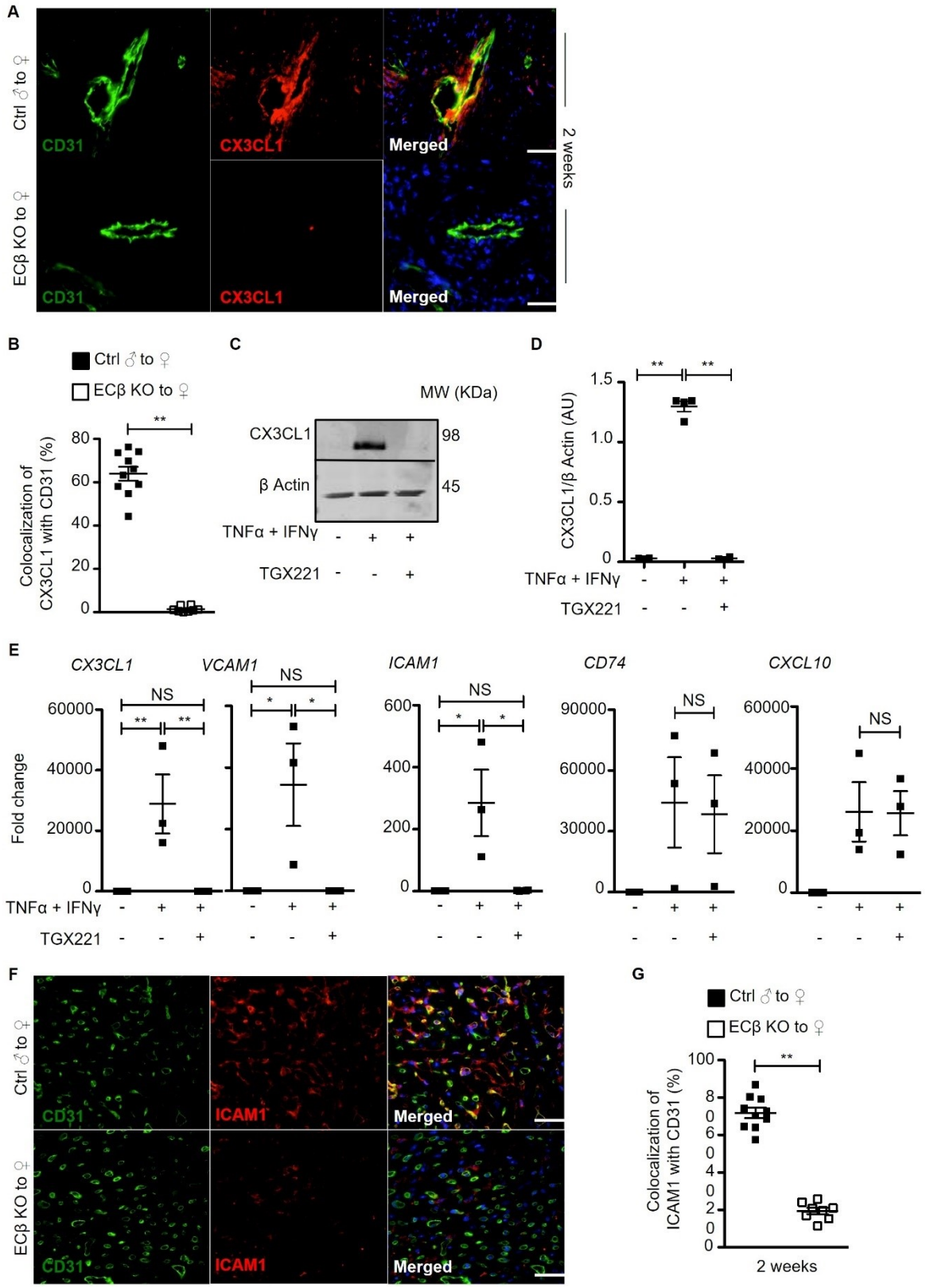
A
Ctrl ♂ to ♀
ECβ KO to ♀
CD31
CX3CL1
Merged
2 weeks
B
Ctrl ♂ to ♀
ECβ KO to ♀
Colocalization of CX3CL1 with CD31 (%)
C
MW (KDa)
CX3CL1
β Actin
98
45
TNFα + IFNγ
TGX221
D
CX3CL1/β Actin (AU)
E
CX3CL1
VCAM1
ICAM1
CD74
CXCL10
Fold change
NS
F
ICAM1
G
Colocalization of ICAM1 with CD31 (%)
2 weeks

Figure 4.5: EC-specific PI3 kinase-β inactivation blocks pro-inflammatory molecule expression in vivo.

(A) Expression of CX3CL1 by intramyocardial coronary artery EC from ECβKO or wild-type donor hearts at two weeks post-transplant. Confocal photomicrographs (1 μm optical section) of endothelial CD31 (green, left panels), CX3CL1 (red, center panels), and the merged images (right panels). Scale bars: 50 μm. **(B)** Quantification of the fraction of $CD31^+$ EC co-stained with CX3CL1 in the groups (n=8-10 mice/ group) in (A). Mean ± SEM; **P < 0.01 by Student's t-test. **(C)** Primary human aortic ECs stimulated with both TNF-α (10 ng/ml) + IFN-γ (50 ηg/ml) TGX221 (100 nM). Cell lysates were collected at 48 h and then immunoblotted for CX3CL1. **(D)** Quantification of CX3CL1 expression relative to β actin (n=4 independent experiments). **(E)** Human aortic ECs were treated as in (C), and expression was quantified by qRT-PCR (n=3 independent experiments). Mean ± SEM; *P < 0.05, **P < 0.01, NS, non-significant by 1-way ANOVA with Bonferroni's post hoc test. **(F)** Expression of ICAM1 by myocardial microvessel EC from ECβKO or wild-type donor hearts at two weeks post-transplant. Confocal photomicrographs of endothelial CD31 (green, left panels), ICAM1 (red, center panels), and the merged images (right panels). (Scale bars: 50 μm). **(G)** Quantification of the fraction of $CD31^+$ EC co-stained with ICAM1 in the groups (n=8-10 mice/ group) in (F). Mean ± SEM; **P < 0.01 by Student's t-test.

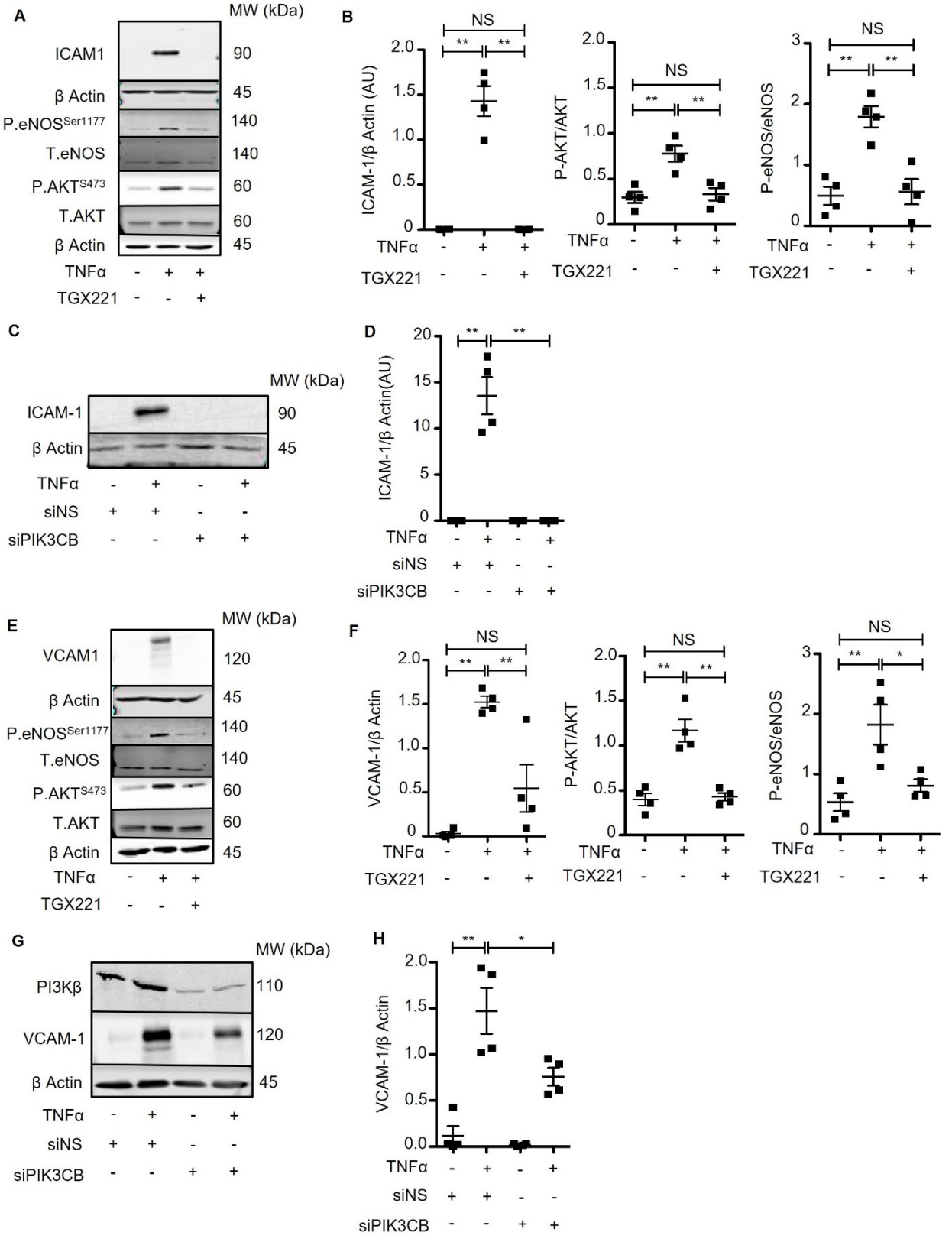
A
MW (kDa)
ICAM1
90
β Actin
45
P.eNOSSer1177
140
T.eNOS
140
P.AKTS473
60
T.AKT
60
β Actin
45
TNFα - + +
TGX221 - - +
B
NS
**
**
ICAM-1/β Actin (AU)
P-AKT/AKT
P-eNOS/eNOS
TNFα
TGX221
C
MW (kDa)
ICAM-1
90
β Actin
45
TNFα - + - +
siNS + + - -
siPIK3CB - - + +
D
ICAM-1/β Actin(AU)
E
MW (kDa)
VCAM1
120
β Actin
45
P.eNOSSer1177
140
T.eNOS
140
P.AKTS473
60
T.AKT
60
β Actin
45
TNFα - + +
TGX221 - - +
F
VCAM-1/β Actin
P-AKT/AKT
P-eNOS/eNOS
*
G
MW (kDa)
PI3Kβ
110
VCAM-1
120
β Actin
45
TNFα - + - +
siNS + + - -
siPIK3CB - - + +
H
VCAM-1/β Actin

Figure 4.6: EC PI3 kinase-β inactivation blocks TNF-α-stimulated adhesion molecule expression in vitro.

(A) HUVECs were stimulated with TNF-α (10 ng/ml) TGX221 (100 nM). Cell lysates were collected at 12 h, then immunoblotted for ICAM1, phospho-Akt^{S473}, and phospho-eNOS^{ser1177}. **(B)** Quantification of ICAM1, phospho-Akt^{S473}, and phospho-eNOS^{ser1177} expression (n=4 independent experiments). **(C)** HUVECs were transfected with PIK3CB- or scrambled siRNA as described in Methods, then stimulated with TNF-α as in (A). Cell lysates were immunoblotted for ICAM1. **(D)** Quantification of ICAM1 expression (n=4 independent experiments). **(E)** HUVECs were stimulated with TNF-α (10 ng/ml) and TGX221 (100 nM). Cell lysates were collected at 24 h, then immunoblotted for VCAM1, phospho-Akt^{S473}, and phospho-eNOS^{ser1177}. **(F)** Quantification of VCAM1 expression (n=4 independent experiments). **(G)** HUVECs were transfected with PIK3CB or scrambled siRNA, then stimulated with TNF-α as in (E). Cell lysates were immunoblotted for VCAM1. **(H)** Quantification of VCAM1 expression (n=4 independent experiments). Mean ± SEM; *P < 0.05, **P < 0.01, NS, non-significant by 1-way ANOVA with Bonferroni's post hoc test.

