

Regulation of Irf2bp2 and deficiency in sepsis-induced QT prolongation

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Abstract

The innate immune system is the body's first line of defence against viral and bacterial pathogens, and the inflammatory response is crucial in maintaining a healthy cellular environment. Interferon regulatory factor 2 binding protein 2 (IRF2BP2), a zinc finger transcriptional corepressor highly expressed in macrophages, suppresses the interferon response and inflammation. In humans, individuals who carry two copies of a genetic variant (rs3045215) that deletes 9 nucleotides from the long 3'UTR of IRF2BP2 have lower IRF2BP2 protein expression in white blood cells and increased risk of coronary atherosclerosis and calcification. The first objective of this thesis was to determine how expression of IRF2BP2 is regulated by its 3'UTR and how this deletion variant leads to lower protein expression. For this, a combination of cellular and molecular techniques were used. First, we showed that the deletion variant is unable to interact with eukaryotic initiation factor 4H (eIF4H), a translation initiation factor that is required for the unwinding of GC-rich mRNA secondary structures. The 9-nucleotide deletion disrupts a proposed stem-loop structure, which is critical for interaction with eIF4H. siRNA knockdown of eIF4H preferentially disrupts translation of the non-deletion variant of the IRF2BP2 3'UTR, but not the deletion allele, revealing an essential role for the 9-nucleotide sequence in interacting with eIF4H. Previous work from our lab demonstrated that expression of IRF2BP2 is downregulated in response to LPS in bone marrow-derived macrophages (BMDM), attenuating the anti-inflammatory function of IRF2BP2 in the presence of endotoxins. We further uncovered a role for IRF2BP2 protein in binding to its own 3'UTR in the cytoplasm in response to LPS, suggesting an autoregulatory function. This response is lost in the rs3045215 deletion variant, demonstrating the importance of the 9-nucleotide sequence in the 3'UTR in regulating its own expression. We also confirm that a single nucleotide polymorphism (rs7545855) located in exon 1 of IRF2BP2 that is co-inherited with rs3045215 does not confer any functional differences with respect to the autoregulatory mechanism. Because IRF2BP2 deficiency leads to pro-inflammatory macrophages, we asked whether mice lacking IRF2BP2 in their bone marrow-derived

macrophages would display a heightened response to lipopolysaccharides with respect to their cardiac electrophysiology, which is sensitive to inflammatory signaling. By using *LysMCre/Irf2bp2^{flx/flx}* mice whose macrophages are prone to inflammation, we revealed that female mice lacking IRF2BP2 in their BMDMs are uniquely protected against severe LPS-induced QT prolongation, an indicator of delayed ventricular repolarization. These female mice displayed an upregulation of growth differentiation factor 3 (GDF3) mRNA and protein, a member of the TGF β family of signaling molecules that protects mice against LPS-mediated cardiac dysfunction. Our ECG studies revealed that pre-treating male wild type mice with recombinant GDF3 (rmGDF3) 12 hours prior to exposure to LPS similarly protected them from severe LPS-induced QT prolongation. Mechanistically, we discovered that short term exposure to LPS results in the upregulation of the sodium-calcium exchanger (NCX) in the mouse myocardium, which is reduced in mice pre-treated with rmGDF3. Our *in vitro* studies revealed that rmGDF3 blocks the uptake of toll-like receptor 4 (TLR4) and TRIF signaling in HL-1 cardiomyocytes and neonatal rat ventricular myocytes, providing insight into the possible mechanisms underlying this protection. Together, the data provide valuable insight into the mechanisms governing expression of IRF2BP2 in response to endotoxins. As well, our findings reveal sex-specific differences in the response to IRF2BP2 deficiency, with males showing an increased risk of coronary artery calcification, while females are protected from LPS-induced QT prolongation through the action of increased GDF3. Our results suggest that GDF3 should be further investigated as a potential therapeutic for protecting patients against QT prolongation in the face of inflammatory diseases, who face an increased risk of ventricular arrhythmias and sudden cardiac death.

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List of Abbreviations

A

AF	Atrial fibrillation
APD	Action potential duration

B

BMDM	Bone marrow-derived macrophages
BSA	Bovine serum albumin

C

CVID	Common variable immunodeficiency disorder
CAD	Coronary artery disease

D

DMEM	Dulbecco's Modified Eagle Medium
------	----------------------------------

E

EAD	Early afterdepolarizations
ECG	Electrocardiogram
eIF	Eukaryotic translation initiation factor

F

FBS	Fetal bovine serum
-----	--------------------

H

HCM	Hypertrophic cardiomyopathy
hiPSC-CM	Human induced pluripotent stem cell-cardiomyocytes

I

i.p.	Intraperitoneal
------	-----------------

K

KO	Knockout
----	----------

L

LD	Linkage disequilibrium
LQTS	Long QT Syndrome
LPS	Lipopolysaccharides

N

NRVM	Neonatal rat ventricular myocytes
------	-----------------------------------

P

PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PPR	Pattern recognizing receptors

Q

QTc	Corrected QT
-----	--------------

R

ROS	Reactive oxygen species
-----	-------------------------

S

SICM	Sepsis-induced cardiomyopathy
------	-------------------------------

T

TBST	Tris-buffered saline with Tween 20
TLR	Toll-like receptor

U

UTR	Untranslated region
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W

WT	Wildtype
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Copyrighted Contents

This dissertation contains findings from the following publications:

Duong, A., et al. (2026). "A 3'UTR polymorphism disrupts IRF2BP2 autoregulation through an eIF4H translational enhancer." Frontiers in Genetics.

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Vilmundarson, R. O., et al. (2022). "Savior Siblings Might Rescue Fetal Lethality But Not Adult Lymphoma in Irf2bp2-Null Mice." Frontiers in Immunology **13**.

1. Introduction

1.1. Cardiovascular disease

Cardiovascular diseases (CVD) are conditions that affect the heart and blood vessels, including heart attacks, strokes, coronary artery disease and hypertension. Globally, the World Health Organization estimates that CVDs represent 32% of all deaths, making it the leading cause of mortality. Risk factors for CVD can include diet, physical activity, tobacco or alcohol use, environmental conditions, economic status and stress. While lifestyle plays a large part in determining the onset of CVDs, genetics can also determine a patient's risk for developing certain cardiovascular complications, making it crucial to better understand the genes involved and the molecular mechanisms at play.

1.1.1. Inflammation and cardiovascular disease

Systemic and local inflammation are associated with the development and pathogenesis of a range of cardiovascular diseases, including atherosclerosis, arterial calcification, myocardial infarction, and are known to cause cardiac remodeling and disrupt cardiac electrophysiology (Boyalla et al., 2023). Inflammation is a highly complex cascade of cellular signaling pathways that is activated in response to a stressor such as infection or injury and is the body's primary defense and tissue repair mechanism. Inflammation can be triggered by the invasion of exogenous pathogens, such as viruses or bacteria, which are detected by host immune cells, including macrophages, dendritic cells and mast cells, which initiate a defensive inflammatory response to eradicate the pathogens (Medzhitov, 2008). Alternately, sterile inflammation can arise in the absence of microorganisms and is triggered in response to damaged or injured cells (Chen & Nunez, 2010). When properly regulated,

inflammation is a protective and beneficial response, however when inflammation is chronic or dysregulated, this can lead to sepsis, tissue injury and a range of diseases (Medzhitov, 2008).

Atherosclerosis is the buildup of plaques inside the arterial walls, leading to a progressive thickening of the arterial walls and a loss of elasticity. It is a chronic inflammatory disease in which low-density lipoproteins (LDLs) infiltrate the tunica intima layer of the endothelium, activating endothelial cells (ECs), causing the recruitment of macrophages to the subendothelial space (Tasouli-Drakou et al., 2025). There, the LDLs are oxidized into low-density lipoprotein cholesterol (oxLDL-C) by reactive oxygen species and taken up by macrophages to ensure tissue cholesterol homeostasis. The cholesterol is then typically released to high-density lipoproteins (HDL), which carry it back to the liver to be excreted in bile salts. In environments where cholesterol uptake by the macrophages is excessive, or when cholesterol efflux to the HDLs is impaired, the macrophages become lipid-laden foam cells (Ouimet & Marcel, 2012). Over time, these foam cells accumulate, forming fatty streaks that mature into plaques (McLaren et al., 2011), which then gradually become calcified in the endothelium.

1.2. IRF2BP2

Variants of interferon regulatory factor 2-binding protein 2 (IRF2BP2) have been discovered in patients with elevated plasma total cholesterol (Teslovich et al., 2010), LDL-C (Lettre et al., 2011) and ischemic heart disease (Murray et al., 2012). Previous research from our lab established IRF2BP2 as a key player in the progression of atherosclerosis through its regulation of the anti-inflammatory transcription factor Krüppel-like factor 2 (KLF2).

IRF2BP2-deficient macrophages were shown to adapt an inflammatory phenotype prone to foam cell formation and mice lacking IRF2BP2 in their bone marrow-derived macrophages had higher total serum cholesterol levels and larger atherosclerotic lesions (H.-H. Chen et al., 2015). Together, this data demonstrates that IRF2BP2 is a key regulator of macrophage inflammatory polarization.

1.2.1. Gene and protein structure

Interferon regulatory factor 2-binding protein (IRF2BP) is a family of transcriptional regulators encoded by the genes IRF2BP21, IRF2BP2 and IRF2BP2L. The gene encoding IRF2BP2 is localized on 1q42.3 and contains a single intron, while IRF2BP1, located on chromosome 19q13.32, and IRF2BPL, found on chromosome 14q24.3, both lack introns. IRF2BP2 is a 61 kDa nuclear protein present in two splicing isoforms, A and B, and was first described as a transcriptional co-repressor that works in conjunction with IRF2 to inhibit enhancer-activated and basal transcription (Childs, 2003). Structurally, IRF2BP2 A and B share two conserved regions: an N-terminus C4 zinc finger domain, which mediates homo- or heterodimerization/multimerization between IRF2BP family members (Yeung et al., 2011) and a C-terminus CH3C4 real interesting new gene (RING) domain, which is responsible for binding to partner proteins such as IRF2 (Childs, 2003) and NFAT (Carneiro et al., 2011). Evidence shows that binding to VGLL4 and IRF2 occurs via an interaction between the sequence just outside of the RING domain of IRF2BP2 and a highly conserved RxSVI motif on VGLL4 and IRF2 (Wang et al., 2024). The nuclear localization signal is found between the RING domain and the zinc finger and is also highly conserved between organisms (Figure 1.1) (Teng et al., 2011). The mRNA of IRF2BP2 also has a long 3'UTR, which is targeted by miR-155-5p for transcriptional downregulation (H. F. Li et al., 2020).

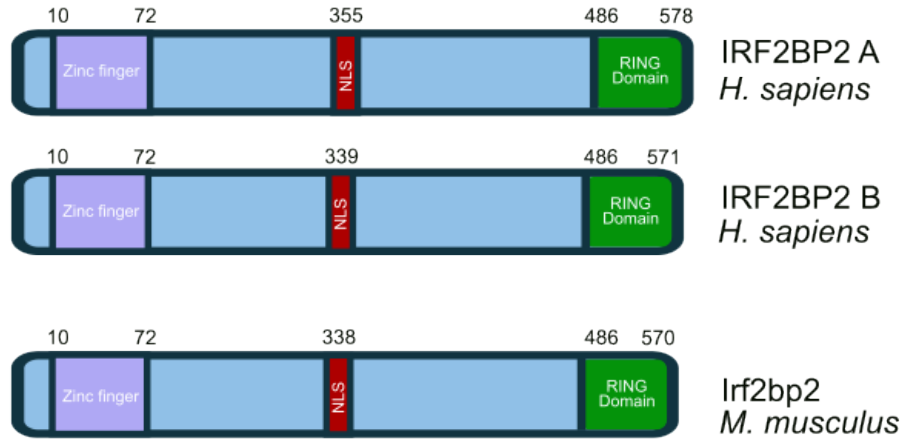


Figure 1.1 Protein structure of IRF2BP2 in humans and mice. The human IRF2BP2 A and B isoforms contain a zinc finger domain at the N-terminus and a RING domain at the C-terminus. The nuclear localization signal (NLS) is found between these two domains. These motifs are highly conserved in humans and mice.