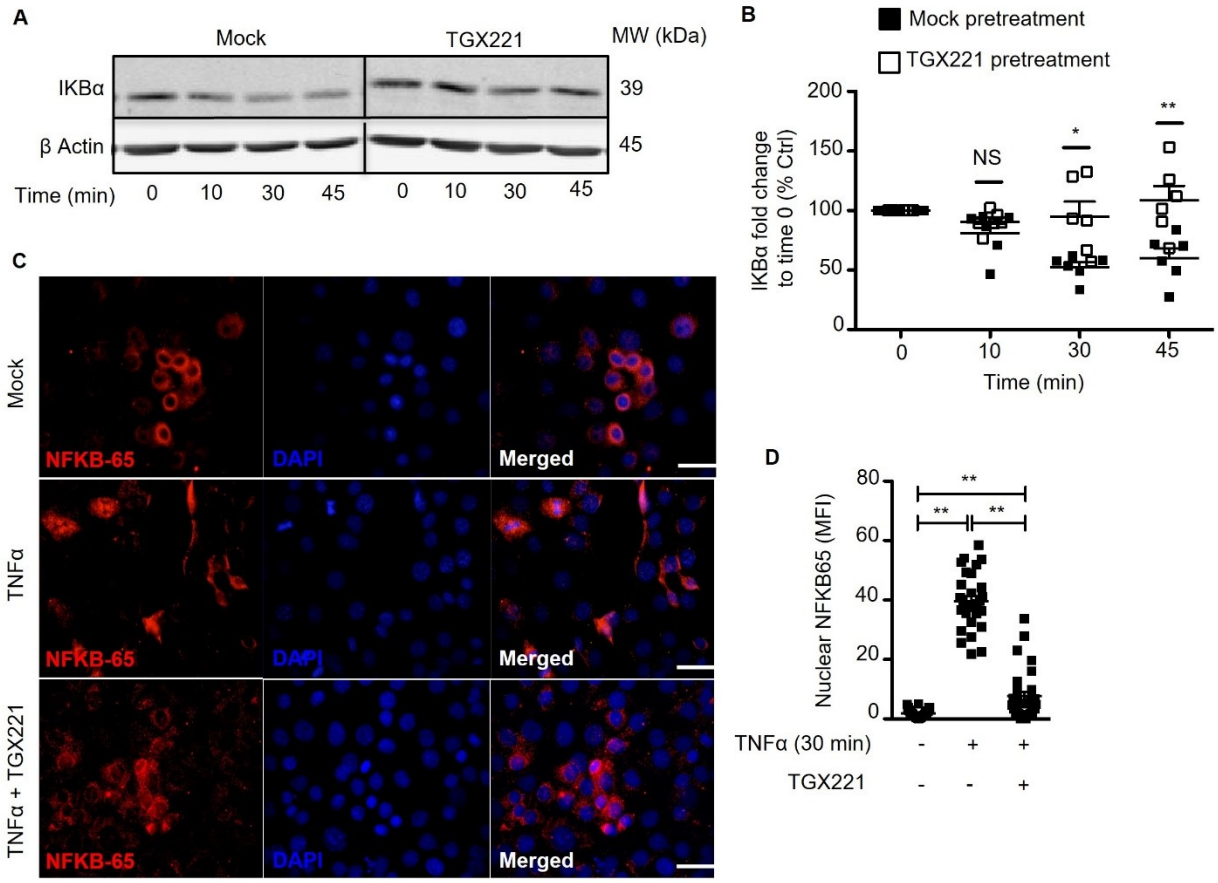

Figure 4.7: EC PI3 kinase-β inactivation blocks the canonical NF-kB pathway.

(A) Mouse microvascular heart ECs were stimulated with TNF-α (10 ng/ml) and TGX221 (100 nM). Cell lysates were collected at the indicated times, then immunoblotted for IKBα. **(B)** Quantification of IKBα abundance (n=4 independent experiments). TGX221 treatment protected against IKBα degradation ($P<0.01$ by 2-way ANOVA). Mean ± SEM; *$P < 0.05$, **$P < 0.01$, NS, non-significant by ANOVA. **(C)** Mouse Heart Endothelial cells (MHECs) were stimulated with TNF-α (10 ng/ml) and TGX221 (100 nM), then imaged at 30 min. Confocal photomicrographs of endothelial NF-kB p65 (red, left panels), DAPI (blue, center panels), and the merged images (right panels). Scale bars: 50 μm. **(D)** Co-localization of NF-kB p65 with the $DAPI^+$ nucleus was

quantified as the mean fluorescent intensity of NF-kB p65 in the nucleus region. Mean ± SEM; **P<0.01 by 1-way ANOVA with Bonferroni's post hoc test.

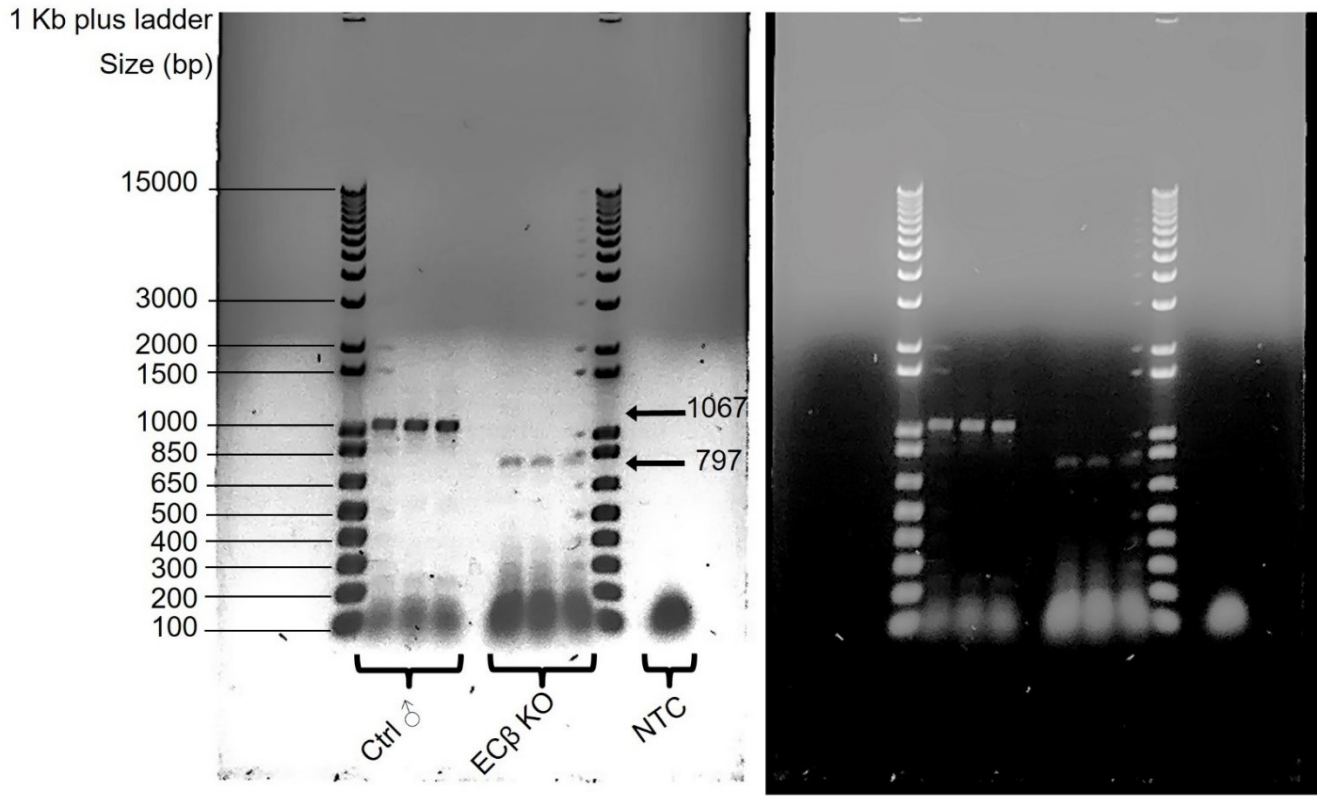

Figure 4.8: Expression of the mutant *Pik3cb* in lung EC isolated from donor mice.

Endpoint PCR products from the ECβKO mice (n = 3) showed the truncated *Pik3cb* at 797 base pairs, while the floxed control mice (n = 3) showed the full-sized *Pik3cb* at 1067 base pairs.

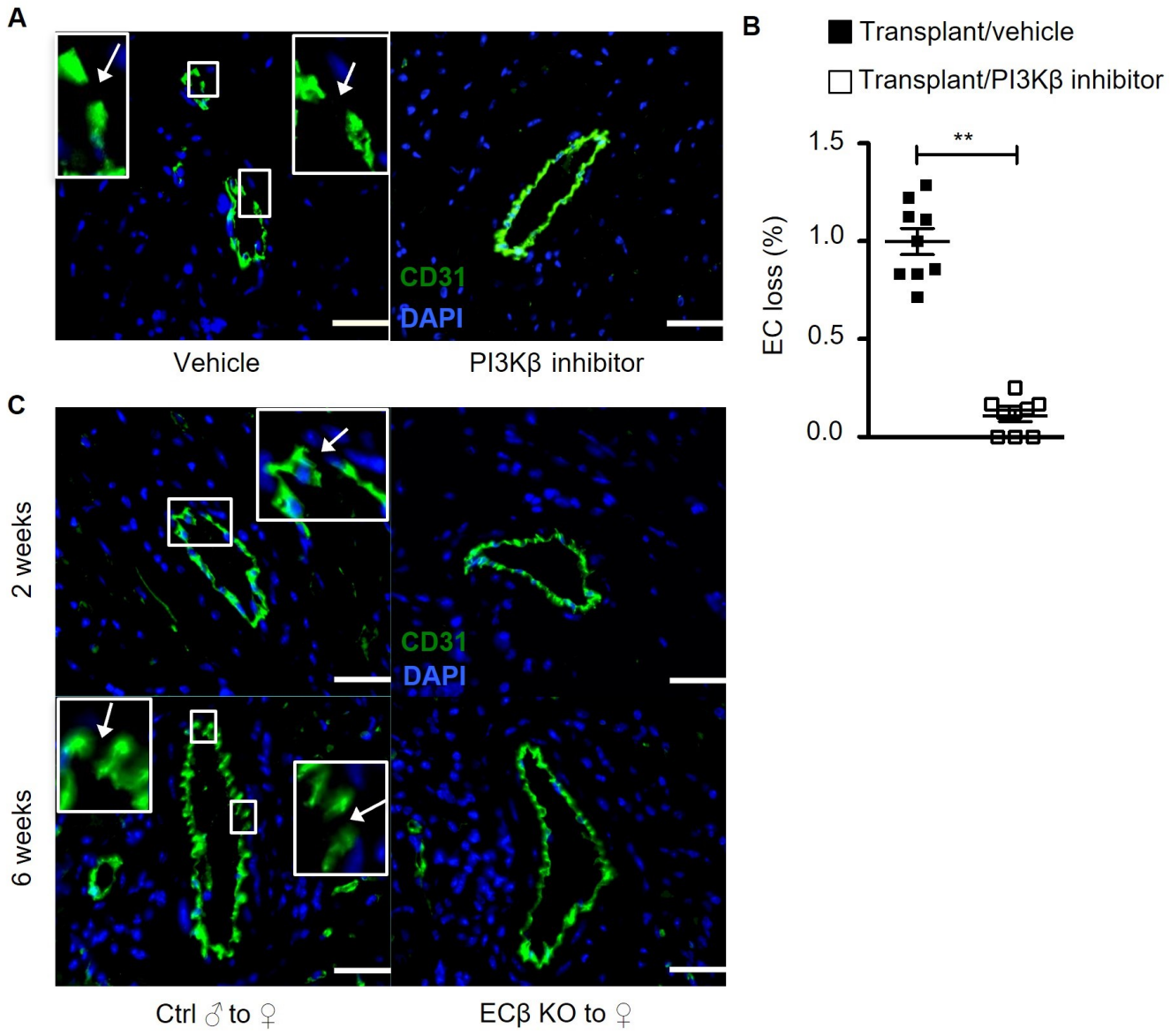

Figure 4.9: Graft arterial injury post-transplantation.

(A) Transplanted hearts from vehicle or GSK2636771(GSK)-treated mice (n=9) were recovered six weeks after transplantation. Loss of continuity of the arterial endothelium in the transplanted hearts is shown (CD31; PECAM1; green) with DAPI nuclear staining (blue). Arrows indicate areas of focal endothelial loss/gaps in the endothelium. **(B)** Quantification of arterial EC gaps in (A; n=9). **(C)** Representative photomicrographs showing the loss of continuity of the arterial

endothelium in ECβKO vs wild-type hearts recovered at 2- or 6-weeks post-transplant. Mean ± SEM; **P<0.01 by Student's t-test. Scale bars: 50 μm.

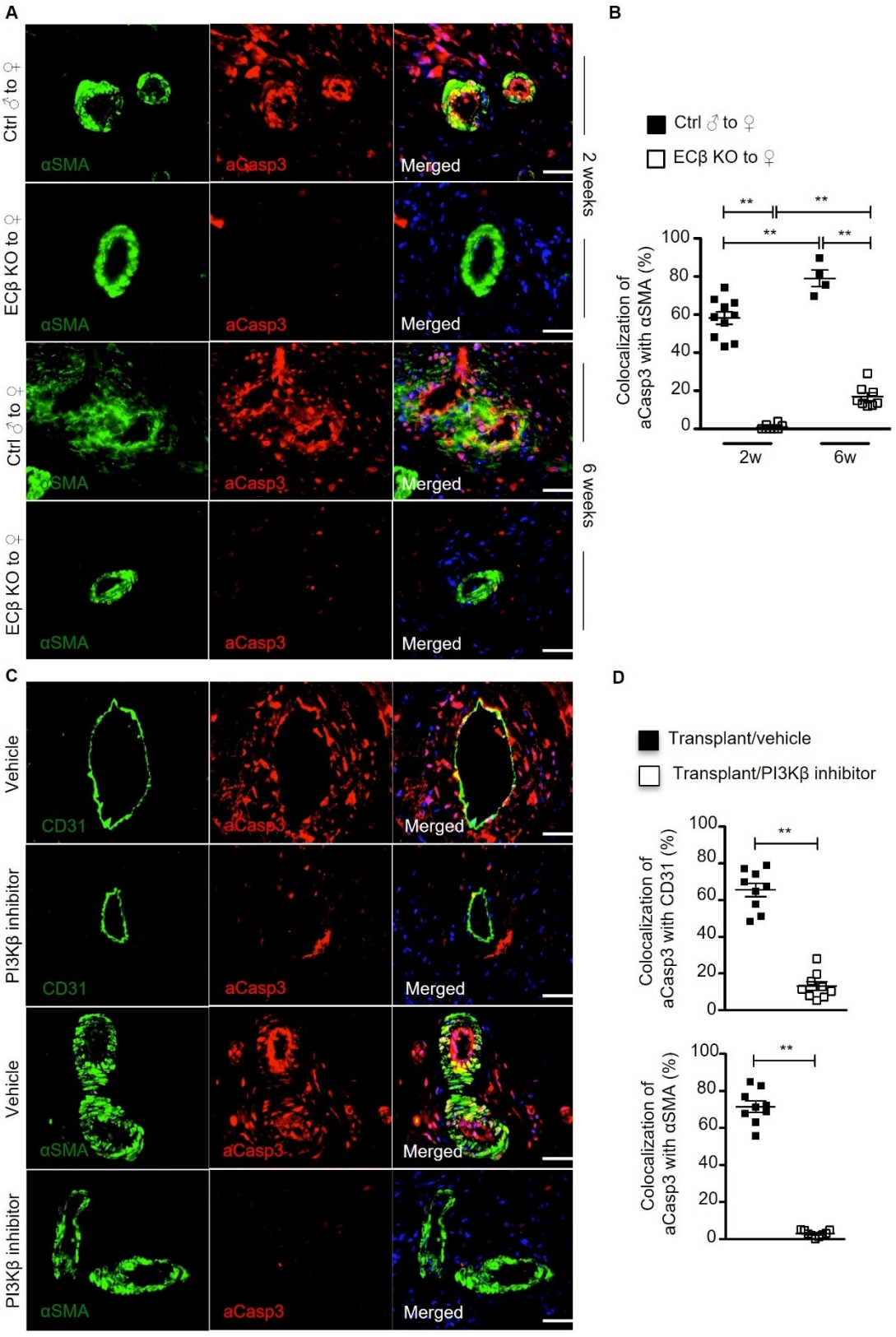

Figure 4.10: Arterial apoptosis post-transplantation.

Transplanted ECβKO or wild-type hearts (n=4-10) were recovered at two- or six-weeks post-transplantation. **(A)** Confocal photomicrographs show double immunofluorescent staining of the VSMC marker α smooth muscle actin (αSMA, green) with cleaved caspase 3 (aCasp3; red). Co-localization is quantified, as shown in **(B)**. **(C)** Transplanted hearts from vehicle or GSK2636771(GSK)-treated mice were recovered six weeks after transplantation. Arterial endothelial and VSMC apoptosis in the hearts of vehicle (n = 9) or GSK2636771-gavaged mice (n = 9) recovered six weeks after transplantation. Confocal photomicrographs show double immunofluorescent staining of endothelial (CD31; green) or VSMC (αSMA, green) with cleaved caspase 3 (aCasp3, red). Colocalization is quantified as shown in **(D)**.

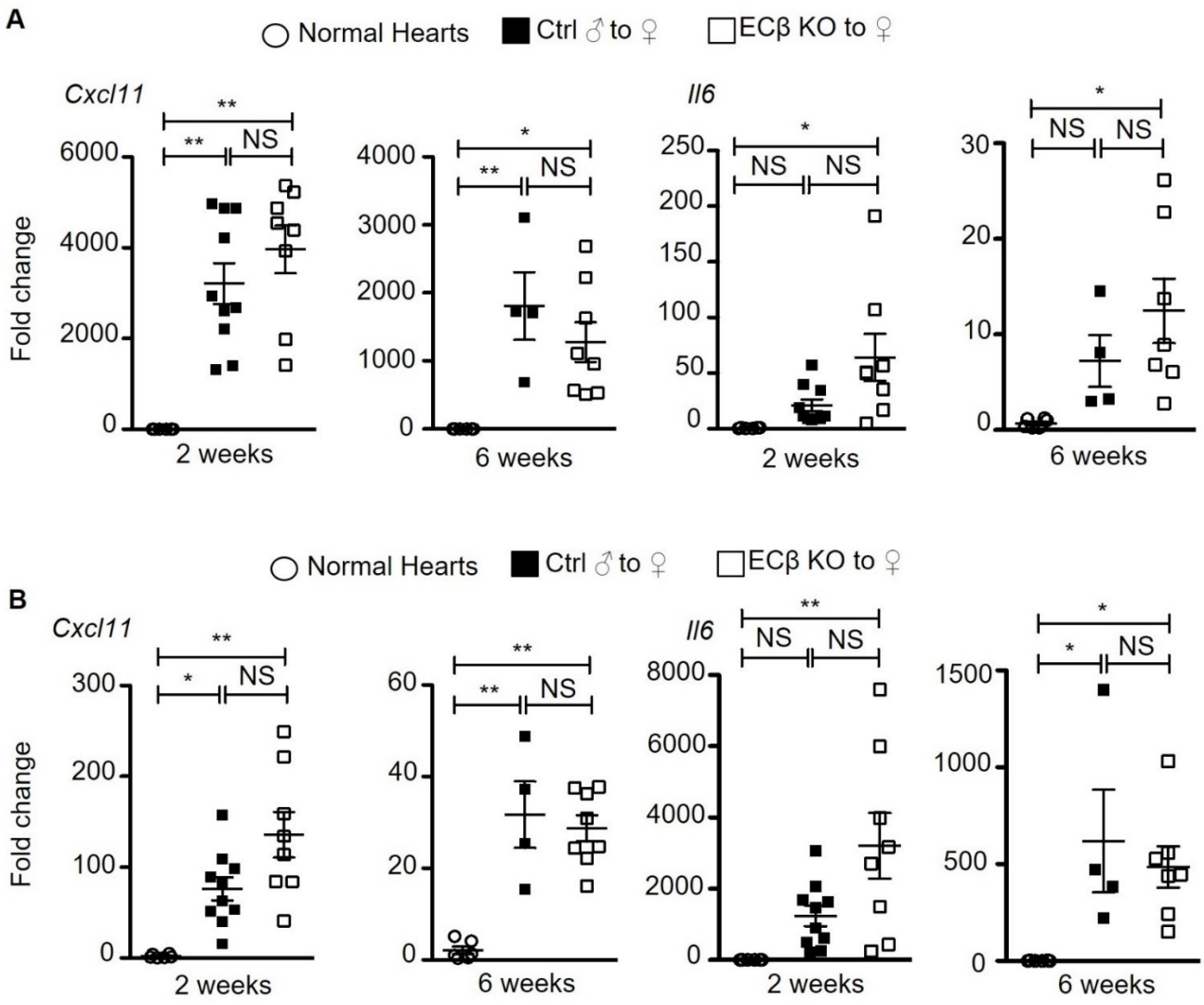

Figure 4.11: Inflammatory molecule expression in graft coronary artery or myocardium.

Transplanted ECβKO or wild-type hearts (n=4-10) were recovered at 2- or 6-weeks post-transplant. Coronary artery **(A)** or myocardial samples **(B)** were processed for qRT-PCR and quantified relative to expression in normal hearts. Mean ± SEM; *P<0.05, **P<0.01 and NS=nonsignificant by one-way ANOVA with Bonferroni's post-hoc test.

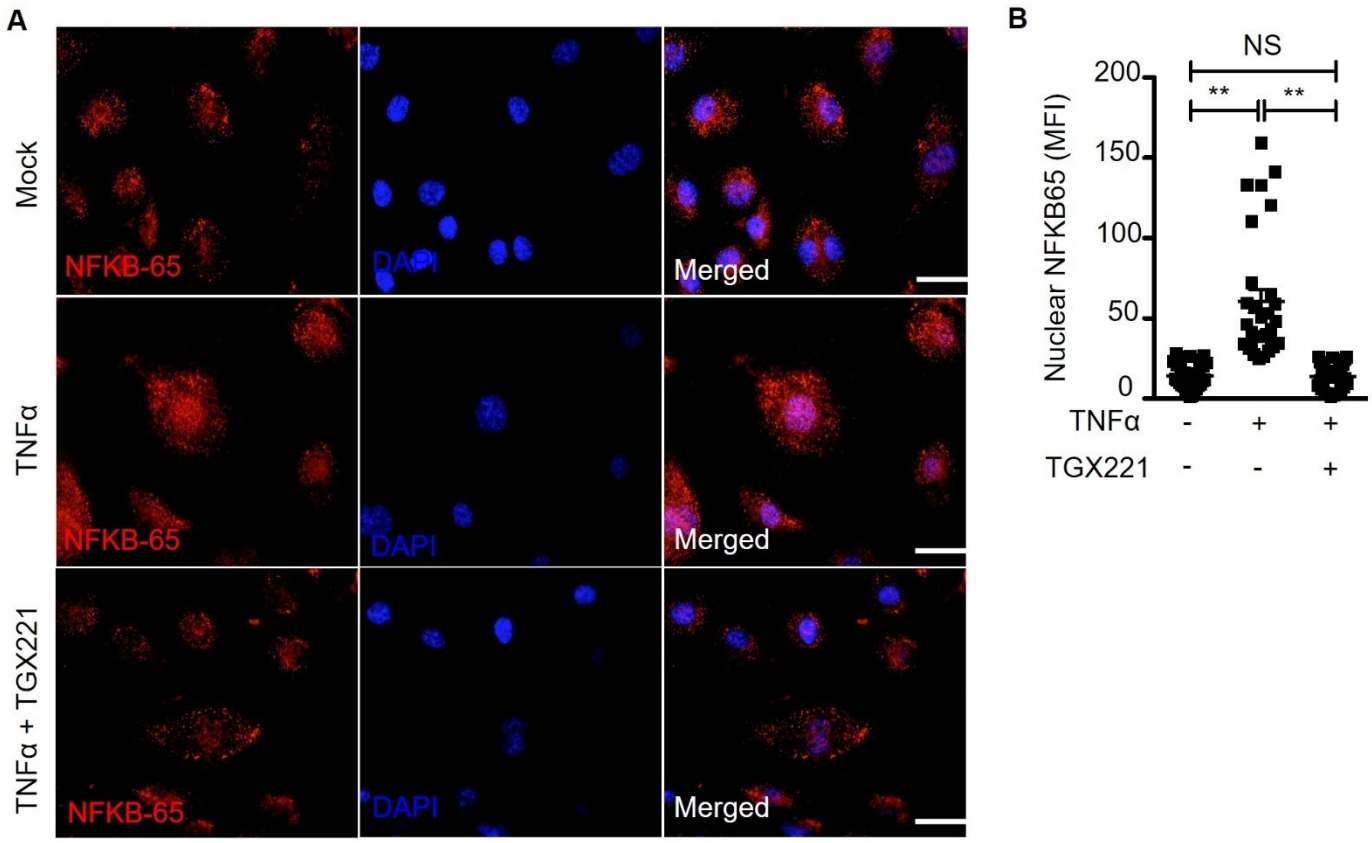

Figure 4.12: EC PI3 kinase-β inactivation blocks the TNF-α-stimulated canonical NF-kB pathway in cultured human endothelial cells (ECs).

(A) Human aortic endothelial cells were stimulated with TNF-α (10 ng/ml) TGX221 (100 nM), then imaged at 30 min. Confocal photomicrographs of endothelial NF-kB p65 (red, left panels), DAPI (blue, center panels), and the merged images (right panels). Scale bars: 50 μm. **(B)** Co-localization of NF-kB p65 with the $DAPI^{+}$ nucleus was quantified as the mean fluorescent intensity of NF-kB p65. Mean ± SEM; **P<0.01 by 1-way ANOVA with Bonferroni's post hoc test.