1.2.2. IRF2BP2 as a co-repressor of IRF1

IFN-regulatory factor 1 (IRF1) is maintained at low levels in human cells and is activated by the JAK/STAT pathway in response to infection stimuli such as interferons (IFN) and nuclear factor kappa-B (NF- κ B) (J. Wang et al., 2020). Following activation, IRF1 moves into the nucleus, a process which is mediated by the nuclear localization signal (NLS) found at amino acid residues 117-141 in human IRF1 (Feng et al., 2021). Transcriptional activity is reliant on the 115 amino acid N-terminal DNA-binding domain which is highly conserved across all 9 mammalian variants of IRF (Feng et al., 2021). IRF1 also interacts with other transcriptional regulators in the nucleus through its C-terminal IRF-association domain, which is unique to IRF1. The result is the activation of pro-inflammatory pathways of the innate immune response, including transcription of type I and type III interferons to combat

viral infections(J. Wang et al., 2020), activation of apoptosis through caspase-1 (Karlsen et al., 2000; Kim et al., 2002) and promotion of the NLRP3 inflammasome and cell death (Kuriakose et al., 2018). While IRF1 traditionally works to activate inflammatory pathways in response to viral infections, IRF2 and IRF2BP2 are constitutively expressed in cells and work to block the action of IRF1 in basal conditions. Initially described by Childs and Goodbourn, IRF2BP2 was found in a yeast two-hybrid system to interact with the C-terminus of IRF2, which recruits the protein to DNA promoter regions, resulting in the repression of IRF1-regulated genes (Figure 1.2) (Childs, 2003).

Since this study, other groups have demonstrated the role of IRF2BP2 in IRF2-independent pathways and functions. Nuclear factor of activated T cells (NFAT) is a family of transcription factors that are critical in the regulation of many immune cells and the expression of cytokines including IL-2, IL-4, TNF- α and IFN- γ (Viola et al., 2005). In studies looking at the interaction between IRF2BP2 and NFAT1, co-transfection of IRF2BP2 with NFAT1 reduces the expression of TNF- α , IL-2 and IL-4 compared to transfection with NFAT1 alone (Carneiro et al., 2011). In addition, overexpression of IRF2BP2 in CD4 T cells decreased endogenous levels of IL-2 and IL-4. These results establish the role of IRF2BP2 in repressing NFAT-mediated gene expression.

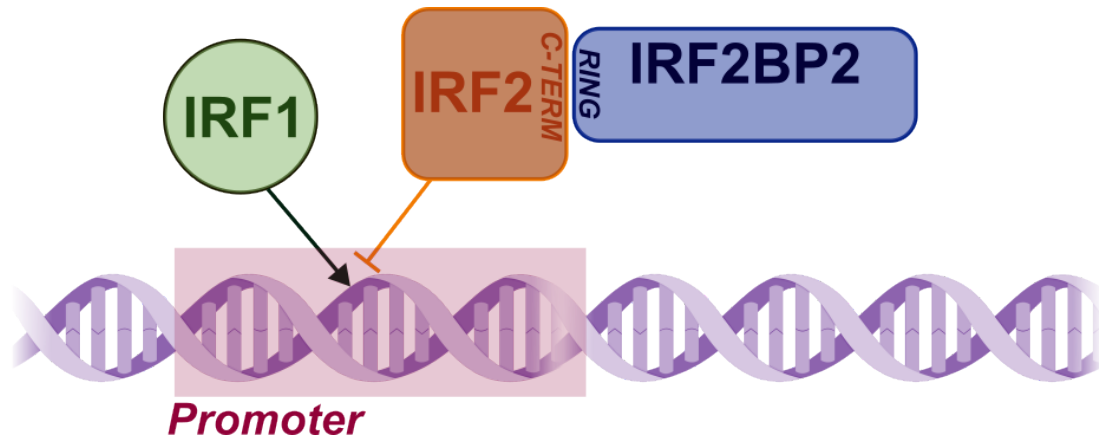


Figure 1.2 Interaction of IRF2 and IRF2BP2 blocks expression of IRF1 target gene sequences. IRF2BP2 binds to the C-terminus of IRF2 and acts as a transcriptional co-repressor, blocking the expression of IRF1-targeted genes.

1.2.3. IRF2BP2 in human disease

Due to its importance in many cellular processes, such as proliferation (Secca et al., 2016), (Feng et al., 2020), (Yan et al., 2022), apoptosis (Max Koeppel et al., 2009), migration (Feng et al., 2020) and regulation of immune cells (Secca et al., 2016), IRF2BP2 is also an important player in tumor development and the microenvironment. In a study from Koeppel et al. (2009), IRF2BP2 was shown to be a direct target of p53, the tumor suppressor protein that determines cell fate. In particular, the authors showed that in response to genotoxic insult, p53 binds to the IRF2BP2 promoter, upregulating its expression. In addition, overexpression of IRF2BP2 stabilizes promoter complexes containing IRF2, increasing the transcription of the histone H4-gene and increasing the number of cells in early S-phase. When exposed to doxorubicin, these cells have a delayed onset of apoptosis. Conversely, in cells lacking IRF2BP2, doxorubicin treatment leads to increasing numbers of apoptotic cells.

Together, this data indicates the importance of IRF2BP2 in determining cell fate in conjunction with p53.

IRF2BP2 is also critical in regulating cells of the adaptive immune system. In CD4 T cells, IRF2BP2 gene expression is lowered with cell activation, while overexpression reduces interleukin-2 receptor alpha chain (CD25) levels at the cell surface, impairing cell survival and proliferation *in vitro* and *in vivo* (Secca et al., 2016). Additionally, overexpression of IRF2BP2 in Th cells led to reduced IL-4 and pro-TGF- β production (Secca et al., 2016). Interestingly, patients with multiple sclerosis (MS) have reduced expression of IRF2BP2 in CD4 T cells during progression of the disease, with expression levels being restored in patients in remission, suggesting that IRF2BP2 is critical in regulating inflammatory activity in MS (Arruda et al., 2015).

Other studies have highlighted the importance of IRF2BP2 in suppressing the Hippo pathway, a highly conserved regulator of cell growth and tumor development. In a study by Feng et al. (2020), the authors describe an inhibitory feedback loop whereby IRF2BP2 expression is repressed by YAP and TEAD4 (Feng et al., 2020), a transcriptional complex of the Hippo pathway critical in hepatocellular carcinoma (Fu et al., 2022). Conversely, overexpression of IRF2BP2 inhibits the activity of YAP through VGLL4, leading to the downregulation of several YAP-targeted genes. Furthermore, overexpression of IRF2BP2 in HepG2 and Huh7 cells dramatically decreased the levels of cell growth and tumor formation (Feng et al., 2020), pointing to a role for IRF2BP2 in tumor suppression. Interestingly, in patients with hepatocellular carcinoma (HCC), IRF2BP2 expression is conversely correlated

with YAP expression, and those with higher levels of IRF2BP2 expression had better survival rates (Feng et al., 2020).

Germline autosomal dominant mutations in IRF2BP2 have been found in familial common variable immunodeficiency disorder (CVID) (Keller et al., 2016). In somatic cells, loss-of-function mutations in IRF2BP2 are found in human promyelocytic leukemia (Jovanovic et al., 2017), primary mediastinal large B cell lymphoma (Chapuy et al., 2019), T cell lymphoma (Watatani et al., 2019) and acute myeloid leukemia (Fischer et al., 2024). However, studying the effects of *Irf2bp2* deficiency *in vivo* is complicated by the important role it plays in fetal development. Mice with a global ablation of *Irf2bp2* result in impaired erythropoiesis and development of the erythromyeloid lineage in the fetal liver, leading to embryonic lethality (Stadhouders et al., 2015). Our experiments in mice with germline ablation of *Irf2bp2* confirmed this fetal lethality, however in the 2nd and 3rd litters of hemizygous *Irf2bp2*^{+/-} mice a few viable *Irf2bp2*-null mice were found carrying *Irf2bp2*-positive myeloid cells in their livers (Vilmundarson et al., 2022) (Appendix 5.2).

Microchimerism is the presence of exogenous, genetically distinct cells in embryonic tissues, which can originate from the mother or prior siblings. These cells populate the fetal tissue during pregnancy, and can persist into adulthood (Maloney et al., 1999). In some instances, microchimerism can be beneficial to the offspring. For example, patients with type 1 diabetes (T1D) have higher levels of maternal microchimerism compared to unaffected siblings, and these maternal microchimeric cells contribute to increasing the number of islet β cells (Nelson et al., 2007). Our study demonstrated the presence of *Irf2bp2*- and Y-chromosome-positive cells in the livers of female *Irf2bp2*-null mice, suggesting the occurrence of sibling microchimerism, which rescues the fetal erythropoiesis and lethality (Vilmundarson et al.,

2022) (Appendix 5.2). Interestingly, these *Irf2bp2*-deficient chimeric mice had a significant presence of lymphoma, with males and females developing enlarged spleens and livers. In humans, lymphoma can arise due to mutations in *Irf2bp2* activating endogenous retroviral sequences, such as human T-cell leukemia virus (HTLV-1) (Kataoka et al., 2015). This data suggests that loss of function or deficiency of *Irf2bp2* increases susceptibility to lymphomagenesis.

1.2.4. LysMCre mouse line

The hematopoietic stem cell (HSC) system constitutes of the group of cells in the bone marrow that give rise to all the blood and immune cells in the body. These cells are capable of multilineage differentiation into cells that make up the two branches of the hematopoietic lineage: the common lymphoid progenitors (CLP) and the common myeloid progenitors (CMP) (McGrath et al., 2015). The cells derived from the CLP form dendritic cells, B and T cells and NK cells. The CMP gives rise to platelets, erythrocytes, granulocytes, macrophages as well as dendritic cells (McGrath et al., 2015). These cells of the myeloid lineage have important roles in the innate immune system and are also known to play a role in the acquired immune system through cytokine signaling (Medzhitov & Janeway, 1998). To study the function of myeloid cells and the regulation of genes expressed in the myeloid lineage, Clausen et al. (1999) generated a line of *Cre/loxP* mice that express *cre* under the control of the murine M lysozyme gene (Clausen et al., 1999), which is specific to macrophages/monocytes and neutrophils, creating a valuable tool for researchers to study selective mutagenesis or knockdown in these cells.

1.2.5. Role of IRF2BP2 in atherosclerosis

Although several reports have focused on the role of IRF2BP2 as a transcriptional repressor, work from our lab has also highlighted the role of IRF2BP2 as a transcriptional activator. In a yeast 2-hybrid screen of human cardiac cDNA, IRF2BP2 was found to bind to the TEAD4/VGLL4 transcription factor complex, which regulates the expression of vascular endothelial growth factor A (VEGFA) in muscle (Teng et al., 2010). Co-expression of IRF2BP2 with TEAD/VGLL4 increased expression of VEGFA in C2C12 cells, and expression of IRF2BP2 was increased in ischemic skeletal and cardiac muscle cells, suggesting it plays a role in ischemia-induced activation of VEGFA (Teng et al., 2010). Our group has also established the role of IRF2BP2 in regulating the inflammatory phenotype of myeloid cells. In LysMCre mice with a myeloid-specific deletion of IRF2BP2, bone marrow-derived macrophages (BMDM) adopted an M1 inflammatory phenotype with increased levels of inflammatory markers such as IL-1 β , TNF- α and iNOS, and decreased anti-inflammatory markers, including Mgl-1 and Arg1 (H.-H. Chen et al., 2015). These mice also had reduced cholesterol efflux from their BMDM, higher total cholesterol and increased atherosclerotic lesion size. This study also showed that IRF2BP2 is required for the activation of Kruppel-like factor 2 (KLF2), an anti-inflammatory transcription factor, and that overexpression of KLF2 in IRF2BP2-deficient macrophages is sufficient for restoration of anti-inflammatory markers.

Work from our lab has also identified a 9-nucleotide polymorphism (rs3045215) in the 3'UTR of IRF2BP2 that disrupts a conserved sequence. Genotyping of the variant demonstrated that homozygous carriers of the deletion allele had a higher risk of coronary artery disease (CAD) once adjusted for known risk factors, such as smoking status,

hypertension, sex, LDL-C and HDL cholesterol (H.-H. Chen et al., 2015). Interestingly, the deletion variant was also found to be correlated with coronary calcification in homozygous male carriers with a minimal burden of atherosclerosis (Ragnar O. Vilmundarson et al., 2021) (Appendix 5.1). As well, CAD patients homozygous for the deletion variant had reduced levels of IRF2BP2 and KLF2 protein in peripheral blood mononuclear cells, and a reporter construct bearing the ~3000 nucleotides of the 3'UTR of IRF2BP2 had significantly lower luciferase activity when carrying the 9 nucleotide deletion compared to the reporter with the non-deleted sequence, indicating that this sequence is critical in post-transcriptional regulation.

1.2.6. IRF2BP2 genetic variations and their role in CAD

While our data shows that rs3045215 is associated with an increased risk of coronary artery disease and calcification in men (Appendix 5.1), identifying the function of a polymorphism is complicated by haplotypes. A haplotype is a set of closely linked genetic variations that are typically inherited together from a single parent, rather than undergoing genetic recombination. Specific adjacent regions of the chromosome are more likely to be inherited together, reducing the likelihood that polymorphisms in these regions will be separated by chromosomal crossover, leading to genetic linkage.

While genetic linkage describes the tendency of specific genes to be co-inherited in an individual, linkage disequilibrium (LD) refers to the non-random association of genes at separate loci on a population level. Linkage disequilibrium is a reflection of past evolutionary events, population history, mutation, natural selection along with many other forces that can

shape population genetics. In short, the coefficient of linkage disequilibrium, D_{AB} , is defined as

$$D_{AB} = \rho_{AB} - \rho_A \rho_B$$

where ρ_A is the allele frequency of allele A and ρ_B is the allele frequency of allele B (Slatkin, 2008). If two alleles are independently inherited in a population, then by definition ρ_{AB} is equal to $\rho_A \rho_B$ and D_{AB} is zero. In a population where the allele frequencies of A and B are equal, the coefficient of linkage disequilibrium will be 1 and the alleles will be in complete LD (Slatkin, 2008).

Several variants in IRF2BP2 have been found to be co-inherited and are associated with several cardiovascular risk factors (Table 1). In addition to the deletion (rs3045215) variant, our lab has also identified a single nucleotide polymorphism (rs7545855) in the first exon of IRF2BP2 that is in complete linkage disequilibrium with the polymorphism in the 3'UTR. While this SNP exchanges a proline for a serine at position 78 close to the C4 zinc finger domain, the functional effects of this variant have not been documented.

Table 1.1 Linkage disequilibrium of 7 variants in IRF2BP2 and the associated phenotypes

D' VALUES	RS1329128	RS6672925	RS112096491	RS10910476	RS7545855	RS3045215	RS8823
RS1329128	-	1	1	0.9659	1	1	1
RS6672925	1	-	1	0.5419	1	1	1
RS112096491	1	0.5317	-	1	0.5317	0.5317	0.5317
RS10910476	0.9667	0.5419	1	-	0.5419	0.5419	0.5419
RS7545855	1	1	0.5317	0.5419	-	1	1
RS3045215	1	1	0.5317	0.5419	1	-	1
RS8823	1	1	0.5317	0.5419	1	1	-

GENE	POLYMORPHISM	MAJOR ALLELE	MINOR ALLELE	ASSOCIATED PHENOTYPE
IRF2BP2	rs1329128	C	A	Diastolic blood pressure
	rs6672925	A	G	Osteoporosis
	rs112096491	C	T	Red blood cell count
	rs10910476	C	T	LDL cholesterol levels
	rs7545855	C	T	Undocumented
	rs3045215	ATTATAACT	-	Coronary artery disease
	rs8823	A	G	Highest math class taken

1.3. Molecular determinants of inflammation

Inflammatory signaling is triggered in response to harmful stimuli and is the body's defence mechanism against pathogens and tissue damage. At the level of tissues, inflammation results in pain, redness, swelling, heat and loss of function. These symptoms are the result of dynamic molecular signaling and communication between immune cells that regulate the onset, progression and resolution of inflammation.

1.3.1. Innate inflammatory signalling

Within the innate immune system, pattern recognizing receptors (PPR) play a crucial role in binding viruses, bacteria, and other invasive pathogens by recognizing conserved regions known as pathogen-associated molecular patterns (PAMP). Toll-like receptors (TLR) are a family of PPRs often found in the first line of defence against invading pathogens and are widely expressed on leukocytic cells, including macrophages, dendritic cells, natural killer cells, as well as non-immune cells (Fitzgerald et al., 2020). One of the most highly studied PAMPs are Lipopolysaccharides (LPS), which are large glycolipids found on the outer membrane of gram-negative bacteria that form a protective barrier in their phospholipid layer, protecting against the entry of antibiotics and small, hydrophobic molecules (Park et al., 2013). Due to their presence on a large number of bacterial pathogens, the innate immune system has evolved to recognize and bind the Lipid A domain of LPS, which is highly conserved across strains and species (Bertani et al., 2018). Upon infection by a bacterial pathogen, immune cells expressing TLR4 can bind LPS at the cell surface and induce an inflammatory response with the aim of eradicating the pathogen. However, in some scenarios, uncontrolled regulation of this response can result in excessive inflammatory signaling, leading to detrimental effects on the host.

TLR4 is a transmembrane receptor found on the surface of immune cells, cardiomyocytes and cells of the central nervous system (Ciesielska et al., 2021). Its structure is composed of a 608-residue extracellular domain that binds LPS and other accessory molecules, and a 187-residue intracellular domain that binds secondary messenger molecules to transduce the signal into the cell. The structure of TLR4 is highly conserved through evolution in both vertebrate and invertebrate species (Purcell et al., 2007). While TLR4 is the

main receptor for LPS, its presence alone on the cell surface is not enough to respond to LPS and association with other accessory molecules is required. LPS binding protein (LBP) is a soluble protein that binds LPS aggregates and transports them to the cell surface where they are recognized by CD14 (Ciesielska et al., 2021). CD14, a GPI-anchored co-receptor of TLR4, aids in binding LPS monomers with TLR4 and is required for initiation of the cellular response. Physical association between myeloid differentiation 2 (MD2), a membrane bound receptor lacking an intracellular domain, and TLR4 is also required for induction of the signal cascade (Tan et al., 2015). After binding of LPS to TLR4, two TLR4/MD2 complexes undergo dimerization and from there two distinct cellular pathways are initiated - the MyD88 pathway and the TRIF pathway (Husebye et al., 2006).

After dimerization of the TLR4/MD2 complexes, the receptor moves laterally towards phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂)-rich regions of the cell membrane, which aid in recruiting the cytoplasmic TIR domain containing adaptor proteins (TIRAP) (Park & Lee, 2013). The intracellular Toll/IL-1R homology (TIR) domain of TLR4 interacts with TIRAP, leading to the recruitment of myeloid differentiation primary response gene 88 (MyD88) through TIR-TIR domain interactions. Once MyD88 is recruited to the submembrane signalling complex, it then binds and activates interleukin-1 receptor-associated kinase 1 and 2 (IRAKs) and together these form the myddosome (Balka et al., 2018). The signal cascade then continues through TRAF6 and TAK1 kinase, leading to the nuclear translocation of NF- κ B and AP-1 and expression of genes encoding pro-inflammatory cytokines (Park & Lee, 2013).

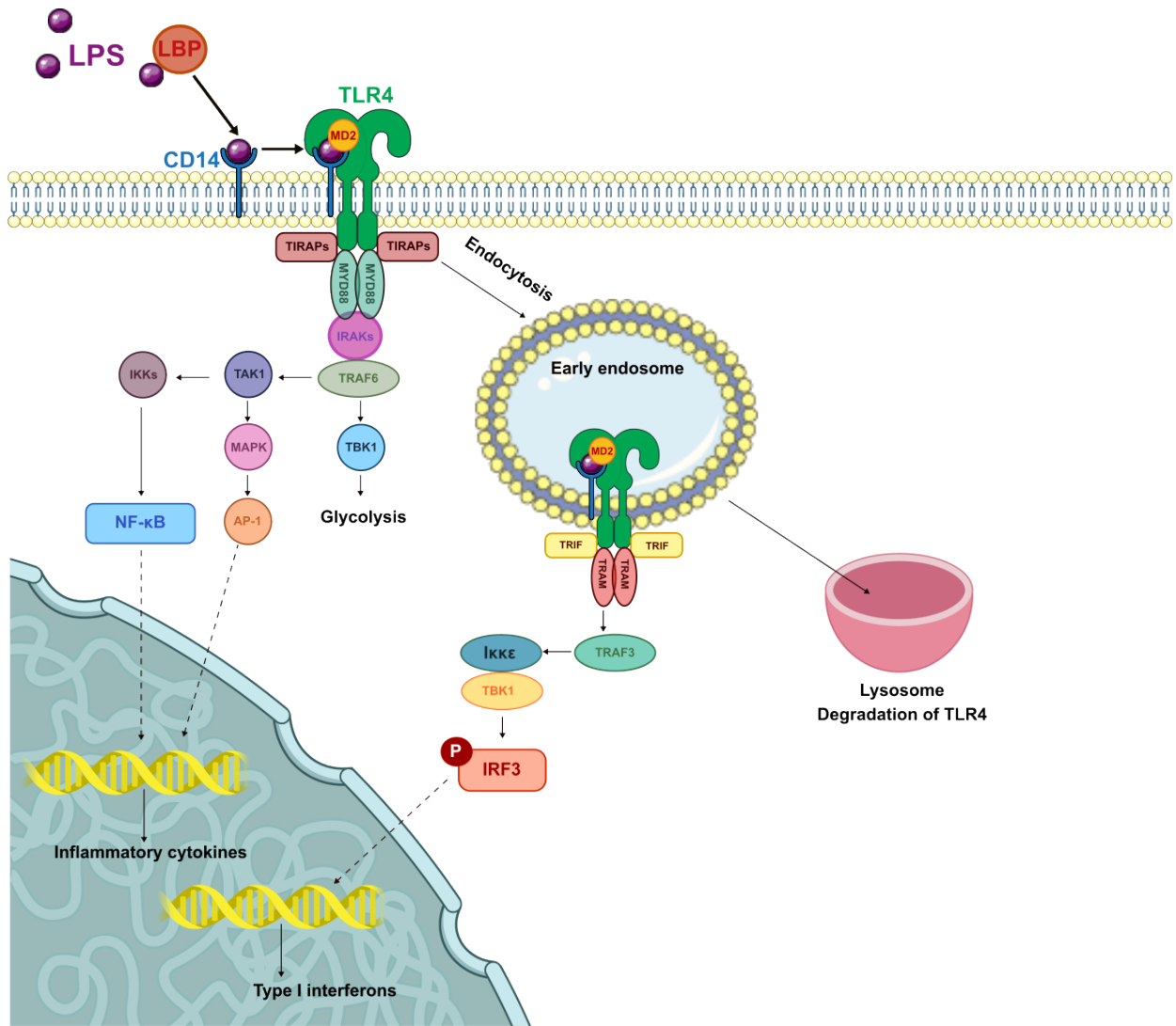


Figure 1.3. Signalling pathway of TLR4. Binding of LPS to TLR4 activates the MyD88 pathway at the cell membrane, leading to expression of pro-inflammatory cytokines, and endocytosis of TLR4 and LPS activates the TRIF pathway, leading to expression of Type I interferon genes. *(Image created using art from NIH BioArt and Smart Servier Medical Art.)*

The second TLR4 signaling pathway is activated upon endocytosis of TLR4 and LPS from the membrane (Tan et al., 2015). Once TLR4 is internalised by endosomes, TIRAP and MyD88 dissociate from the receptor and a second set of TIR-adaptor proteins, TRAM and TRIF, are able to bind to TLR4. Once these adaptor proteins bind, the TRIF-dependent

pathway proceeds through the activation of the ubiquitin ligase TRAF3 and the activation of non-canonical IKK kinases, including TANK binding kinase 1 (TBK1) and IKK ϵ (Ciesielska et al., 2021). TBK1 phosphorylates interferon regulatory factor 3 (IRF3), allowing it to translocate into the nucleus and activate transcription of Type I interferon genes (Rajaiah et al., 2015). The TRIF pathway also leads to the expression of anti-inflammatory IL-10, providing a resolution for the pro-inflammatory signalling (Ciesielska et al., 2021).