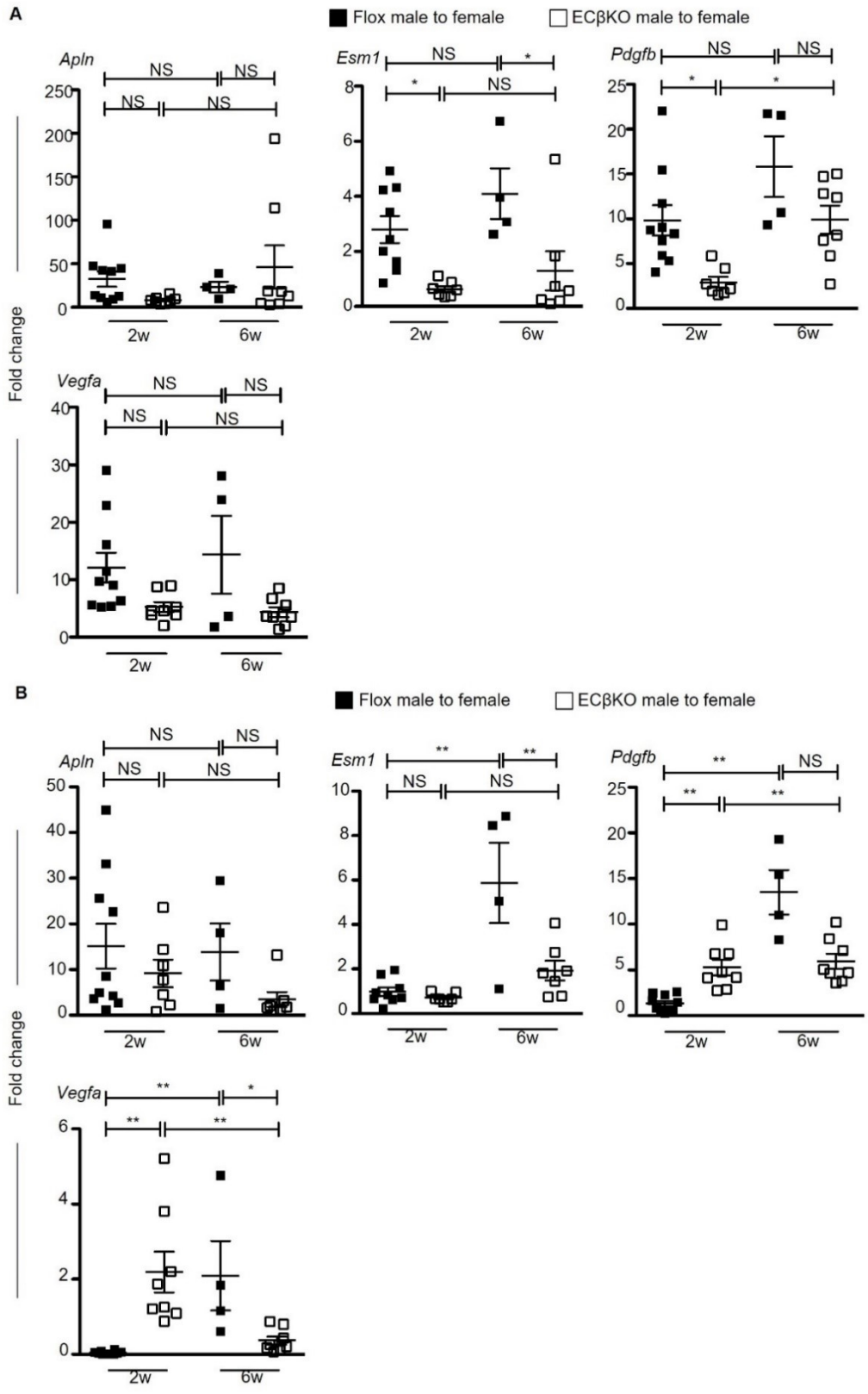
A
Flox male to female
ECβKO male to female
Apln
Esm1
Pdgfb
Vegfa
Fold change
NS
*
2w
6w
B
Flox male to female
ECβKO male to female
Apln
Esm1
Pdgfb
Vegfa
Fold change
NS
**
*
2w
6w

Figure 4.13: Vascular tip-cell EC repair gene expression analysis following PI3 kinase-β inactivation in graft coronary artery or myocardium.

Transplanted ECβKO or wild-type (Flox) hearts (n=4-10) were recovered at two- or six-weeks post-transplant. Coronary artery **(A)** or myocardial samples **(B)** were processed for qRT-PCR and quantified. Mean ± SEM; *P<0.05, **P<0.01 and NS=nonsignificant by one-way ANOVA with Bonferroni's post-hoc test.

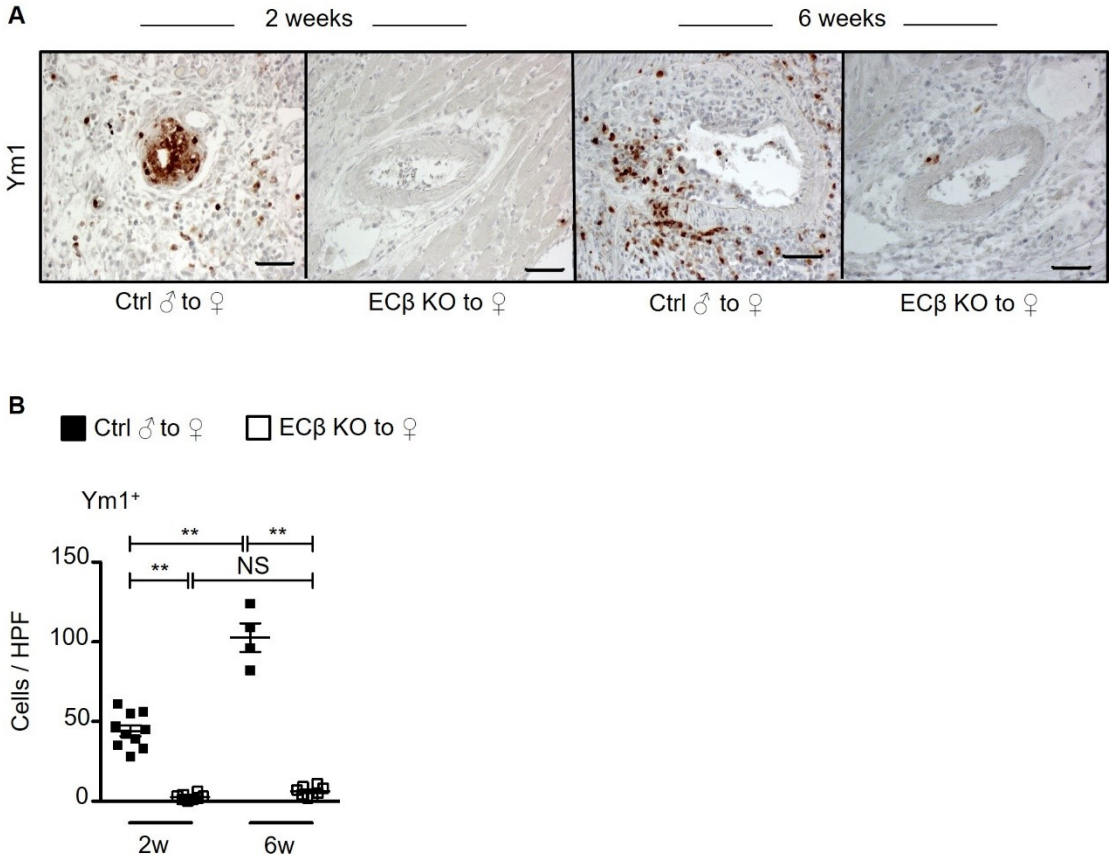

Figure 4.14: M2 macrophage infiltration of heart allografts.

(A) Photomicrographs of the $Ym1^+$ macrophages (top panels) among the heart from ECβKO or wild-type donors (n=4-10 mice/ group) recovered at 2- or 6-weeks post-transplant. **(B)** Quantification of the number of myocardial-infiltrating $Ym1^+$ in (A). Mean ± SEM; **P < 0.01 and NS, non-significant by 1-way ANOVA with Bonferroni's post hoc test; n=4-10 mice/group). HPF = high-power field. Scale bars: 50 μm.

Chapter 5 : Sacubitril Treatment Suppresses the Chronic Allograft Vasculopathy in Mouse Heart Allografts

Abstract

Heart failure dwindles individuals' functionality and quality of life while putting financial strains on healthcare resources. Heart transplantation definitively treats heart failure. Patients, however, must wait extended periods to avail of whatever is attainable of donors' hearts on long waitlists. Along, despite advances in immunosuppression, transplanted heart allografts fail eventually through the development of Chronic Allograft Vasculopathy (CAV), the primary cause of death succeeding the first-year post-transplantation. There is currently no treatment for CAV. Hence, testing novel therapeutic targets to prevent vascular immune injury and promote repair could preserve such precious transplants.

Pathologically, CAV starts with immune-mediated vascular Endothelial-Cell (EC) injury, which becomes mediated by infiltrating immune-inflammatory cells with subsequent immune activation of the allograft ECs. Consequently, ECs initiate maladaptive vascular repair responses in allografts' supplying vascular trees (i.e., along coronary arteries and microvessels), resulting in fibro-intimal thickening and near luminal obliteration, cutting off the allograft blood supply with eventual ischemia/failure.

Exploiting minor-histocompatibility-(HY)antigen-mismatched heterotopically-transplanted mice heart allografts, we demonstrated apelin, a novel tip-EC gene required for vascular development and forming new blood vessels from pre-existing ones (angiogenesis), is essential for directing vascular repair while alleviating CAV-injury by controlling nitric-oxide abundance. Therefore, inhibiting the rapid degradation of endogenous vasoactive peptides like apelin by neutral

(Metallo)endopeptidases like neprilysin represents a viable method for developing possible CAV therapies.

A clinically approved drug for heart failure and a neprilysin inhibitor, Sacubitril is an attractive opportunity for research into its effects on CAV vascular injury repair. We observed a significant CAV suppression after daily Sacubitril oral gavage in mice harbouring heart allografts. Sacubitril oral-gavage treatment starts at week two post-transplantation, when CAV develops in this transplantation model, until week six. Utilizing Apelin knockout mice can directly link Sacubitril CAV-protective effects to apelin signalling. Sacubitril could alleviate chronic transplant rejection or other immune-mediated vascular diseases (e.g., autoimmune vasculitis).

Introduction

Despite significant breakthroughs in heart failure management, which is a life-threatening condition (Benjamin et al., 2018; Ponikowski et al., 2014), heart transplantation (HTx) remains the best treatment for people who have reached an advanced stage of their heart failure disease (Toyoda et al., 2013). Donor hearts, nevertheless, are in short supply, transplant rates have remained constant, and many potential patients die while waiting (Jansen et al., 2015; Shemie et al., 2021). Maximizing the survival of heart allografts is thus a crucial goal of care.

Chronic vascular immune injury of the transplanted heart is currently a primary cause of death, even in the first year following transplantation, and continues to be a barrier to long-term graft survival (Costello et al., 2013b; Khush et al., 2021; Loupy et al., 2020; Lund et al., 2013) despite breakthroughs in immunosuppression regimens to circumvent acute rejection (Radio et al., 1996; Taylor et al., 2006). Chronic allograft vasculopathy (CAV), preceded by alloimmune vascular injury and endothelial apoptosis (Jansen et al., 2015), gradually occludes epicardial and branch

arteries (Hiemann et al., 2007; Mehra et al., 2010). Higher CAV grades correlate to progressive deterioration of left ventricular function (Reddy et al., 2022). The vascular endothelium of the transplanted hearts emerged as a critical target of the immune response to HTx, leading to CAV injury in arteries and microvessels (Chih et al., 2016; Loupy et al., 2016). However, the processes relating vascular endothelial injury to increasing artery occlusion are elusive.

We previously reported apelin as a critical factor to direct vascular immune injury repair in mice with minor histocompatibility HY-antigen mismatched heterotopic heart transplants (A. G. T. Masoud et al., 2020). This preclinical model reiterates many features of transplantation vasculopathy among solid organ and bone marrow transplant human patients (Merola et al., 2017). Moreover, apelin emerged as a member of the endothelial gene biomarkers that promote the reparative differentiation of endothelial cells in the arterial and microvessel compartments of the murine cardiac allografts (A. G. T. Masoud et al., 2020). Interestingly, this became consistent between mice and humans, according to studies that used two separate tissue banks from human heart transplants and a third from hearts after myocardial infarction (A. G. T. Masoud et al., 2020; Zhang et al., 2021).

Aside from its cardioprotective functions like atherosclerosis protection, cardiovascular regeneration, antihypertensive, and positive cardiac inotropic effects in heart failure (Barnes et al., 2010; Ureche et al., 2019), endothelial apelin became upregulated in CAV. Further, the APLN-17 analogue has successfully conferred protection from CAV immune injury. These effects became confirmed via a loss-of-function approach that exploits apelin-deficient heart transplants from donor gene-knockout mice. This approach resulted in enhanced vascular injury features compared to wild-type heart allografts. Besides, apelin considerably promoted the endothelial cell repair response in a gain-of-function experiment by injecting APLN-17 synthetic

analogue peptide that resists endogenous degradation by proteinases with an extended half-life (Fischer et al., 2020). Furthermore, genetic deletion of apelin in mice worsened the unfavourable myocardial remodelling following infarction and ischemia-reperfusion injury (Wang et al., 2013b).

The endothelial Nitric Oxide Synthase (eNOS) activity, as a net effector molecule downstream of apelin signalling, modulated most apelin effects to repel mononuclear immune cells' adherence to the vascular endothelium. Additionally, inhibiting eNOS through administering N(ω)-nitro-L-arginine methyl ester (L-NAME) abolished the APLN-17 protective effects in vivo in CAV. Hence, augmenting endogenous apelin's relatively short plasma half-life and efficacy through inhibiting its proteinase degradation emerges as a novel therapeutic strategy for vascular immune injury diseases (Japp et al., 2008; Zhen et al., 2013).

An excellent candidate for testing herein in CAV is Sacubitril, a clinically approved drug for treating heart failure to lessen rates of cardiovascular deaths (Abumayyaleh et al., 2021; Docherty et al., 2020; King et al., 2015). It confers inhibition of neprilysin, neutral endopeptidase activity that hinders the endogenous vasoactive peptides abundance and efficacy (e.g., the apelin) (Chang et al., 2020; Gu et al., 2010; Hubers & Brown, 2016; McMurray et al., 2014; Writing Committee et al., 2016).

Of note, the plasma membrane metalloprotease neprilysin is zinc-modulated and is also called Cluster of Differentiation 10 (CD10), Neutral EndoPeptidase 24.11 (atriopeptidase, or enkephalinase; NEP), or the Common Acute Lymphoblastic Leukemia Antigen (CALLA) (Campbell, 2018; Schiering et al., 2016). It degrades various vasoactive and cardioprotective peptides (e.g., adrenomedullin (ADM), bradykinin (BK), and natriuretic peptides) (Cruden et al.,

2004; Rademaker et al., 1996; Wilkinson et al., 2001) and its recently discovered target peptide apelin through proteolysis (McKinnie et al., 2016; Wang et al., 2016). Distinctly, neprilysin degenerates and inhibits apelin by targeting the "RPRL" motif at the N-terminus, whereby removing any of the amino acids from this motif/sequence abolishes the apelin's binding capacity to its cognate receptor (ApelinR) (El Messari et al., 2004; Gerbier et al., 2015).

Essentially, inhibiting neprilysin confers protection from maladaptive cardiovascular remodelling (Kuhn, 2004; Maric et al., 2006). Additionally, hindering neprilysin promotes vasodilation and natriuresis (Campbell, 2018). Further, neprilysin gene knockout studies using wild-type B6-strain mice demonstrated a substantial effect on bradykinin and Substance-P, whereby basal vascular permeability was modulated (Campbell, 2018; Geppetti, 1993; Skidgel et al., 1984). Thus, neprilysin inhibitors (NEPi) emerged as an attractive research topic in cardiovascular disease contexts to maintain endogenous levels of vasoactive peptides (Mangiafico et al., 2013). However, optimizing a calibrated therapeutic dose that balances the Sacubitril efficacy in inhibiting neprilysin, thus maximizing the augmentation of other vasoactive peptides like bradykinin (Campbell, 2017), would be essential to avoid potential side effects. These side effects include angioedema (Schmaier, 2018) (Banerji et al., 2017; Bas et al., 2015; Nussberger et al., 1998) and other potential therapeutic limitations of NEPi (Nicolas et al., 2018).

Results

The longevity of the heart allografts is limited by the development of vascular injury and maladaptive repair. The disease condition is marked by gradual pathological growth of the artery intima, obliteration of the vascular lumen, and a loss of microvessels. Previously, our research work identified that the apelin- ApelinR signalling helps promote vascular repair. This effect was confirmed using a gain-of-function approach. We treated heart allograft-bearing recipient

mice with the proteinase-resistant APLN-17 synthetic analogue with a long plasma half-life to rescue heart allografts from CAV injury and maladaptive repair. We inhibited the endothelial Nitric Oxide Synthase with N-Nitro-L-Arginine Methyl Ester (LNAME), and the APLN-17 analogue protective effects in CAV heart allografts became abrogated. As a result, we investigated the potential role of conserving endogenous apelin levels in rescuing CAV allografts from vascular immune injury. By administering Sacubitril, we inhibited neprilysin, a neutral endopeptidase that promotes the rapid degradation of various vasoactive peptides, including apelin. Notably, Sacubitril is a clinically approved treatment that can repurpose to treat immune-mediated vascular diseases besides heart failure, based on our initial findings.

We used male-to-female heart transplantation in mice, which is known to cause an indolent immune response and vascular disease akin to human transplant vasculopathy (Merola et al., 2017). In the first set of trials, recipient mice were given Sacubitril via oral gavage every day starting two weeks after transplantation and continuing for four weeks until week six post-transplantation. Subsequently, we harvested the hearts for further histological evaluation of the CAV findings. Heart allografts recovered from the vehicle treatment recipients showed significant thickening of the coronary artery intima, substantially occluding the arterial lumen (Figure 5.1 A, C). In contrast, arterial intimal expansion in Sacubitril-treated mice's hearts substantially reduced intimal proliferation, as measured by intima area quantification visualized with Van Gieson elastin staining.

Furthermore, we discovered that following Sacubitril treatment, the microvessel density of the myocardial compartment became exceptionally conserved following the alloimmune injury directed against the vascular endothelium. Sacubitril, therefore, protected the microvasculature

from immune injury (Figure 5.1 B, D). This finding implies that treating the allograft-harbouring recipient mice with Sacubitril helped reduce the vascular immune-mediated disease pathology.

Vasculopathy starts following immune-cell infiltration and consequent damage to the transplant vasculature. As a result, we looked at how Sacubitril affected $CD8^+$ lymphocyte and Mac-2^+ (M1) macrophage infiltration to the transplanted heart allografts. Compared to vehicle-treated heart allograft-harbouring mice, Sacubitril successfully decreased infiltrating inflammatory immune cells ($CD8^+$ lymphocyte and Mac-2^+ macrophage) to the heart allografts (Figure 5.2), indicative of its potential characteristics to control vascular inflammation.

Conclusion

Despite advances in immunosuppression, the vascular immune injury and the subsequent maladaptive vascular repair response of the chronic allograft vasculopathy (CAV) limit the transplanted allografts and recipients' survival (Loupy et al., 2020). In development, apelin-ApelinR signalling was deemed indispensable for vascular developmental angiogenesis (Cox et al., 2006; Kasai et al., 2008). Distinctly, genetic deletion of apelin from zebrafish yielded an abrogated process of sprouting angiogenesis and a blunted tip-cell phenotypic commencing by endothelial cells (Helker et al., 2020). In our previous study, we characterized the role of apelin in vascular injury and the repair of CAV. We used the same heterotopic cardiac transplantation of minor histocompatibility HY-peptide mismatched mouse heart allografts. Notably, apelin is a tip-cell endothelial gene involved in vascular development, and its genetic deletion established vascular defects (Kasai et al., 2008). We identified apelin as an indispensable factor for vascular repair following the immune-mediated vascular injury of CAV. This effect became confirmed by employing loss-of-function approaches through genetic deletion (i.e., Apln knockout mice).

Further, we investigated the effects of apelin's gain of function after administering the proteinase-resistant APLN-17 synthetic analogue with a much longer plasma half-life to rescue heart allografts from CAV progression. Further, we reported that apelin treatment successfully accelerated the healing of the injured endothelial monolayer in vitro following scratch wounding, besides suppressing the adhesion of monocytes to the cultured human endothelial cell monolayer, indicative of apelin's vascular endothelial regenerative and anti-inflammatory roles. These reported vascular protective effects of apelin were dependent on nitric oxide. Distinctly, cotreatment of the heart allograft-harbouring mice with an endothelial Nitric Oxide Synthase (eNOS) inhibitor (i.e., the Nω-Nitro-L-arginine methyl ester hydrochloride-LNAME) abolished the protective features and effects of the co-administered APLN-17 synthetic analogue peptide in these mice of cardiac transplantation.

Our current study aimed to characterize the effect of augmenting endogenous apelin abundance and efficacy by conserving it from rapid degradation by neprilysin. Neprilysin breaks down vasoactive and cardioprotective peptides like bradykinins and natriuretic peptides to promote vasoconstriction as a neutral endopeptidase. We investigated if this strategy can rescue the transplanted heart allografts from vascular immune injury and track the inflammatory phenotype. Sacubitril is a well-tested, a clinically approved drug treating heart failure patients with reduced left ventricular ejection fraction. It can inhibit neprilysin and, thus, to a greater extent, preserve endogenous apelin levels and efficacy to respond to allogeneic vascular immune injury and promote vascular repair. A protected vascular tree can then perfuse the heart allograft substantially with hindered vascular intimal expansion and luminal obliteration to safeguard the graft blood supply.

While inhibiting neprilysin, Sacubitril demonstrated modulation of vascular inflammation in Apo-E deficient mice to study atherosclerosis, whereby Sacubitril treatment hindered atherosclerotic plaque development when added to valsartan (Zhang et al., 2019). Sacubitril also demonstrated cardiovascular protective functions to benefit heart failure patients (McMurray et al., 2014; Ruilope et al., 2010). Furthermore, Sacubitril treatment helped reduce the genes of vascular inflammation (e.g., *Il6* and the monocyte chemoattractant protein-1 gene-*Ccl2*) in carotid plaques of established atherosclerosis in ApoE knockout mouse models. This effect denotes the Sacubitril's potential to alleviate endothelial dysfunction and hinder inflammation in numerous cardiovascular disease pathologies. Additionally, Sacubitril treatment demonstrated advanced superiority over traditional therapies in patients with congestive heart failure (Gu et al., 2010; Hayashi et al., 2012; Kusaka et al., 2015; McMurray et al., 2014; Solomon et al., 2012).

Considering other used approaches to alleviate the degradation of endogenous apelin in the Unilateral Ureteral Obstruction (UUO) animal model of tissue fibrosis, the use of Losartan that inhibits the Angiotensin II receptor and preserves apelin resulted in hindered renal fibrosis, reduced macrophage infiltration, enhancement of apelin mRNA levels, promoted Protein Kinase B (PKB; also known as AKT) as well as eNOS phosphorylation and suppressed myofibroblast aggregation in this model (Nishida et al., 2012).

In the current study, we identified a significant reduction in the infiltrating inflammatory immune cells to the transplanted heart allograft following sustained treatment with daily Sacubitril, whereby CAV vascular injury became established. In response to Sacubitril treatment, $CD8a^{+}$ T lymphocytes and Mac-2^{+} pro-inflammatory monocyte macrophages were recruited significantly lower to arterial-vascular and myocardial tissue compartments (as assessed per high power fields) or tissue sections from vehicle-gavaged control mice. This finding comes in agreement with the

previously reported effects of Sacubitril on inflammatory cell infiltration (e.g., macrophages) to the atherosclerotic plaque. Subsequently, Sacubitril treatment reduced the inflammatory cytokines in atherosclerotic tissues and plasma of treated mice (Zhang et al., 2019).

The potential preservation of the apelin-ApelinR pathway's activity through inhibiting neprilysin helped reduce cardiovascular injury by promoting angiogenesis (Kunduzova et al., 2008). This approach helped provoke mitochondrial normalization to balance cellular metabolic needs (Pisarenko et al., 2010). It also inhibited mitochondrial autophagy (Pisarenko et al., 2010) and hindered endothelial and cardiac cellular apoptosis in SARS-CoV-2 patients with lung injury (Bellis et al., 2020; Heusch et al., 2008). These observations show Sacubitril's effectiveness in controlling injured endothelial apoptosis activation. Moreover, Sacubitril could potentially hinder cellular oxidative stress, mitophagy, and cardiotoxicity with enhanced mitochondrial functional preservation (Dindas et al., 2021; Vaskova et al., 2020; Yeh et al., 2021).

The current study reports the potential protective effects of Sacubitril treatment to ameliorate vascular immune-mediated injury in chronic allograft vasculopathy of the vascularized heart allografts. This approach might help future characterization of the repair signals' activation towards developing a translational plan to introduce Sacubitril into clinical trials for further testing on patients undergoing chronic organ transplantation rejection, considering its clinical approval as an effective therapy for heart failure.

The current CAV protective effects following neprilysin inhibition by Sacubitril could be modulated through other vasoactive peptides downstream neprilysin aside from apelin. Hence, we would pursue further confirmatory testing to directly correlate apelin to the observed protective CAV findings following neprilysin inhibition. This direct correlation can be confirmed by utilizing the *Apln* knockout mice to test the CAV outcomes following Sacubitril treatment.

Figures

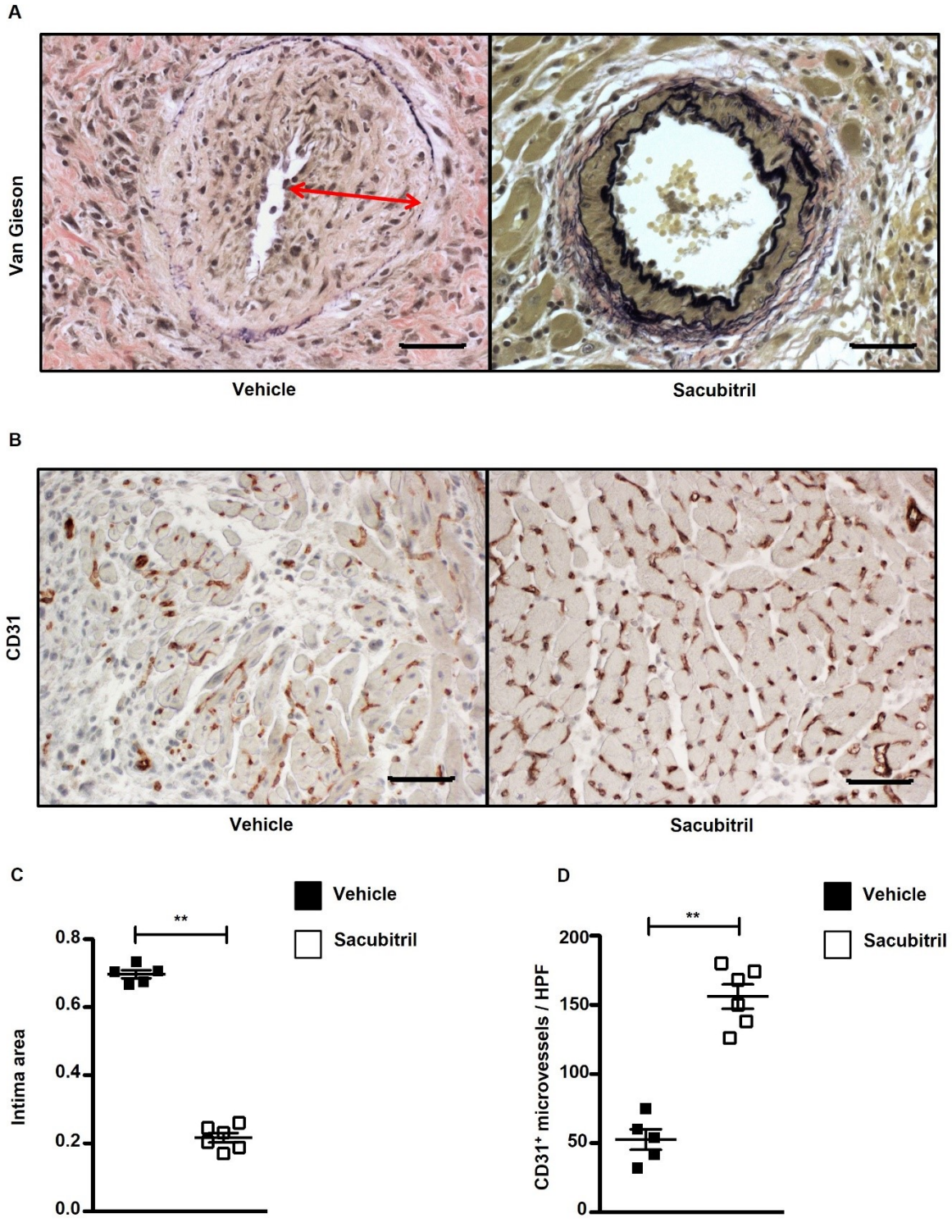

Figure 5.1: Sacubitril mitigates vasculopathy in transplanted hearts.