TLR4 signaling also involves the activation and recruitment of spleen tyrosine kinase (SYK), a non-receptor tyrosine kinase that is expressed in myeloid, endothelial, neuronal and epithelial cells and fibroblasts (Zarbock & Ley, 2011). Syk is highly conserved between species and consists of two SH2 domains at the N terminus, which bind TLR4, and a kinase domain at the C terminus that catalyzes the phosphorylation of tyrosine residues found on a variety of intracellular signaling cascade molecules (Xue et al., 2013). Activation of Syk occurs rapidly in RAW264.7 cells after stimulation with LPS, with increased levels of phosphorylated Syk occurring seconds to minutes after LPS exposure (Lee et al., 2009). In macrophages, phosphorylation of Syk leads to the activation of several downstream effector molecules, such as NF- κ B and IKK, resulting in expression of proinflammatory genes, including TNF- α and iNOS (Dennehy et al., 2008; Yi et al., 2014). Conversely, studies looking at the effects of Syk inhibition or knockdown have produced inconsistent results. Several plant compounds are known to suppress the inflammatory response in macrophages through the inhibition of Syk. Extracts from *Cinnamomum cassia*, *Evodia lepta* and *Artemisia asiatica* were shown to inhibit the LPS-stimulated production of NO, TNF- α and PGE₂ in macrophages (Jeong et al., 2014; Yoon et al., 2013; Yu et al., 2012). Piceatannol, a Syk inhibitor, also reduces the production of NO, TNF- α and PGE₂ in RAW264.7 cells in an

I κ B α -dependent manner (Lee et al., 2009). Contrary to these results, a study looking at bone marrow-derived macrophages deficient in Syk demonstrated that these cells have an increased production and secretion of proinflammatory TNF- α , IL-6 and IL-12 in response to LPS (Hamerman et al., 2005). The discrepancy in these results could be explained by different doses of TLR ligands used between these studies. In particular, the study by Hamerman et al. used a lower dose of LPS compared to other studies and their results did not demonstrate a dose-dependent response of inflammatory cytokines with LPS.

1.3.2. Internalisation of TLR4

Endocytosis of TLR4 is triggered by the binding of LPS and can proceed through both clathrin-independent and clathrin-dependent pathways (Husebye et al., 2006). Budding of the early endosomes transporting TLR4 into the cell depends on dynamin (Rajaiah et al., 2015) and once inside the cell, the receptor is targeted by ubiquitination for degradation at the lysosome (Wang et al., 2007), terminating the signal cascade and the pro-inflammatory response. While the binding of LPS is essential for activation of the receptor, internalisation of TLR4 is independent of its ability to transduce the signal intracellularly. In TLR4 mutants lacking the TIR domain essential for formation of the myddosome, uptake of the receptor is uninhibited in bone marrow derived macrophages (BMDM) (Tan et al., 2015). Rather, regulation of TLR4 uptake is highly dependent on the activation of CD14, and knock-out of CD14 in mice has been shown to eliminate the LPS-induced TLR4 uptake and subsequent TRIF-dependent signal cascade in the BMDM of these animals, while preserving the MyD88 pathway (Zanoni et al., 2011). As well, in cells that have naturally low expressions of CD14 at the plasma membrane, TLR4 uptake does not occur and surface levels remain unchanged in response to LPS (Zanoni et al., 2011). Notably, CD14 is part of a constitutive uptake and

repopulation pathway, while TLR4 endocytosis occurs mainly in response to LPS and its levels at the plasma membrane are not rapidly restored (Tan et al., 2015). Activation of CD14 by LPS initiates the formation of PI(4,5)P₂ in the plasma membrane of macrophages, which accumulate in CD14 localised rafts, and the subsequent hydrolysis/phosphorylation of PI(4,5)P₂ following the recruitment of TIRAP is essential for TLR4 endocytosis (Plociennikowska et al., 2015). Because phosphorylation of PI(5) to PI(4,5)P₂ proceeds via Arf6 and PIP5K1 α , inhibiting expression of these kinases also blocks the endocytosis of TLR4 (Murase et al., 2018) and TRIF-dependent phosphorylation of IRF3 (Van Acker et al., 2014). CD14 uptake of TLR4 also acts via the Syk tyrosine kinase, which activates phospholipase C γ 2 (PLC γ 2) and inhibition of these molecules blocks LPS-stimulated TLR4 endocytosis and TRIF-dependent signalling in murine BMDM (Voss et al., 2016). In addition to CD14, uptake of TLR4 is also dependent on myeloid differentiation factor 2 (MD-2), a non-anchored extracellular protein that forms a complex with TLR4 (Plociennikowska et al., 2015). Upon binding of LPS, MD-2 aids in the dimerization of TLR4, leading to the formation of the myddosome and activation of the MyD88 pathway. However, it is now also known that crosslinking of TLR4 at the plasma membrane is necessary for receptor internalisation, and MD-2 mutants with impaired ability to induce TLR4 dimerization lack TLR4 endocytosis (Tan & Kagan, 2017).

1.4. Cardiac conduction

Cardiac conduction is the propagation of electrical activation between cardiomyocytes that triggers their synchronous contraction. Dysfunction to this system, such as inflammatory diseases that affect the myocardium, can impair the cardiac conduction system or cause cardiac remodeling, predisposing patients to arrhythmias or cardiac sudden death.

1.4.1. Phases of cardiac conduction

In order for the heart to effectively pump blood throughout the vascular system, there must be coordination in the phases of contraction and relaxation. Action potentials are initiated by the pacemaker cells of the sinoatrial node (SA node) located in the wall of the right atrium (Grainger & Santana, 2022). During this phase, non-pacemaker cardiomyocytes rapidly depolarize from their resting membrane potential through the opening of voltage-gated Na^+ channels in the cell membrane (Grant, 2009) (Figure 1.5). The influx of sodium ions in the cell rapidly increases the membrane potential towards the Na^+ equilibrium potential. Voltage-gated calcium channels are also triggered to open, further increasing the membrane potential of the cell and opening delayed rectifier potassium channels, which allow potassium ions to leak out of the cell (Dobrzynski et al., 2013). L-type calcium channels and ryanodine receptors, activated by the influx of sodium ions, move calcium ions into the cytoplasm, which then bind Troponin C, activating contraction (Varro et al., 2021). As the cytoplasmic space of cardiomyocytes are linked by gap junctions, this wave of excitation-contraction coupling spreads through the electrically coupled cardiomyocytes, causing a coordinated contraction of the myocardium.

To prevent the initiation of a premature action potential that would disrupt the synchronicity of contraction, cardiomyocytes experience a refractory period whereby cells are unsusceptible to excitatory stimuli (Strobel et al., 1990). During this time, sodium channels are in an inactivated state and cannot open regardless of the strength of the excitatory stimulus. Repolarization describes the phase during which ionic channels work to restore the electrochemical gradients between the intracellular and extracellular spaces and prepare the cell for the next action potential (Nerbonne & Kass, 2005). Specifically, L-type

Ca^{2+} channels close and delayed rectifier potassium channels remain open, allowing positive potassium ions to leak out of the cell. As potassium ions continue to leak out of the cell, the membrane potential repolarizes, resetting the state of the sodium channels (Nerbonne & Kass, 2005). The sodium-calcium exchanger and the sodium-potassium pump work to restore the electrochemical gradients of these ions, pumping calcium and sodium out from the cytoplasmic space and potassium ions back into the cell (Armoundas et al., 2003). This results in the uncoupling of calcium ions from Troponin C and relaxation of the myocyte. Cells are now ready to respond to a new action potential stimulus.

1.4.2. Cardiac ion channels and currents

The generation and propagation of action potentials throughout the myocardium depends on two important properties of ion channels: selective permeability and gating. In channels that do not use ATP, the ion-specific electrochemical gradient is the force that drives the movement of ions across the lipophilic membrane (Grant, 2009). Rather than simply being pores in the cell membrane, ion channels are highly specific for each ion. Selectivity of ion channels is determined by the size, valency and hydration energy of each ion and the distribution of ion channels throughout the cardiomyocyte is based on their selectivity for certain ions (Grant, 2009). The other important property of ion channels is gating. The most common form of gating found in cardiac ion channels is voltage-dependent gating, whereby channels alter their conductance depending on the membrane potential of the cell (Varro et al., 2021). The steepness of conduction can vary from channel to channel, impacting the shape of the action potential. Ion channels also have the ability to close and reduce conductance depending on the voltage of the cell. In their inactivated state, sodium ion channels are unable to respond to any excitatory stimulus, thus maintaining the

synchronicity required for contraction of the myocardium. This is known as the absolute refractory period (Strobel et al., 1990). Ion channels can also open the inactivation gate, while leaving the channel closed, making it possible to activate the channel and initiate another action potential if an incoming stimulus is strong enough. This is known as the relative refractory period (Strobel et al., 1990).

Repolarization is mediated by cardiac potassium channels that are made up of highly conserved α -subunits and multiple β -subunits (Grandi et al., 2017). The α -subunits include KvN.x (N=1 to 4), HERG (*KCNH2*) AND KvLQT1 (*KCNQ1*), which make up the pore of the channel and control selectivity and gating. The β -subunits include ATP-binding cassette transport-related proteins, cytoplasmic proteins (KChAP and KChIP) and single transmembrane spanning proteins (minK) which modulate the trafficking, kinetics and activation of the α -subunits and the channel (Grandi et al., 2017).

There are several different types of cardiac potassium currents, which are controlled by K^+ channels that vary in their voltage-gated activation and inactivation, permeability and conductance. Different currents are activated during specific phases of the action potential and are mediated by specific α -subunits.

Transient outward potassium currents (I_{to}). The I_{to} is activated during the initial rapid depolarizing phase 1 of the cardiac action potential (Jeevaratnam et al., 2018). The activation of these channels causes the net movement of positive K^+ ions out of the cell, resulting in a brief dip of the membrane potential known as early repolarization (Figure 1.5). This change in membrane potential also triggers the activation of L-type Ca^{2+} channels.