(A) Transplanted hearts from vehicle (n = 5) or Sacubitril-treated mice (n = 6) were recovered six weeks after transplantation, stained with the Van Gieson elastin stain immuno-stained for endothelial CD31 (PECAM). Fibro-cellular expansion of the intima, bounded by the internal elastic lamina and the lumen, is marked in a coronary artery from a vehicle-treated heart (red arrow). Little neointima expansion is evident in coronary arteries from transplanted hearts from Sacubitril-treated mice. Rarefaction of myocardial microvessels is evident in vehicle-treated vs Sacubitril-treated hearts **(B)** Transplanted hearts as in A immune-stained for endothelial CD31 (PECAM). **(C)** As described in Methods, the quantification of the neointima area and **(D)** myocardial $CD31^+$ microvessel density of the groups in (A). HPF = high-power field. Scale bars: 50 μm. Mean ± SEM; **P < 0.01 by unpaired student's t-test.

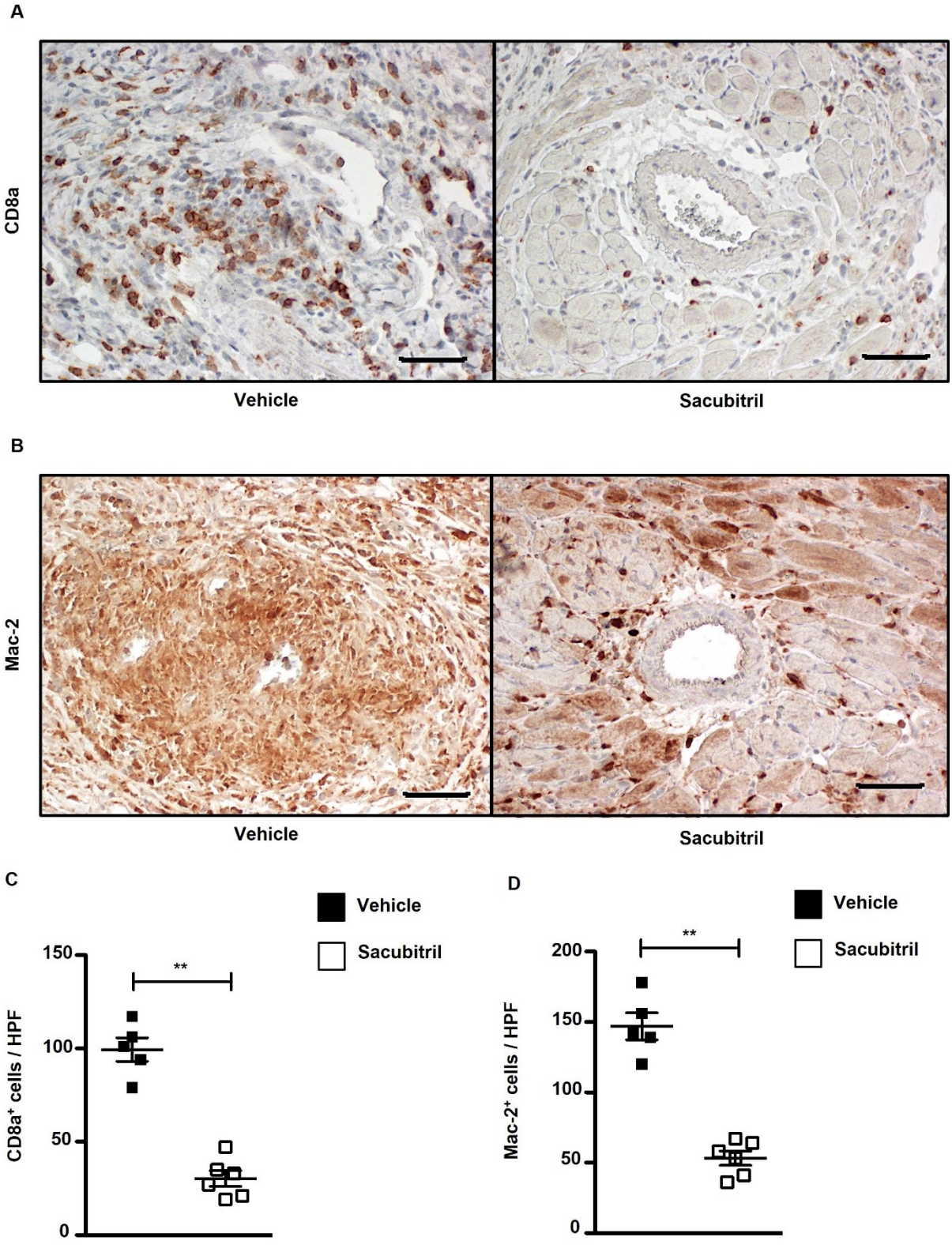

Figure 5.2: Immune cell infiltration of heart allografts following Sacubitril treatment.

(A) Photomicrographs of the $CD8^+$ lymphocytes and **(B)** Mac-2^+ monocyte/ macrophages among the heart allograft from vehicle-treated or Sacubitril-treated recipient mice (n=5-6 mice) when recovered at 6-weeks post-transplant. **(C)** Quantification of the number of myocardial-infiltrating $CD8^+$ in (A) and **(D)** Mac-2^+ cells in (B). HPF = high-power field. Scale bars: 50 μm. Mean ± SEM; **P < 0.01 via Student's t-test.

Chapter 6 General Discussion, Conclusions, Limitations and Future Directions

The current work seeks to elucidate the mechanisms of allograft vascular repair and the potential function of endothelial reparative therapeutic molecular targets in allograft survival. In our previous work, we discovered that apelin/apelin receptor (ApelinR) signalling is indispensable for vascular immune injury repair of the Chronic Allograft Vasculopathy (CAV) (A. G. Masoud et al., 2020). Despite contemporary advances in immunosuppression regimes (Black et al., 2018; Leblanc et al., 2018), the chronic immune onslaught on the allograft vascular Endothelial Cells (ECs) with the resultant vascular injury-maladaptive repair (i.e., CAV) is still the primary cause of heart transplant loss and recipients' mortality (Costello et al., 2013b; Khush et al., 2021; Loupy et al., 2020). Biomarkers of EC injury are associated with markers of EC repair in human transplant vasculopathy (Malyszko et al., 2005). Hence, eventually, disturbed repair contributes to the allograft vasculopathy pathogenesis. There is no CAV therapy; thus, focused reparative therapies are crucial to emerge.

In terms of the CAV EC injury, following heart transplantation, the first alloantigen recognition occurs mainly in the spleen, where T effector lymphocytes are generated (Lakkis et al., 2000; Larsen et al., 1990). In the allograft, ECs and tissue Dendritic Cells (DCs) attract and activate memory or T effector lymphocytes via projections to the luminal surface, and hence interactions at the vessel wall trigger the local alloimmune response (Boardman et al., 2016; Herrera et al., 2004; Honjo et al., 2004; Hornick et al., 2000; Lee et al., 2001; Libby & Pober, 2001; Siu et al., 2018). Additionally, the donor DCs generate exosomes that provoke the directed immune responses against the allografts (Liu et al., 2016). Furthermore, the endothelium is the primary vascular target of cell-mediated lymphocyte cytotoxicity and alloantibody-dependent injury, whereby complement system activation drives macrophage and Natural Killer (NK) cell

migration to the allograft vessels (Cross et al., 2018; Piotti et al., 2014; Pober et al., 2014; Pober et al., 1996; Weis & Cooke, 2003; Zhang & Reed, 2009). Devoid of immunosuppression, the allografts exhibit a gradual endothelial deterioration throughout the blood vessels with electron imaging revealing localized EC loss and exposed basement membrane (Dong et al., 1996; Lai et al., 2003; Savage et al., 1993). Even with immunosuppression, the inactive cytotoxic immune effector pathway promotes EC apoptosis that precedes CAV development (Cailhier et al., 2006; Hillebrands et al., 2003; Krupnick et al., 2002; Schmauss & Weis, 2008). Such transplant models with immunosuppression and those mediated by T helper cells provoke a much more indolent endothelial injury (Colvin & Smith, 2005; Lee et al., 2001; Russell et al., 2011). Current CAV models, driven by T lymphocyte responses, show EC injury, cell proliferation, and matrix buildup, all of which impair artery lumen breadth by enlarging the intima layer, i.e., the neointima (Mitchell, 2009; Pober et al., 2014; Weiss et al., 2008). The primary cell type of the neointima, the Vascular Smooth Muscle Cell (VSMC)-like cells, are recruited chiefly from the media (Campbell et al., 1988; Pober et al., 2014; Thyberg et al., 1990; Zalewski et al., 2002). These VSMCs elicit mild alloimmune responses (Murray et al., 1995) but significant responses to the proliferation and chemotactic factors produced by wounded donor ECs and recipient mononuclear cells (Autieri, 2003; Ip et al., 1990).

Vascular smooth muscle cells dedifferentiate into myofibroblasts capable of migration, proliferation, cytokine, and matrix production (Mosse et al., 1986; Owens et al., 2004; G. Wang et al., 2015; Yoshida et al., 2008). Some proximal cues that trigger VSMC migration and myofibroblast differentiation became identifiable. The Transforming Growth Factor (TGF) family members (Booth & Bishop, 2010; Jain et al., 2000; Majesky et al., 1991) and the Platelet-Derived Growth Factor (PDGF) (Abramsson et al., 2003; Armulik et al., 2005; Heldin et al.,

1998; Jawien et al., 1992; Waltenberger, 1997) are two of the recognized mediators causing VSMC maladaptive de-differentiation (Owens, 1995; Owens et al., 2004).

The PDGF-BB isoform generated by angiogenic ECs is required to drive pericyte and VSMC migration throughout development. Furthermore, failure of EC-VSMC crosstalk in *Pdgfb* knockout animals is deadly (Leveen et al., 1994). In vitro, PDGF-BB boosts myofibroblast dedifferentiation of mature VSMCs (Holycross et al., 1992). PDGF-AA, the major isoform generated by leukocytes and platelets, on the other hand, is anti-migratory for VSMCs (Koyama et al., 1992). Although neutralizing antibodies to PDGF-AA fail to prevent CAV, widespread inhibition of PDGF receptors reduces both experimental CAV and mechanical arterial injury, indicating that PDGF-BB dominates the artery's maladaptive response (Bilder et al., 1993; Davies et al., 2000; Gocht et al., 2018; Golomb et al., 1996; Hart & Clowes, 1997). Genetic approaches have revealed that the PDGF-BB isoform directly signals to the VSMC PDGF receptor-β, causing intimal hyperplasia following mechanical balloon injury (Caglayan et al., 2011; Deguchi et al., 1999). As a result, blocking the endothelium PDGF-BB signal is a potential target for preventing maladaptive intimal growth, although specific treatments do not exist.

In vascular EC repair contexts, the Endothelial Progenitor Cells (EPCs) in established large arteries move laterally to fill an endothelial defect (Miller-Kasprzak & Jagodzinski, 2007; M. Zhang et al., 2014). Multiple local EC progenitor clones repopulate the endothelium in an aortic EC mechanical denudation model (McDonald et al., 2018). Other paracrine effector mechanisms emerged to highlight different ways the EPCs contributed to vascular repair (C. W. Chen et al., 2018; Li et al., 2016; Ma et al., 2015; Yan et al., 2022; Yi et al., 2019). The angiogenic response of the heart microvasculature becomes blunted, and healing is restricted (Kocijan et al., 2021). Resident EC progenitors have lately emerged as candidates for EC injury repair, although they

did not undergo complete testing for function and their debatable reservoirs (Chambers et al., 2021).

Microvascular angiogenesis requires the production of novel genes to empower vascular sprouting from existing blood vessels. The components of this unique EC developmental pathway become shared with the endothelial regeneration of wounded arteries (Evans et al., 2021; A. G. Masoud et al., 2020; Udan et al., 2013). The endothelial cell differentiates from a resting "phalanx" EC during angiogenesis to acquire motile and invasive properties of the angiogenic "Tip-cell" phenotype (del Toro et al., 2010). In response to growth factor signals, the tip EC drives the invasion of the extracellular matrix (Eilken & Adams, 2010a).