I_{to} is made up of two distinct currents: I_{to1} and I_{to2} . While I_{to1} is calcium-independent outward potassium current, I_{to2} is a Cl^- current that is sensitive to extracellular Ca^{2+} (Niwa & Nerbonne, 2010). I_{to1} can be further separated into two kinetic variants: fast I_{to} ($I_{to1,f}$) and slow I_{to} ($I_{to1,s}$), which requires a longer recovery time. $I_{to1,f}$ is mediated by the Kv4.2 (*KCND2*) and Kv4.3 (*KCND3*) α -subunits and the KChIP2 (*KCNIP2*) and MiRP2 (*KCNE3*) β -subunits. $I_{to1,s}$ is mediated by the Kv1.4 (*KCNA4*) α -subunit. The distribution of both kinetic variants of I_{to1} also varies throughout the heart. While I_{tof} and I_{tos} are both expressed in the ventricles, I_{tof} is the predominant variant found in the atria (Pongs, 2009).

Rectifier K^+ channels. Certain potassium channels are able to regulate the strength of conductance depending on the direction of ion flow, commonly referred to as rectification (Jeevaratnam et al., 2018). For example, for the same absolute voltage difference across the cell membrane, inward potassium rectifier channels will pass a stronger current into the cell at a membrane voltage negative to the K^+ equilibrium potential than an outward current when the membrane voltage is more positive than the K^+ equilibrium potential. The mechanism of rectification in these channels is dependent on blockade by magnesium ions and polyamines that occupy the channel pore at positive potentials (Pongs, 2009).

Rapid delayed rectifier K^+ currents (I_{kr}). The I_{kr} is rapidly activated during depolarization and is mediated by the hERG α -subunits (*KCNH2*) and MiRPI (*KCNE2*) β -subunits (Guo et al., 2009). Although the channel is activated at positive membrane potentials during depolarization, the rate of conductance is much lower than when the membrane potential becomes negative. During this phase, conductance is much higher,

resulting in a net K^+ efflux and repolarization of the cell. I_{kr} is highly expressed in the left atrium and ventricles.

Slowly activating delayed rectifier K^+ currents (I_{ks}). I_{ks} is mediated by the Kv1.7.1 α -subunits (*KCNQ1*) and minK β -subunits (*KCNE1*) (Nerbonne & Kass, 2005). As its name suggests, the I_{ks} slowly activates when the membrane potential is higher than -20 mV, allowing it to play a significant role in phase 3 repolarization. Compared to I_{kr} , I_{ks} inactivation is much slower, making it an important current in regulating the action potential duration (APD) when heart rate increases. I_{ks} is also an important current in the repolarization reserve (Nerbonne & Kass, 2005).

Inward rectifier potassium current (I_{k1}). I_{k1} is regulated by the Kir2.1 and Kir2.2 α -subunits (*KCNJ2*, *KCNJ12*) (Nerbonne & Kass, 2005). Unlike the other potassium channels found in the heart, these channels are not voltage-dependent and permeability is controlled primarily by rectification through magnesium ions and polyamines. During phase 0, 1 and 2 of the action potential, the membrane potential is more positive than the equilibrium potential of K^+ , reducing the conductance of I_{k1} . As the membrane potential becomes more negative during repolarization, conductance of the channel starts to increase, further driving phase 3 repolarization (Nerbonne & Kass, 2005). As the channels regulating I_{k1} are not voltage-gated, the resting membrane potential is largely influenced by I_{k1} and as a result is close to the equilibrium potential of K^+ (Jeevaratnam et al., 2018).

Sodium-calcium exchanger current (I_{NCX}). In addition to potassium channels, cardiac repolarization is also influenced by the current generated by the sodium-calcium exchanger

(NCX). The cardiac isoform of the sodium-calcium exchanger, NCX1, is a transmembrane protein that exchanges 3 sodium ions for every calcium ion moved across the plasma membrane (Liao et al., 2012). Two calcium binding domains are found within a large cytoplasmic loop of NCX1, allowing the exchanger to be allosterically regulated by intracellular calcium (Ottolia et al., 2009). NCX1 is a bidirectional channel, operating in either forward or reverse mode depending on the sodium and calcium gradients at the cell membrane and the cell's membrane potential. The driving force of NCX1 can be described as $E_m - E_{Na/Ca}$, where E_m is the membrane potential and E_{Na} and E_{Ca} are the Na and Ca equilibrium potentials, respectively (Ottolia et al., 2013). In forward mode, NCX1 moves Na^+ into the cell, resulting in an extrusion of Ca^{2+} ions from the cytoplasm. In the reverse mode, Na^+ is moved out of the cell in exchange for Ca^{2+} influx. The exchanger predominantly acts in the forward mode, when the membrane potential is lower than the equilibrium potential of the exchanger. In this state, NCX1 removes the calcium influx generated by the opening of the L-type calcium channels (LCCs) during excitation-contraction coupling, contributing to the gradual repolarization of the myocardium. While calcium also enters the cytoplasm through ryanodine receptors found on the sarcoplasmic reticulum, studies have established that NCX extrudes the same amount of calcium brought in by LCCs, maintaining cellular balance (Bridge et al., 1990).

Repolarization reserve. Repolarization is an important component controlling the synchronicity of contraction through the myocardium, and thus there are redundancies in the system to guard against its disruption. The repolarization reserve refers to the overlapping functions and activation of I_{kr} , I_{ks} and I_{kl} . This ensures that repolarization is not under the

control of a single current and that other channels can take over if one fails (Nerbonne & Kass, 2005).

1.4.3. Trafficking of repolarization channels in ventricular myocytes

The function and density of voltage-gated K⁺ channels in the plasma membrane of cardiomyocytes is highly regulated through posttranslational modifications and channel trafficking. The α -subunits of channel proteins are synthesized and assembled in the endoplasmic reticulum (Kosolapov & Deutsch, 2003; Kosolapov et al., 2004), after which they are exported to the Golgi apparatus with the help of COPI and Rab proteins (Murshid & Presley, 2004; Wang et al., 2012). Importantly, inhibition of Rab1 in ventricular myocytes impairs the movement of Kv4.2 to the cell membrane, leading to a decrease in $I_{to,f}$. As well, accessory proteins, such as KChIPs and β -subunits also aid in post-translational trafficking from the ER to the Golgi (Hasdemir et al., 2005). From the Golgi, targeting of potassium channels to the cell surface involves motor proteins and the cytoskeleton, with distinct channels being transported by specific motor proteins. The trans-Golgi network dictates the plasma membrane domains into which the channels will be inserted by sorting channels into the appropriate vesicles (Capera et al., 2019; Z. Wang et al., 2004). Glycosylation also takes place in the Golgi apparatus, a process which can affect the gating function of certain voltage-gated potassium channels, their protein stability, cell surface protein levels and intracellular retention (Gong et al., 2002; Watanabe et al., 2004). SNARE proteins then mediate the fusion of exocytotic vesicles with the plasma membrane, resulting in the insertion of channels to the cell surface (Capera et al., 2019).

Regulation of potassium channel expression at the plasma membrane involves balancing the rates of anterograde channel trafficking and channel endocytosis. Internalisation predominantly involves the activity of Rab-positive endosomes and Rab GTPases are known to play a critical role in clathrin-mediated endocytosis and early endosome formation. For example, internalisation of Kv1.5 involves Rab5-mediated pathways (Zadeh et al., 2008), while Kv4.2 endocytosis relies on Rab4 and Rab5 (Wang et al., 2012). Overexpression of Rab11, which plays a role in anterograde and retrograde trafficking of vesicles, increases $I_{to,f}$ current density, although expression of a Rab11 dominant negative does not decreased $I_{to,f}$ (Wang et al., 2012). Dynamin also regulates the expression of Kv channels at the cell surface by catalysing the scission and endocytosis of clathrin-coated vesicles from the plasma membrane (Choi et al., 2005). Proteins are then be subjected to ubiquitin-dependent proteosomal degradation.

1.4.4. Abnormalities in ventricular repolarization

Ventricular repolarization is a crucial phase of electrical propagation through the myocardium. On a cellular level, this phase of the action potential resets the electrochemical gradients that enable the cell to respond to a new action potential stimulus. Because cells are unable to respond to an incoming depolarizing stimulus during this time, this period acts as a refractory period and enables a coordinated contraction and relaxation of the myocardium. On an electrocardiogram, the spread of depolarization and repolarization through the ventricles is indicated by the duration between the start of the QRS complex and the end of the T wave and is known as the QT interval (Figure 1.7). Abnormalities in repolarization are captured by the QT interval and can occur in a variety of diseases and can have many different underlying causes. A major concern in QT prolongation is the onset of abortive

action potentials before the completion of repolarization, known as early afterdepolarizations (EADs), which can lead to the generation of premature ventricular contractions (PVCs) and arrhythmias (Zhang et al., 2021). The impact of prolongation of the QT interval is an increased risk of ventricular arrhythmias, such as Torsades de Pointes, and sudden cardiac death (Panoulas et al., 2014).

Long QT Syndrome (LQTS) can be classified as acquired or congenital. In the hereditary form of the disease, mutations in the genes encoding cardiac ion channel proteins or subunits, such as *KCNQ1* (Barsheshet et al., 2012), *KCNH2* (Mehta et al., 2014), *KCNE1* (Harmer et al., 2010) or *KCNE2* (Roberts et al., 2017) form the genetic basis of the disease. The most common genetic mutation associated with LQTS is in the *KCNQ1* gene, which accounts for almost half of patients with LQTS variant 1 (LQT1) (Crotti et al., 2008). LQTS is also found in 13% of patients with hypertrophic cardiomyopathy (HCM), and while structural changes related to HCM can account for abnormalities in the QT interval, recent evidence suggests that a subset of these patients also have mutations in LQTS genes that may be the underlying cause (Cava et al., 2023). In acquired QT prolongation, certain drugs can disrupt the balance of electrolytes as a side effect, leading to disturbances in various ion channel currents in the heart (Kannankeril et al., 2010). Inflammatory diseases also impact the QT interval and more evidence is now drawing a link between the immune system and QT prolongation.

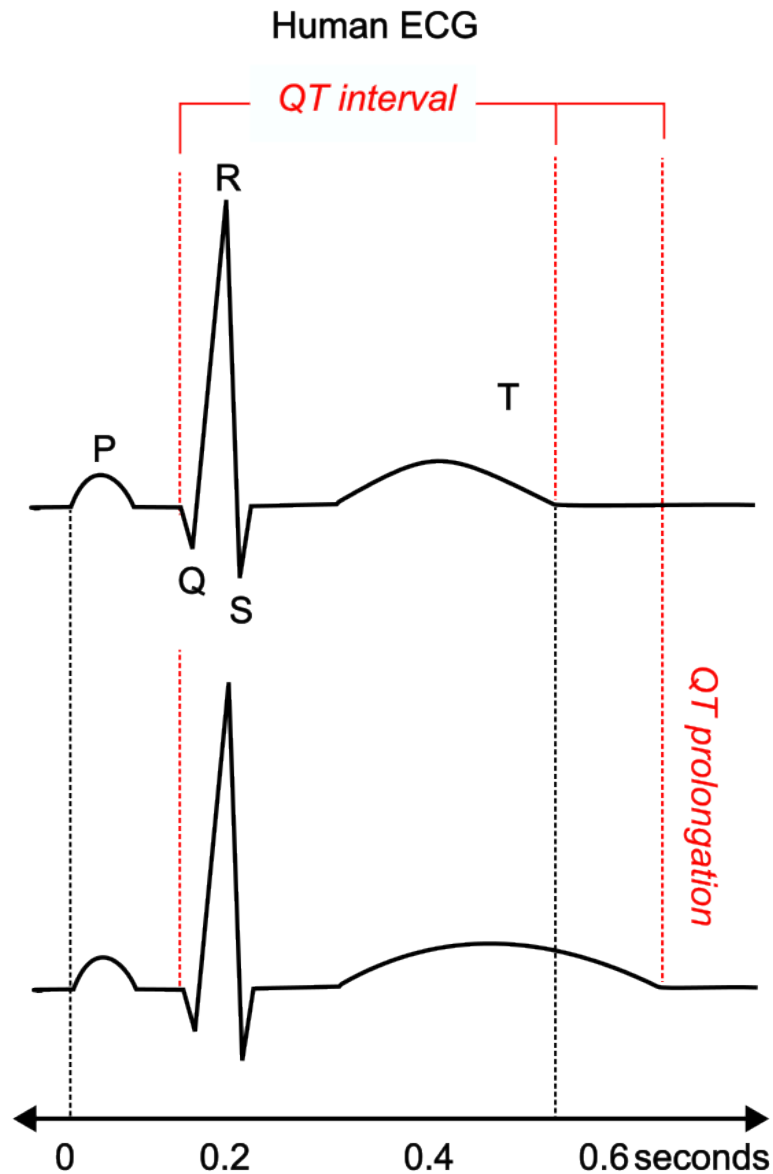


Figure 1.4 QT prolongation on an ECG. As the depolarization and repolarization currents spread through the myocardium, they are picked up by an electrocardiogram (ECG) and represented as different phases: P-wave (atrial depolarization). QRS (ventricular depolarization), QT (ventricular depolarization and repolarization). Delays in ventricular depolarization and repolarization are captured by the QT interval. Time scale is approximate.