The Vascular Endothelial Growth Factor (VEGF) generated at a tissue level has been identified as the primary proangiogenic cue (Apte et al., 2019; Claesson-Welsh & Welsh, 2013a). However, redundant signals such as Receptor Tyrosine Kinase (RTK)- and G-Protein Coupled Receptor (GPCR)-ligands, all of which become critically linked to PhosphoInositide 3-Kinase (PI3K) activity, may also initiate sprout formation (Hara et al., 2013; Kasai et al., 2008; Oladipupo et al., 2014). The endothelial tip-cell has a higher VEGF-Receptor-2 expression (Gerhardt et al., 2003; Jarad et al., 2017). VEGF signalling is adequate but insufficient to produce EC tip cells, microvessel sprouts or the production of Notch ligands by tip EC, inhibiting tip differentiation in neighbouring EC (A. G. Masoud et al., 2020). Thus, pro-angiogenic cues that signal through the GPCRs (e.g., apelin, SDF1, S1P, and others) work to round off the RTK's signalling input (e.g., the classic VEGF signalling). Such a reciprocal interaction modulates effective CAV repair, amplifies the RTK-mediated signalling magnitude and stimulates vasculogenesis (Figure 6.1) (Balaji Ragunathrao et al., 2019; De Francesco et al., 2017; A. G. Masoud et al., 2020; Odent Grigorescu et al., 2017; Reddy et al., 2008). Moreover, RTK signalling can transactivate the

GPCR or any of its downstream signalling molecules (e.g., the heterodimeric G-proteins, the Adaptor-proteins, and other effector molecules) (Cattaneo et al., 2014; Kilpatrick & Hill, 2021). However, researchers should weigh the advantages and disadvantages of promoting transactivation among these dominant cellular signalling pathways (Araldi et al., 2018; A. K. Azad et al., 2020; Y. Cheng et al., 2017; Di Liberto et al., 2019).

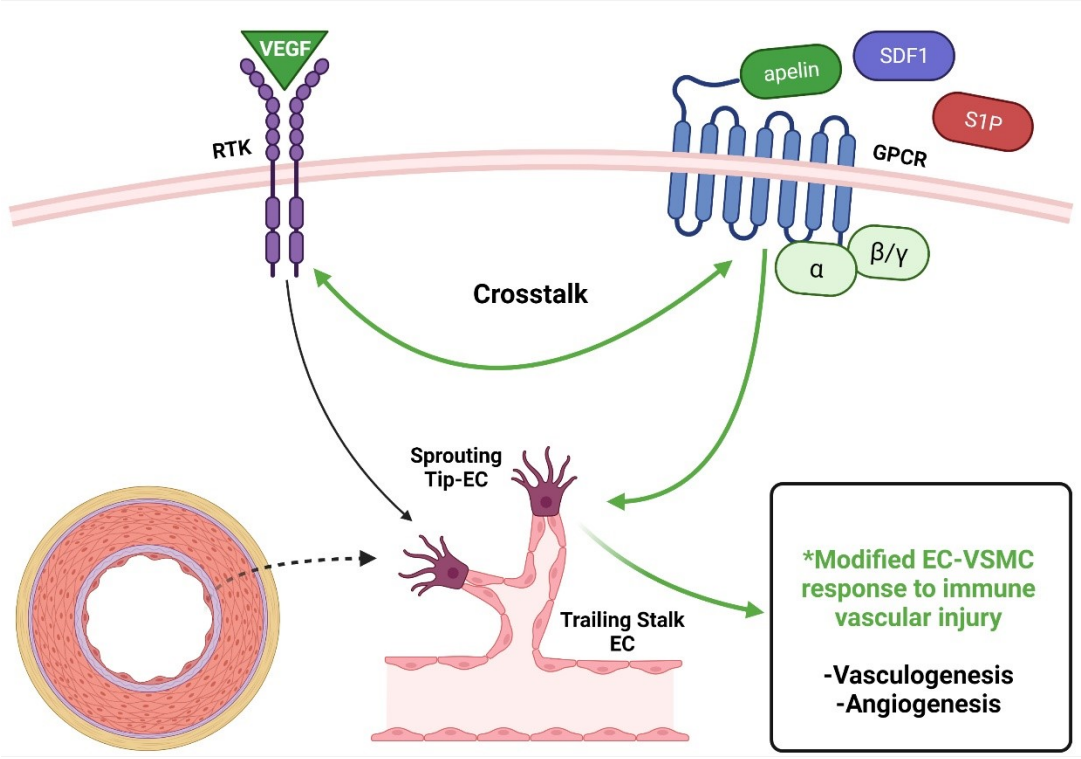

Figure 6.1: GPCR pro-angiogenic signalling complements RTK typical signalling to cue vascular repair.

A depiction that shows the pro-angiogenic GPCR signalling is crucial to complete RTK signalling to generate and maintain a functional tip-EC phenotype and promote vascular EC and VSMCs protection with modified response CAV immune-mediated vascular injury. **(Picture is created by using the available icons in Biorender).**

6.1 Apelin Signaling Is Indispensable for Vascular Repair Following Immune-mediated Vascular Injury of The Chronic Allograft Vasculopathy

Apln, an X-chromosome-linked EC-restricted gene, is constitutively expressed in the coronary endothelium (M. M. Chen et al., 2003; Cox et al., 2006; del Toro et al., 2010; Kawamata et al., 2001; Lee et al., 2000; Tian et al., 2013; Wysocka et al., 2018). According to the current study, apelin deficiency worsens CAV. Both *Apln* and its receptor (*Aplnr*) genes become activated in tip endothelial cells in vivo (del Toro et al., 2010). Further, *Apln* EC deletion in mutant mice is associated with defective embryonic and pathological angiogenesis (Charo et al., 2009; Eyries et al., 2008; Kasai et al., 2008). Nonetheless, examples of these mice are alive and well (Ishida et al., 2004; Kuba et al., 2007a).

Notably, tip-cell endothelial apelin communicates to nearby ECs in the endothelial vascular sprout to boost EC migratory activities and the VSMCs and cardiomyocytes (Eyries et al., 2008; Hashimoto et al., 2006). Loss of EC apelin synthesis in myocardial infarction and damaged kidney glomerular microvasculature is functionally associated with unsuccessful vascular repair and eventual organ failure (Haddad, Zhabyeyev, Farhan, Zhu, Kassiri, et al., 2014; Wang et al., 2013b).

The apelin GPCR (ApelinR) expression is evident in various tissues, including ECs, VSMCs, fibroblasts, and cardiomyocytes (Kleinz et al., 2005; Pope et al., 2012). Loss of the ApelinR produces embryonic and early postnatal mortality in mice (Kang et al., 2013) and aberrant vasculature deficient VSMCs (Kleinz et al., 2005). In arteries, apelin communicates with phalanx EC (through PI3Kβ/Akt/eNOS to create nitric oxide) and bordering VSMCs that express the apelin receptor continuously (del Toro et al., 2010; Kalin et al., 2007; Kasai et al., 2004; Mughal et al., 2018). Loss of apelin signalling has lessened neointimal growth in atherosclerosis and

arterial ligation models (Kojima et al., 2010), although there is a differing indication that it worsens vasculopathy in pulmonary hypertension (Alastalo et al., 2011; Chandra et al., 2011). In vitro investigations reveal that apelin has intricate variable effects on VSMC proliferation, migration, calcification, and phenotypic transitioning (Samura et al., 2016; Shan et al., 2011). Additionally, apelin limits the expansion of vascular smooth muscle cells and the proliferation of fibroblasts from the adventitia and controls the levels of various chemoattractant factors (Brash et al., 2012; Leeper et al., 2009; H. Zhang et al., 2014).

We investigated the development of CAV by exploiting the heterotopic mouse heart transplantation model to characterize arterial neointimal and microvascular pathologies. Transplantation of male hearts to female major histocompatibility antigen-matched recipients elicited alloimmune responses against the minor HY histocompatibility antigen produced by male hearts. This delayed response results in T-cell-mediated alloimmune rejection (Bedi et al., 2010; Corry et al., 1973a). *Apln* knockout or littermate wild-type heart transplants into wild-type female mice were employed to examine the role of apelin. We identified allograft vascular injury at two weeks post-transplant. We see a significant increase in tip EC genes such as *Apln*, *Esm1*, and *Pdgfb* in both micro-dissected left anterior descending arteries and microvascular compartments of the wild-type vascular tissues.

In contrast, *Apln*-knockout heart allografts do not express tip endothelial cell genes. Remarkably, both groups had a substantial induction of *Vegfa* expression. Six weeks after transplantation, arterial neointimal proliferation had advanced to occlude 50% of the vascular arterial lumen in wild-type hearts, while *Apln* knockout hearts had 90% occlusion.

Furthermore, microvessel density is reduced by 30% in *Apln* knockout hearts. These findings suggest that in response to immune-mediated endothelial injury, *i*) the graft ECs, as well as a

fraction of the VSMCs, upregulates the expression of apelin, and ii) apelin production is essential to elicit and maintain future EC reparative tip cell differentiation. Failed differentiation is associated with maladaptive arterial repair deterioration and loss of microvascular density.

We exploited APLN-17, a synthetic apelin receptor agonist analogue resistant to serum proteolytic enzyme degradation (W. Wang et al., 2019), to treat heart transplant recipients beginning two weeks after transplantation, when early vascular damage is manifest. Surprisingly, APLN-17 slows the course of arterial neointima thickening and boosts microvessel density compared to control mice. These findings suggest that apelin receptor signalling is rate-determining in vivo under pathophysiologic circumstances, owing to the rapid destruction of native apelin by (inducible) proteolytic enzyme activity. However, while the apelin receptor features a broad expression by vascular cells, it is not shown that the advantageous CAV-protection effects are due to EC signalling.

The immune response to the heart allograft was studied in the systemic (spleen) and within the allograft. We found a comparable rise in HY-alloreactive lymphocytes in apelin knockout and control grafted mice. However, we see an enhanced signal for lymphocyte interferon-γ and interferon-γ-dependent chemokines (e.g., CXCL11) in the *Apln* deficient heart allografts two weeks post-transplant. APLN-17 supplementation, on the other hand, decreased inflammation. This observation suggests that apelin reduces the local activation of recipient effector lymphocytes, either directly or indirectly.

6.2 Endothelial Phosphoinositide 3-Kinase β Modulates Vascular Adhesion Molecules' Expression Following Inflammatory TNF-α Stimulation by Controlling The Classical NF-κB Pathway Activation

For the first time, we identified the role of vascular endothelial PI3Kβ in modulating the NF-κB canonical pathway activation. We observed that the inactivated PI3Kβ hindered the IκBα degradation and suppressed the NF-κB-65 nuclear translocation (indicators of the NF-κB canonical pathway activation). Furthermore, the inactivation of the PI3Kβ blocks the arterial endothelial fractalkine expression (the CX3CL1 chemokine, in vitro and in vivo). Moreover, inactivating the PI3Kβ hinders the expression of inflammatory immune-modulated vascular adhesion molecules (e.g., ICAM-1 and VCAM-1) (in vitro and in vivo). These findings were consistent in our CAV model and the proinflammatory TNF-α-stimulated cultured human and mouse endothelial cells. Notably, such adhesion molecules (e.g., ICAM-1, P-selectin, and VCAM-1) significantly increased during allograft rejection (Briscoe et al., 1991; Koskinen & Lemstrom, 1997; Tanio et al., 1994). Further, these vascular adhesion molecules have been considered predictors of CAV development in patients with cardiac allograft vasculopathy, indicative of the prognostic importance of the endothelial activation status in CAV to speculate on future graft failure (Labarrere et al., 1997; Labarrere et al., 2000). Moreover, the inflammatory cytokines released from the infiltrating immune-inflammatory and the activated endothelial cells (e.g., the IL-1β, TNF-α, and others) are significantly involved in the adhesion molecule upregulated expression. They affect coronary arterial VSMC activation and proliferation (Autieri, 2003; Densem et al., 2000; Gullestad et al., 1999). In the current study, we identified the effects of endothelial PI3Kβ inactivation on protecting the allograft vascular arterial ECs and VSMCs from the infiltrating inflammatory immune cells (deemed fully functional to deliver an effective allogeneic immune response). These inflammatory cells' adhesion and arrest on the vascular

endothelium with the subsequent endothelial activation became modulated by the PI3Kβ through controlling the expression of arterial CX3CL1 Fractalkine chemokine and the correlated vascular adhesion molecules' expression during immune injury. This effect can open new avenues in repurposing the PI3Kβ-selective inhibition to combat vascular (immune) inflammation.

We speculate that the PI3Kβ activity might interact with the NF-κB pathway at the level or upstream of the IKKs within the TNF-α-stimulated canonical NF-κB signalling cascade.

Previously, the activation of PI3Kγ emerged to stimulate the VSMC proliferation, phenotypic transition, and neointimal expansion in a tested rat aortic allograft model of transplantation arteriosclerosis (Yu et al., 2018). Furthermore, in vitro silencing of the PI3K/IKK/NF-κB-65 axis hindered the TNF-α-induced atherosclerosis progression, positioning the PI3K (not a specific isoform) upstream of the IKK molecule to regulate the NF-κB pathway activation in vascular inflammation (Dai et al., 2019). Extending our study, we can further evaluate the effects of PI3Kβ inactivation on modulating the expression of vascular adhesion molecules and EC in different but CAV-relevant, pro-inflammatory endothelial pathways (e.g., the IL-1β/NF-κB/ICAM-1 or VCAM-1 cascade). Additionally, we can test the impact of PI3Kβ inactivation on modulating the stress kinases' proteins (e.g., the P38 MAPK, which demonstrated protection from CAV when inhibited (Ollinger et al., 2008)) to assess the stress response in vascular inflammation following the PI3Kβ inactivation. This effect can lay the foundations to test the PI3Kβ as a secondary messenger molecule downstream of endothelial pro-inflammatory stimuli (e.g., TNF-α in the current study) and upstream of the P38 MAPK or the IKK within the canonical NF-κB-modulated pathways. Hence, future experiments testing the role of PI3Kβ in controlling the stress-activated proteins could control vascular inflammation activation pathways.

6.3 Rescuing Endogenous Vasoactive Peptides, Including Apelin, From Neprilysin-mediated Degradation Protects the Transplanted Vascularized Heart Allografts From Vascular Immune-mediated Injury With Suppressed Chronic Allografts Vasculopathy.