1.4.5. Differences between human and mouse cardiac action potential

While mice are commonly used as animal models for studying human diseases and therapeutics, there are several key differences between the human and mouse action potential that should be highlighted. Mice have a much higher heart rate than humans (500-700 bpm for mice compared to 60-100 bpm for humans) and this results in a much faster action potential duration (Boukens et al., 2014). As a general rule, the duration of the cardiac action potential increases with larger body sizes. In mice, the action potential is roughly 50 ms in the ventricles (Danik et al., 2002), compared to a 250 ms action potential duration in humans (Edvardsson et al., 1984). As well, the mouse action potential lacks the calcium plateau found in human cardiomyocyte action potentials, indicating that different currents contribute to the shape and duration of the action potential between mice and humans. Furthermore, even when the same ion channels are expressed in mouse and human ventricular myocytes, their contribution to the morphology of the action potential can differ.

Both murine and human ventricular action potentials start with a rapid depolarization phase triggered mainly by the opening of rapidly activating sodium channels, resulting in a I_{Na} upstroke (Figure 1.7) (Bers, 2000). In humans, the opening of L-type calcium channels also contributes to the depolarization phase (phase 0) and the plateau phase (phase 2), however this current is less active in the mouse heart and they instead have a gradual repolarization phase (Ismaili et al., 2022). Mouse cardiac myocytes also lack the characteristic repolarizing “notch” of human cardiac action potentials characterized by the rapid inactivation of the Na^+ current and activation of the I_{to1} current. With respect to repolarization, I_{ks} and I_{kr} are the predominant currents found in human cardiomyocytes and are active during phase 3 repolarization (Nerbonne & Kass, 2005). However, in murine

ventricular myocytes, these currents are negligible and do not contribute considerably to repolarization. Instead, the transient $I_{to,f}$ and $I_{to,s}$ currents and the slowly inactivating K^+ current ($I_{K,slow1}$ and $I_{K,slow2}$) are much more pronounced in murine myocytes and contribute more strongly to ventricular repolarization (Schouten & ter Keurs, 1985). While we know that the contribution of NCX1 to calcium removal from the cytosol differs between murine and human cardiomyocytes, there is conflicting evidence when it comes to quantifying the role of NCX1. Some studies have suggested that in rabbit and human ventricular myocytes, NCX1 is responsible for 28% of cytosolic Ca^{2+} removal, with SR Ca^{2+} -ATPase removing the other 70%, while murine cardiomyocytes only remove 8% of cytosolic Ca^{2+} through NCX1 due to a higher density of SR Ca^{2+} -ATPase ion pump units expressed in the cell (Xu et al., 2012). While the authors of this study used complete removal of Ca^{2+} and Na^+ to block NCX activity, a separate study using an NCX-specific inhibitor (SEA0400) conversely saw a larger effect size in APD90 shortening of rodent cardiomyocytes compared to human (Bogeholz et al., 2017). *In vivo* studies have also attempted to study the role of NCX1 in calcium regulation by bypassing inhibitors, which can have incomplete specificity, and global knockout, which is embryonic lethal, by generating tamoxifen-inducible cardiac-specific NCX1 KO mice (Lotteau et al., 2021). The authors of this study report that knockout of NCX1 in cardiomyocytes led to a shortening of APD90 after 1 week, which carried through to the 4-week time point. Because of its bidirectional nature, the effects of blocking NCX1 activity are also dependent on the experimental intra- and extracellular concentrations of Na^+ and Ca^{2+} . In a study looking at the effects of NCX inhibition on the APD in canine cardiomyocytes, which display similar electrophysiological characteristics to human myocytes, the authors showed that inhibition of NCX by XIP at 5 mmol/L $[Na^+]_i$ resulted in shortening of APD₉₀, while 15 mmol/L $[Na^+]_i$ displayed lengthening of APD₉₀ in response to

XIP (Armoundas et al., 2003). Therefore, when comparing studies on NCX, it is important to take into consideration the differences in species, protocol and experimental conditions.

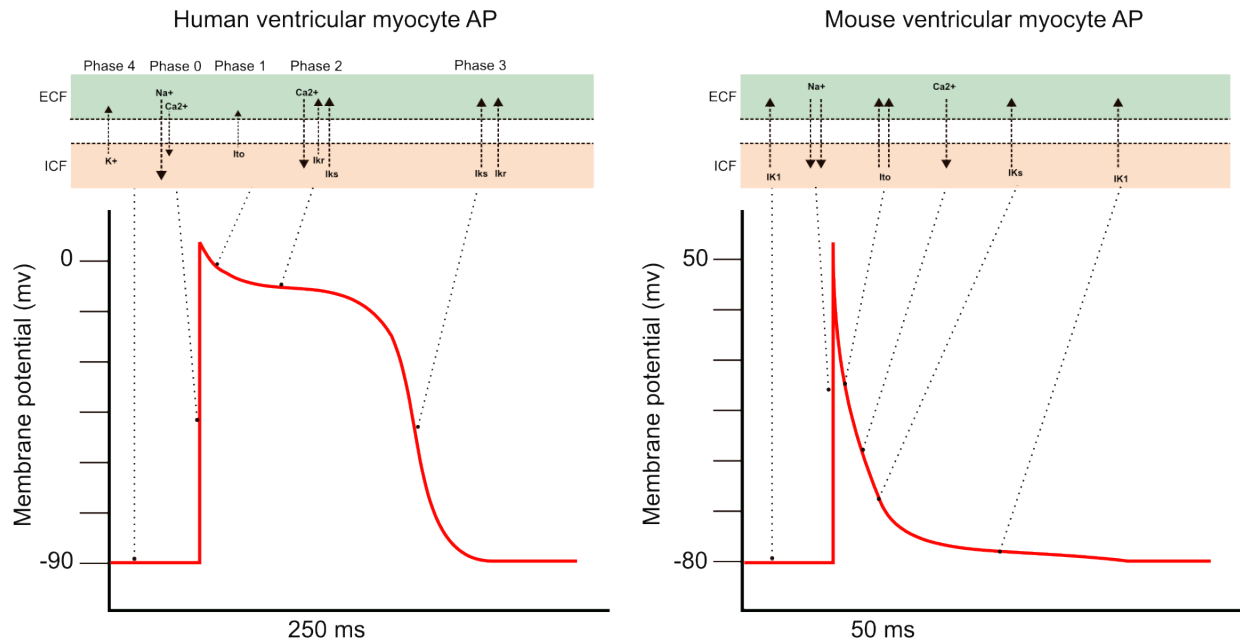


Figure 1.5 A comparison of the human and murine ventricular myocyte action potential. The key differences between the action potentials of humans and mice are the presence of the calcium plateau phase in humans compared to the gradual repolarization in the murine action potential. Different currents are also responsible for each phase between the two species.

In humans, ECGs are typically recorded from three limb leads recording from the right arm (R), left arm (L) and left leg (F). In mice, the electrodes are typically inserted into the left (L) and right (R) arms and the left groin (F). In both of these setups, leads I, II and III can be calculated, as represented by the Triangle of Einthoven (Figure 1.6).

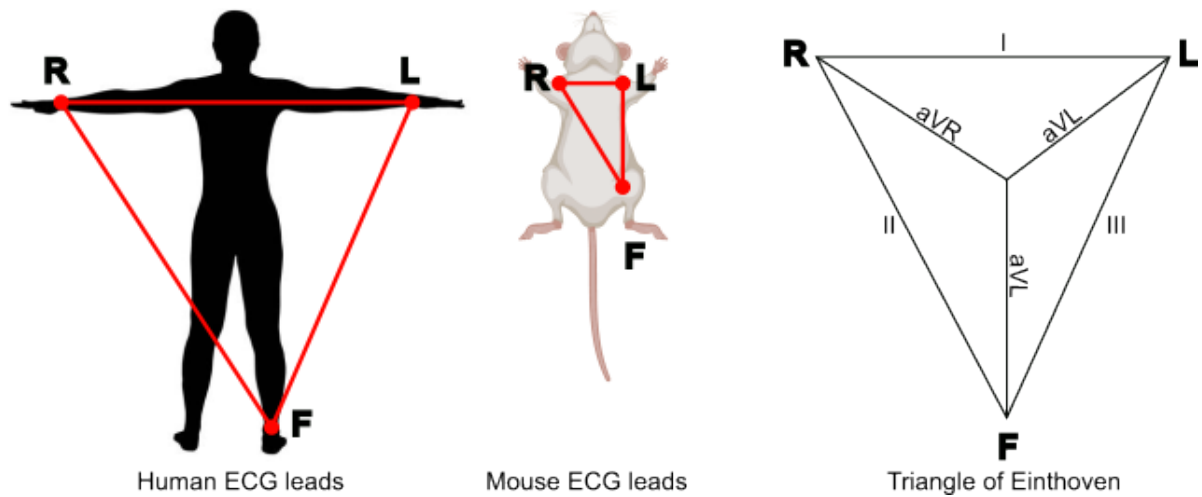


Figure 1.6 Six lead ECG on a human and mouse. Schematic of the lead positions on a human and mouse and the triangle of Einthoven measuring the electric field.

The key differences in the human and murine action potentials are reflected in their ECGs as well. Atrial depolarization is represented by the P wave deflection from the isoelectric line in both species (Figure 1.6), and both species have an isoelectric PQ interval that results from conduction through the AV node and Purkinje fibers. The QRS interval reflects ventricular depolarization in both species, however the QRS complex in mice is followed immediately by the J wave, which represents early repolarization (Boukens et al., 2014).

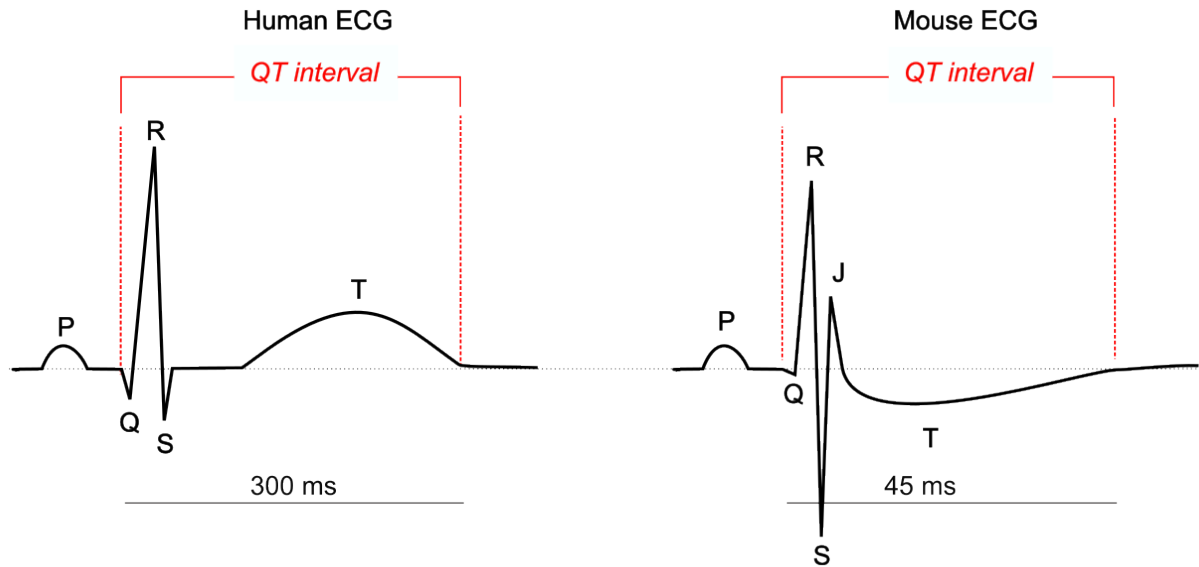


Figure 1.7 The electrocardiogram of a human compared to a mouse. The major differences between the murine and human electrocardiogram are the morphology of the QRS complex and the inclusion of the J wave.

1.5. Inflammation and cardiac electrophysiology

1.5.1. Effects of inflammation on cardiac function

Sepsis is an exaggerated, dysregulated inflammatory response to infection and can affect several systems in the body. Myocardial dysfunction can be a severe consequence of sepsis and is associated with significantly increased mortality (Geri et al., 2019). In a study on 76 patients with septic shock, 74% presented with cardiac dysfunction, with 30% of patients experiencing LV systolic dysfunction during the first 72 hours of septic shock (Beraud et al., 2014). Ejection fraction was also found to be abnormal in 35% of patients and RV function was impaired in 31% of patients. Interestingly, many of these variables return to normal post-septic patients, suggesting that the effects of sepsis-induced myocardial dysfunction are reversible (Kan & Finkel, 2003).

In addition to detrimentally impacting cardiac function, sepsis can also lead to electrical remodelling. Sepsis-induced cardiomyopathy (SICM) can predispose patients to arrhythmias, such as sinus tachycardia and atrial fibrillation (AF). Reductions in the QRS duration and amplitude (Rich et al., 2002) are typically found in patients with sepsis and can be a negative predictor of mortality. As well, new-onset QT prolongation is a risk factor found in sepsis, and Liu et al. (2024) (Liu et al., 2024) reports an incidence rate of 22.9% in septic patients without a history of QT prolongation or QT-influencing medications. The onset of QT prolongation presents alongside increased atrial and ventricular tachyarrhythmias and is a marker of 30-day mortality. QT prolongation was also found to be associated with disease severity and multiple organ dysfunction (Liu et al., 2024). Indeed, a meta-analysis by Bosch et al. (2019) revealed that factors associated with acute sepsis, such as C-reactive protein levels, fungal infection and heart catheterization, are strongly correlated risk factors for new-onset arrhythmias (Bosch et al., 2019). This suggests that sepsis, independent of traditional risk factors for arrhythmias, can induce cardiac changes that lead to cardiac arrhythmias.

Inflammatory heart diseases, such as myocarditis, also present with QT prolongation, which can increase the severity of the disease, leading to a higher risk of in-hospital mortality. For example, patients with fulminant myocarditis have significantly prolonged QTc intervals compared to those with non-fulminant cases, which correlates with higher rates of arrhythmias and mortality (Hung et al., 2016). In mice infected with Chagas disease, a form of myocarditis caused by the *Trypanosoma cruzi* parasite, QTc duration was shown to be significantly correlated with the severity of cardiac inflammation and lasted throughout the duration of the disease (Eickhoff et al., 2010). Studies have also suggested that the

occurrence of QT prolongation in patients with myocarditis can be as high as 25% (Ukena et al., 2011), demonstrating the prevalence of this condition.

Abnormalities in the QT interval are also present in sterile systemic inflammatory diseases such as rheumatoid arthritis (RA), a chronic autoimmune disease. Indeed, patients with RA have a 50% increased risk of cardiovascular mortality and sudden cardiac death (Meune et al., 2009) and have a higher chance of developing QT prolongation (Chauhan et al., 2015). The authors of this study also excluded patients using medications known to cause QT prolongation, demonstrating the idiopathic nature of this condition. Mortality in individuals with diabetes is also strongly associated with cardiovascular disease, and prolonged QT is found in 66.7% of patients with type 1 diabetes 51.3% in type 2 diabetic patients (Z. Lu et al., 2017). In addition to predisposing these patients to arrhythmias, this study also found that 62% of type 1 and 2 patients with prolonged QT had a reduced left ventricular ejection fraction (LVEF) at follow-up, suggesting a correlation. Prolonged QT at baseline is also correlated with an increased QT interval during severe hypoglycemic episodes in patients with type 2 diabetes (Cha et al., 2016), and insulin-induced hypoglycemia has been shown to delay the QT interval in non-diabetic patients (Gastaldelli et al., 2000; Kacheva et al., 2017). Notably, 82% of these individuals also developed hypokalaemia, further highlighting the role of insulin in regulating the distribution of potassium between the intracellular and extracellular compartments.

Prolongation of the QT interval and sudden cardiac arrhythmias are also risk factors in patients with acute inflammatory viral infections due to the risk of septic shock. Patients hospitalized with acute infections can often develop novel cardiac arrhythmias, putting them

at an increased risk of sudden cardiac death and in-hospital mortality (Leoni & Rello, 2017). QT prolongation has been found in patients presenting with COVID19 (Pornwattanakavee et al., 2021), pneumonia and even urinary tract infections (Lazzerini et al., 2022). In certain cases, sudden cardiac arrest can occur in the absence of signs of shock or respiratory failure (Carr et al., 2012). Because ECG monitoring is only done on a subset of these patients (Carr et al., 2012), this data highlights the importance of monitoring for new-onset arrhythmias, which can be indicated by the presence of rate-corrected QT prolongation.

1.5.2. Inflammatory pathways affecting cardiac conduction

Many studies have shown that inflammatory cytokines can increase APD by disrupting the function or expression of various ion channels. Transgenic mice and rats overexpressing TNF- α have longer APD and QT durations (Fernandez-Velasco et al., 2007; Grandy & Fiset, 2009; Kawada et al., 2006; Petkova-Kirova et al., 2006), and myocytes treated with TNF- α display a reduction in potassium voltage-gated currents, including I_{to} (Fernandez-Velasco et al., 2007; Kawada et al., 2006). As well, left ventricular myocytes incubated in TNF- α have reduced levels of Kv4.2 and Kv4.3 expression (Petkova-Kirova et al., 2006) and inhibition of KChIP2 (Fernandez-Velasco et al., 2007). In addition to reducing I_{to} , TNF- α can also prolong APD by reducing the densities of I_{Kr} and I_{Ks} . In Hek293 cells stably expressing the human *ether-a-go-go*-related gene, TNF- α decreases the HERG current by impairing the function of the HERG channel in a dose-dependent manner (Jingxiong Wang et al., 2004). The authors of this study also showed similar effects on I_{Kr} in canine cardiomyocytes. Interestingly, the data from this study revealed that TNF- α reduces the function of these potassium channels by stimulating reactive oxygen species (ROS), but not their expression.

While activation of TLR4 and uptake of the receptor has mainly been studied in immune cells, internalisation of TLR4 and LPS has also been shown to occur rapidly in rat ventricular cardiomyocytes after just 30 minutes (Cowan et al., 2001). Myocardial dysfunction is a common complication in patients with endotoxemia and is a significant contributor to mortality (Hoffman et al., 2019; Nguyen et al., 2023). To that end, many studies have examined the direct effects of LPS on cardiomyocytes in human and animal models. In HL-1 cells, incubation with LPS (1 ug/ml) increases action potential duration and decreases firing rate after just 20 minutes (Wondergem et al., 2012) and this increase in APD was found to be correlated with an inhibition of I_{Kr} , while L-type Ca^{2+} currents were not affected over this time course (Wondergem et al., 2010). Similarly, human induced pluripotent stem cell-cardiomyocytes (hiPSC-CMs) also display an inhibition of I_k after treatment with LPS along with an increase in APD (Wang et al., 2018; Yucel et al., 2017). In mice, treatment of isolated myocytes with LPS (1 ug/ml) reduced I_{to} amplitude as well as Kv4.3 and KChIP2 mRNA levels, while Ca^{2+} transients and I_{CaL} were not affected (Monnerat-Cahli et al., 2014). The reduction in I_{to} amplitude was shown to be prevented by inhibition of IRF3, but not IRAK4, suggesting that this effect is associated with the IRF3 pathway and independent of the MyD88 pathway.

While many studies focus on the impacts of inflammatory signaling on potassium currents, the effects of these pathways on sodium and calcium currents are also crucial to our understanding of inflammation and cardiac repolarization. As calcium handling is heavily regulated by cardiomyocytes, inflammatory cytokines can disrupt cardiomyocyte excitation by impacting Ca^{2+} release or reuptake. Several reports have shown that inflammatory cytokines such as TNF, IL-1B and IL-6 can disrupt Ca^{2+} transients, leading to arrhythmias.

Exposure of rodent cardiomyocytes to TNF- α results in elevated diastolic [Ca²⁺] and prolongation of the Ca²⁺ transient, leading to an increase in the action potential duration and generation of ventricular tachycardias (London et al., 2003)(Saba et al., 2005)(Lee et al., 2007). Cardiomyocytes acutely exposed to IL-1 β also experience a prolongation of the Ca²⁺ transient and a decrease in the Ca²⁺ transient amplitude after 3 hours. This was observed to likely be the result of an increase in asynchronous local Ca²⁺ sparks leading to Ca²⁺ leaks from the SR and an overall reduction in the SR Ca²⁺ content (Duncan et al., 2010). Importantly, these studies also shown an increase in the activity of NCX in response to cytokine exposure.

Due to the bidirectional nature of NCX, and the role that SR Ca ATPases (SERCAs) play in returning intracellular calcium to the SR, the effects of overexpression of NCX and increased NCX activity are not easily described. While some groups have reported that constitutive overexpression of NCX did not affect contractility *in vivo* (Adachi-Akahane et al., 1997), voltage clamp studies have shown that murine cardiomyocytes overexpressing NCX have an almost 50% reduction in the calcium transient amplitude (Ottolia et al., 2013) as well as a decrease in myocyte shortening (Wang et al., 2009). Transgenic studies have also shown that overexpression of NCX1 leads to increased action potential duration and QT prolongation (Wang et al., 2009)(Scranton et al., 2024)(Pott et al., 2012). These studies and the viability of these transgenic mice suggests that while overexpression of NCX may lead to imbalances *in vitro*, compensatory mechanisms exist to ensure that contractility of the myocardium is preserved.

1.5.3. Role of IRF2BP2 in septic cardiomyopathy

With respect to inflammatory signalling, IRF2BP2 is known to regulate the inflammatory phenotype of bone marrow derived macrophages and deficiency of IRF2BP2 leads to impairment in cholesterol efflux and increased risk of CAD (H.-H. Chen et al., 2015). In the heart, IRF2BP2 protein expression has also been shown to increase in the myocardium following ischemia (Teng et al., 2010). With respect to sepsis, it has also been shown that IRF2BP2 protein is upregulated in the heart following exposure to LPS, as quickly as 6 hours following LPS challenge (T. Li et al., 2020). The authors of this study also posit that overexpression of IRF2BP2 in the myocardium reduces the severity of LPS-induced cardiac dysfunction by measuring parameters such as ejection fraction and fractional shortening. Overexpression of IRF2BP2 in the heart also reduces the levels of IL-1 β , TNF- α AND IL-6 mRNA following LPS challenge and blocks NF- κ B signaling (T. Li et al., 2020). Cell death following LPS challenge was also reduced in mice overexpressing IRF2BP2 in the myocardium. These protective effects were shown to be mediated by AMPK and acetyl-CoA carboxylase (ACC) signaling.