Despite its potent vascular functions, the effects of apelin become hampered by its short half-life in the plasma because of its endogenous enzymatic degradation (Japp et al., 2008). To a certain extent, the Angiotensin-Converting Enzyme type-2 (ACE-2) was correlated to the apelin's metabolism while functioning as a "carboxy-monopeptidase that contains zinc in its structure" to process [Pyr]-apelin-13 into an apelin-12 metabolite, and to a lesser extent, the [Pyr]-apelin-17 into apelin-16 (Vickers et al., 2002; Wang et al., 2016). As a neutral endopeptidase/metalloprotease, Neprilysin degraded the endogenous apelin by shortening the apelin RPRL motif (Arg2-Leu5). This effect makes the final apelin product incapable of binding to its cognate receptor, the apelin GPCR (ApelinR) (McKinnie et al., 2016).

Other vasoactive peptides are prone to degradation by neprilysin, such as the bradykinins, B-type Natriuretic Peptide (BNP), adrenomedullin, and the C-type Natriuretic Peptide (CNP) (D'Elia et al., 2017). Hence, to better interpret the complete picture of CAV protection following neprilysin inhibition by sacubitril treatment, we can utilize the *Apln* knockout mice to retest the sacubitril treatment effect on CAV development. This approach can directly correlate sacubitril's protective action(s) in CAV models to apelin modulation (i.e., establishing causality).

6.4 Study Limitations in Translating The Current Biological Findings To Contribute To Clinical CAV Management

The current biological findings of our study represent state-of-the-art therapeutic targets and the repurposing of clinically approved drugs that can benefit patients undergoing solid organ transplantation or patients suffering from vascular immune diseases (e.g., autoimmune

vasculitis). For example, the investigated apelin effects on orchestrating the expression of the vascular endothelial tip-cell repair machinery in the transplanted heart allografts undergoing immune-provoked injury consistently with the observed anti-inflammatory effects and rescuing of these heart allografts following administering the enzymatic degradation-resistant APLN 17 synthetic analogue peptide represent promising translational findings that can open new avenues for vascular regenerative and inflammation-hindering therapies. In the same direction, apelin has been the seed of many clinical trials that study metabolic diseases, cardiovascular pathologies, and pulmonary artery hypertension (Barnes et al., 2013; Brame et al., 2015; Brash et al., 2018; Japp et al., 2010). Notably, these are just a few examples of the many clinical trials to test apelin clinically, whereby an ideal apelin therapeutic reagent is biased for apelin receptors with less involvement of β-arrestins to minimize receptor desensitization as well as hypotensive effects with chronic use (Besserer-Offroy et al., 2018; Read et al., 2016). Furthermore, apelin has a solid potential to alleviate many cardiovascular diseases as a promising drug target (Zhong et al., 2017).

Still, in many studies of tumour progression and angiogenesis, apelin underwent testing for its expression along with its receptor on many types of tumours, its influence on tumourigenesis fueling, and its targeting to alleviate resistance to anti-angiogenic therapies (Berta et al., 2010; Feng et al., 2016; Hao et al., 2017; Hoffmann et al., 2017; Masoumi et al., 2020; Mastrella et al., 2019; Muto et al., 2014; Uribesalgo et al., 2019). Therefore, clinicians and researchers must apply careful planning and optimization of the potential apelin treatments when trying them on transplant patients with a family history of cancer or undergoing active tumour pathologies.

6.5 Future Directions

1. **Characterization of The Role of The Apelin G-protein Coupled Receptor (ApelinR) In Vascular Repair Following Immune-mediated Vascular Injury of CAV.**

   Our findings link APLN-17 actions on cultured EC to direct repair cues via the apelin receptor. However, the apelin functional modulation to attenuate transplant vasculopathy may be indirect in vivo via signals to different cell types. As a result, we will apply a genetic strategy to selectively eliminate graft vascular endothelial apelin receptor (*Aplnr)* expression in ECs in vivo to see if EC *Aplnr* facilitates vascular protection. Additionally, endothelial single-cell transcriptome analysis (single-cell RNAseq, scRNAseq) will be used to characterize the heart transplant microvascular EC subpopulations and identify the frequency of reparative cells expressing tip cell marker genes. Hearts will be digested and then enriched for single EC by flow cytometry-sorting on CD31 expression. Freshly isolated EC suspensions as pools of three heart samples per group from pre-transplant reference, *Aplnr*-inducible EC-specific knockout, and wild-type heart groups will be processed at the High Content Analysis Core at the University of Alberta. The scRNAseq cDNA expression library samples will be sequenced by Novogene. Bioinformatic comparison with established scRNAseq databases of normal heart vascular cells and cardiomyocytes will be used to identify and exclude non-EC from the analysis. Genes with more than 2-fold change versus the reference will be analyzed for pathway enrichment.

2. **To Study CAV And the Effects of Apelin Following the Loss- or Gain-of-function Approaches In Major Histocompatibility Complex Haplotype-mismatched Heart Transplants, Which Mimic Heart Transplantation In Humans.**

   To utilize an aggravated rejection model that uses the major histocompatibility complex-mismatched heart allograft transplants. In this model, we can further test the effects of *Apln*

knockout/genetic deletion (loss-of-function experiment) as well as the proteinase-resistant APLN-17 daily intraperitoneal treatments beginning from week two till week six post-transplantation (gain-of-function experiment) on CAV outcomes in this accelerated T cell-mediated rejection in vivo model (e.g., the functional assessment of the allograft antigen-presenting cells).

3. **Exploiting Antibody-mediated Rejection Models of CAV To Study the Role of Apelin in Vascular Injury And Repair Within The Context of Antibody-mediated Rejection.**

To utilize Antibody-Mediated Rejection (ABMR) in vivo models to test the effects of *Apln* knockout (loss-of-function experiment) side by side with the proteinase-resistant APLN-17 daily intraperitoneal injections starting from the second week till the sixth-week post-transplantation (gain-of-function experiment) on CAV outcomes in this distinct ABMR rejection model.

Vasculopathy caused by Donor-Specific Alloantibody (DSA) is a primary cause of CAV and graft failure in solid organ transplants (Baldwin et al., 2016; Lefaucheur et al., 2013; Mangiola et al., 2017; Topilsky et al., 2013). Presently no medication is available to lower the levels of antibodies or to effectively alter the pacing of increasing graft and patient mortality in ABMR (Kim & Brennan, 2021; Schinstock et al., 2020). We will be attempting to adjust the tissue response to immunological insult by investigating the role of apelin in immune response modulation and vascular cellular repair in this ABMR model of CAV. The production of DSA necessitates a helper T-cell immune response (Susal et al., 2018), and ABMR is frequently associated with cellular rejection (Haas et al., 2018). Nevertheless, the mechanism(s) of isolated DSA-dependent graft vascular damage may be partly NK cell- or complement system-dependent rather than T cell-dependent (Farkash & Colvin, 2012; Hidalgo et al., 2010; Pontrelli et al., 2020; Sis & Halloran, 2010; Thurman et al., 2019;

Turner et al., 2019; Yazdani et al., 2019). Alloantibody injury, like HY-antigen-dependent cell-mediated reactions, is focused on the graft vasculature (Jane-Wit et al., 2013; Piotti et al., 2014). The DSA, on the other hand, causes EC adaptation, which may attenuate the type of reaction to injury (McCaughan & Tinckam, 2018; Tang & Platt, 2007). Thus far, the role of apelin in modulating ABMR CAV has never undergone full investigation.

4. **Characterization of The Role of PI3Kβ Downstream of The Interleukin-1β Pro-inflammatory Pathway in ECs To Modulate Vascular Adhesion Molecules' Expression In Correlation With The NF-κB Canonical Pathway Activation.**

   To test the role of the Phosphoinositide 3-Kinase beta (PI3Kβ) in controlling adhesion molecules' expression, in correlation to NF-κB activation, following further proinflammatory endothelial stimulation with IL-1β. We can explore if PI3Kβ is conserved as a messenger molecule to regulate both inflammatory signalling pathways in the ECs (i.e., in our currently tested TNF-α pathway and the proposed IL-1β vascular inflammation pathway). This approach can confirm the importance of PI3Kβ in controlling vascular immune injury and inflammation for potential clinical testing in patients.

5. **Studying The Impact of PI3kβ Inactivation on The Stress Kinases' Activation Signalling (e.g., The P38 Stress-activated Mitogen-Activated Protein Kinase-MAPK) Within the Contexts of NF-κB Canonical Pathway Activation.**

   The various roles played by the Mitogen-Activated Protein Kinase (MAPK) subfamilies (e.g., the c-Jun NH2-terminal Kinase (JNK), the Extracellular signal-Regulated Kinase-1/2 (ERK1/2), and the p38 MAPK) in provoking vascular injury during heart transplantation have been a matter of intensive research (Vassalli et al., 2012). Previous research identified a link between P38 MAPK and CAV development, whereby an increased P38 MAPK phosphorylation/activation becomes evident in aortic allografts versus the control aorta. At

the same time, the inactivation of P38 MAPK hindered the development of Chronic Allograft Vasculopathy (CAV) (Ollinger et al., 2008). Furthermore, P38 MAPK is a significant modulator of the inflammatory cytokines (e.g., the TNF-α and the IL-6) secretion, induction, and net effects to provoke ischemic cardiovascular remodelling with depressed cardiac contractility (Bellahcene et al., 2006; Li et al., 2005; Yin et al., 2008). Hence, precisely locating the PI3Kβ (potentially upstream of the P38 MAPK) within the vascular inflammatory NF-κB canonical pathway is potentially therapeutic to target CAV development and progression. Mainly, a mechanistically defined signalling axis that explains the observed vascular CAV protective effects following PI3Kβ inactivation (e.g., the suppressed adhesion molecules' expression) would pave the way to hinder CAV and other clinically significant immune-mediated vascular inflammatory injury diseases (e.g., the autoimmune vasculitis).

6. **Characterization of The Direct Correlation Between Sacubitril and Apelin In Alleviating CAV Progression.**

   We can utilize *Apln* knockout heart allograft transplants in a dedicated in vivo cohort to track the direct impact of Sacubitril (neprilysin inhibitor) treatment on CAV outcomes and see if it still can exert its effects in the absence of *Apln* in these heart allografts. This definitive experiment can confirm the direct effector relationship between Sacubitril and apelin to produce allograft vasculopathy suppression with hindered vascular EC injury. Further, this approach can identify other vasoactive and vascular protective peptide targets of sacubitril in allograft vasculopathy other than the apelin target.

7. **Investigation of CAV Progression in Aged Heart Allografts.**

   To broaden the existing research scope to include researching the role of apelin in vascular senescence, its influence on CAV development in aged heart allografts, and the CAV vascular consequences associated with aging. As a result, we can assess apelin's angiogenic, pro-

reparative, anti-inflammatory, anti-apoptotic, and vascular-preserving properties in transplanted old-donor heart allografts. We will compare the impact of i) the proteinase-resistant APLN-17 synthetic peptide and ii) Sacubitril therapy in CAV to control vehicle-treated age-matched elderly mouse heart transplants. Considering the current study findings towards outlining a GPCR-mediated vascular endothelial repair (senolytic) pathway, our preclinical approach to studying vascular senescence in correlation with CAV would translate novel research approaches to benefit the underrepresented sector of elderly transplantation patients undergoing transplantation rejection.

8. **Characterization of EC Death forms in CAV, Other Than Apoptosis.**

In the current study, we investigated vascularized heart allografts' endothelial injury and assessed the endothelial loss or gaps present alongside the continuity of the endothelial contour in coronary arterial cross-sections among our experimental groups of CAV. We further characterized the process of programmed cell death (i.e., apoptosis), which entails intrinsic and extrinsic pathways of signalling activation with inevitable cell death (McIlwain et al., 2013; Norbury & Hickson, 2001), using the activated caspase-3 marker as an indicator of apoptosis in these heart allograft vascular ECs undergoing immune injury to decipher the observed EC loss patterns better. Still, other forms of EC death in CAV represent a reasonable study target aside from apoptosis (Lukenaite et al., 2022). Examples include the non-programmed cell necrosis (Adigun et al., 2022), autophagy to replenish the cellular elements constantly via autophagosome-mediated transport of misfolded proteins to lysosomes for destruction (Orsi et al., 2012), and the recently identified process of pyroptosis, which is a specialized way of programmed cell necrosis (He et al., 2021). The latter form of cell death entails inflammasome activation, stimulation of the canonical cysteinyl aspartate specific proteinase 1 (caspase 1), stimulation of the non-canonical caspase-4/5/11, cellular

edema, disruption and burst of the cellular membranes, activated NFκB cascades, and heightened inflammation occurring endogenously following cell injury with infections, as examples of pyroptosis triggering factors (Emini Veseli et al., 2017; Hu et al., 2018; Kayagaki et al., 2013; Y. Wang et al., 2019). Pyroptosis can occur in various cell types, such as ECs, VSMCs, DCs, macrophages, and cardiomyocytes (Reisetter et al., 2011; Shi et al., 2021; Yang et al., 2022; Yu et al., 2021). Indeed, studying the vascular cellular impact of pyroptosis can help decipher various disease pathologies and physiological processes, such as EC pyroptosis in atherosclerosis plaque instability and rupture (Hoseini et al., 2018; Xu et al., 2018; Yin et al., 2015), vascular tissue factor expression, coagulation and immunothrombosis (Ryan et al., 2022), control of allograft homeostasis (Burke et al., 2021), autoimmune diseases (You et al., 2022), and various cardiovascular diseases (Ji et al., 2021).

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