1.6. Growth Differentiation Factor-3

Growth differentiation factors (GDFs) are a subset of the transforming growth factor beta superfamily of proteins that also includes bone morphogenetic proteins (BMPs), activins and inhibins (Levine & Brivanlou, 2006a). As their name suggests, GDFs are active during embryonic growth and development, and many continue to play important roles into adulthood. Growth differentiation factor 3 (GDF3), also known as Vg-related gene 2 (Vgr2), is crucial in maintaining markers of pluripotency in human embryonic stem cells by inhibiting BMP signaling (Levine & Brivanlou, 2006b). Contrastingly, in mice where BMP

signaling is required for the maintenance of stem cells (Ying et al., 2003), reduction of GDF3 supports pluripotency (Levine & Brivanlou, 2006b). Maternal GDF3 is also crucial for Nodal signaling in zygotes and knockdown of GDF3 disrupts left-right patterning in zebrafish (Pelliccia et al., 2017), while complete knockout of GDF3 is embryonically lethal in one third of embryos (Andersson et al., 2007). GDF3 signaling during embryogenesis occurs through the ALK4 receptor, which regulates the development of the anterior visceral endoderm and mesoderm (Andersson et al., 2007).

In adulthood, GDF3 signals through the ALK7 receptor and is implicated in metabolic pathways (Andersson et al., 2008). *Gdf3*^{-/-} mice were shown to have significantly lower fat accumulation on high fat diet compared to their WT controls, indicating an important role for GDF3 in energy balance (Andersson et al., 2008). Similarly, *Alk7*^{-/-} mice had lower diet-induced fat accumulation and weight gain compared to controls. In response to insulin release, production of GDF3 by CD11c⁺ adipose tissue macrophages (ATM) acts locally on ALK7-expressing adipocytes to activate PPAR γ and adipose lipases in order to convert excess nutrients to fat (Bu et al., 2018). GDF3 inhibition of BMP signaling is also implicated in insulin resistance and glucose homeostasis. In adipose tissue and skeletal muscle, GDF3 was found to be a transcriptional target of PPAR γ S273 phosphorylation, leading to insulin resistance in mice (Hall et al., 2020). The authors of this study showed that overexpression of GDF3 in C2C12 muscle cells decreased glucose uptake in the presence of insulin, likely due to an inhibition of BMP signaling. Overexpression of GDF3 in mice also lead to impairments in glucose and insulin tolerance tests independent of body weight and food intake. Together, this data demonstrates the role of GDF3 signaling in insulin-sensitizing pathways and energy storage.

1.7. Rationale and objectives

1.7.1. Objective: The objective of this PhD project aims to shed light on the mechanism behind IRF2BP2 self-regulation at the 3'UTR and how disruption of this sequence by the deletion variant rs3045215 reduces expression of IRF2BP2.

1.7.2. Hypothesis: Expression of IRF2BP2 is reduced by the deletion variant of rs3045215 found in the 3'UTR compared to the insertion variant. This deletion variant also correlates with an increased risk of coronary artery calcification and disease in men. Here, I hypothesize that this 9-nucleotide sequence is critical for binding of eukaryotic translation initiation factor-4 (eIF4h), which mediates IRF2BP2 self-regulation and expression.

1.7.3. Objective: The objective of this PhD project is to investigate the role of IRF2BP2 deficiency and GDF3 upregulation in reducing the severity of LPS-induced QT prolongation and identify the signaling pathways involved.

Hypothesis: IRF2BP2 is critical in determining the inflammatory phenotype of bone marrow derived macrophages. Deficiency of IRF2BP2 in female BMDM leads to the upregulation of growth differentiation factor 3 (GDF3), an important player in reducing the severity of LPS-induced cardiac dysfunction. I hypothesize that GDF3 counteracts the effect of LPS on cardiac ion channel regulation and attenuates LPS-induced QT prolongation.

In the first chapter, I discuss the autoregulatory capabilities of IRF2BP2 via its own 3'UTR and the role of eIF4H. The data presented in this chapter reveals the potential of IRF2BP2 as an mRNA-regulating protein found in the cytosol and demonstrates the impact

of LPS on IRF2BP2 self-regulation. In the second chapter, we look at the targeted deletion of IRF2BP2 in the myeloid lineage and the effects of GDF3 on LPS-induced QT prolongation in mice. The data presented in this thesis highlight a novel role for GDF3 in inflammatory signalling and its effects on cardiac electrophysiology.

2. Chapter I: A 3'UTR polymorphism disrupts IRF2BP2 autoregulation through an eIF4H translational enhancer

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Keywords: genetic polymorphism, interferon regulatory factor (IRF), translation regulation, macrophage, eukaryotic translation initiation factor 4H

2.1. Statement of the manuscript

The manuscript “*A 3'UTR polymorphism disrupts IRF2BP2 autoregulation through an eIF4H translational enhancer*” has been published by Frontiers in Genetics (27 May 2026).

2.2. Contribution statement

In this chapter, I performed all of the experimental work and data analysis. I wrote the manuscript along with HHC and AS. HHC and AS provided funding for the studies from research grants.

2.3. Acknowledgements

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2.5. Disclosure

None

2.6. Abstract

Introduction: Interferon regulatory factor 2 binding protein 2 (IRF2BP2) suppresses the interferon response and inflammation. Individuals who carry 2 copies of a genetic variant (rs3045215) that deletes 9 nucleotides from the long 3'UTR of IRF2BP2 have lower IRF2BP2 protein expression in white blood cells and increased risk of coronary atherosclerosis and calcification.

Methods and Results: RNAfold revealed that the deletion variant of IRF2BP2 disrupts an RNA stem-loop structure that can recruit the eukaryotic initiation factor 4H (eIF4H) to facilitate translation. siRNA knockdown of eIF4H reduced expression of endogenous IRF2BP2 protein. Similarly, it impaired translation of a luciferase reporter bearing the whole 3'UTR of IRF2BP2 but had no effect on one bearing the 9-nucleotide deletion variant (rs3045215). This deletion variant happens to be co-inherited with an IRF2BP2 coding variant that changes proline to serine at position 78. Overexpression of either isoform of IRF2BP2 (Pro⁷⁸ or Ser⁷⁸) suppressed translation of the luciferase reporter containing the whole IRF2BP2 3'UTR to the same level but had no effect on the deletion-bearing reporter. RNA gel mobility shift assay using cytosolic extracts of LPS-stimulated THP1 macrophages revealed that the 9-nucleotide deletion variant prevents endogenous IRF2BP2 protein from interacting with its own 3'UTR RNA sequences.

Conclusion: The rs3045215 9-nucleotide deletion that increases the risk of heart disease abolishes IRF2BP2 autoregulation through an eIF4H-dependent translational enhancer.

2.7. Introduction

Interferon regulatory factor 2-binding proteins (IRF2BP) encode a family of transcriptional regulators including IRF2BP1, IRF2BP2 and IRF2BPL. Each member bears a conserved N-terminal C4 zinc finger domain, a nuclear localization signal and a C-terminal CH3C4 RING domain. IRF2BP2 was the first family member described by Childs and Goodbourn (2003) as a transcriptional co-repressor that interacts with IRF2 through its RING domain to inhibit transcription of interferon-inducible genes (Childs, 2003). The IRF2BP factors have non-redundant functions; IRF2BP1 not only represses IRF2-dependent gene expression (Childs, 2003) but also interacts and competes with the TGF-beta-induced homeodomain factor (TGIF) to enhance TGF β signaling (Faresse et al., 2008). IRF2BPL plays a crucial role in neurodevelopment and loss of IRF2BPL causes a severe neuro-regressive disorder due to excessive WNT signaling in neurons (Marcogliese et al., 2022).

IRF2BP2 also plays an important role in several IRF2-independent pathways, including cell survival and protection from apoptosis by repressing p53-mediated transactivation of the apoptosis regulator BAX (Max Koeppel et al., 2009). IRF2BP2 also represses NFAT1-mediated gene expression in Jurkat T cells (Carneiro et al., 2011), YAP/TEAD4 signalling in hepatocytes (Feng et al., 2020) and the JAK/STAT signaling pathway, since STAT1 is activated in peripheral lymphocytes of individuals with truncation mutations in IRF2BP2 (Palmroth et al., 2021). While the preponderance of genes affected by ablation of *Irf2bp2* are upregulated in the liver, skeletal muscle and heart of *Irf2bp2* null mice, suggesting a co-repressor function of IRF2BP2, it may also function as a co-activator of Sirt1, Taf4 and Nkx2-3 (Vilmundarson et al., 2022). We identified IRF2BP2 as a partner of VGLL4 in a yeast two-hybrid screen of the human heart and found that it

activates a vascular endothelial growth factor A promoter when co-expressed with TEAD4 and VGLL4 (Teng et al., 2010). IRF2BP2 was also reported to act as a strong co-activator of the glucocorticoid receptor (GR) and to modulate GR and tumor necrosis factor alpha (TNF) signaling in lung epithelial A549 cells (Manjur et al., 2019).

Congenital mutations in IRF2BP2 have been tied to common variable immunodeficiency (CVID) (Keller et al., 2016; Udemgba et al., 2025). In a recent survey of 34 individuals with mutations in IRF2BP2 from 18 different families, 31 (91%) displayed CVID, 22 (65%) had gastrointestinal diseases (chronic abdominal pain, inflammatory bowel disease, Crohn's disease, and irritable bowel syndrome) and the third most common diagnosis was inflammation in 19 individuals (56%). Somatic loss-of-function mutations have been identified in cases of lymphoma (Bruno et al., 2014; Mazharuddin et al., 2018) and acute promyelocytic leukemia (Alotaibi et al., 2020). In mice, deletion of *Irf2bp2* is lethal due to the loss of an essential function related to fetal myeloid-dependent hepatic hematopoiesis (Stadhouders et al., 2015). However, *Irf2bp2*-null mice that acquire wild type myeloid progenitors from older male siblings through trans-maternal microchimerism are rescued from fetal lethality, but succumb to lymphoma as adults (Vilmundarson et al., 2022). Ablation of *Irf2bp2* in macrophages augments their inflammatory response, increases the susceptibility of mice to atherogenic mutations (H.-H. Chen et al., 2015) and impairs recovery from ischemic brain injury (Cruz et al., 2017). These studies suggest that reduced IRF2BP2 expression may enable chronic inflammation.

Common genetic variants in the vicinity of IRF2BP2 affect traits related to heart disease, including elevated LDL cholesterol (rs10910476) (Richardson et al., 2020) and

diastolic blood pressure (rs1329128), as well as inflammatory bowel disease (rs10910476) (Z. Liu et al., 2023) and osteoporosis (rs6672925) (Morris et al., 2019a), known risks of coronary artery disease (Guo et al., 2025; Laroche et al., 2017). Our lab has identified a 9-nucleotide deletion polymorphism (rs3045215) that works by a recessive model to increase the risk of coronary artery atherosclerosis (H.-H. Chen et al., 2015) and coronary artery calcification in men (Ragnar O. Vilmundarson et al., 2021). In individuals homozygous for the deletion, the level of IRF2BP2 protein in their peripheral blood mononuclear cells is reduced (H.-H. Chen et al., 2015).

Activity studies of luciferase reporters bearing the entire 3' UTR of IRF2BP2 (2,868 nucleotides) but differing only in the 9 nucleotide deletion revealed a translational enhancer at the 3' UTR whose function is disrupted by the deletion (H.-H. Chen et al., 2015). Further, our studies show that this deletion variant does not affect the rate of mRNA decay following actinomycin-D treatment to block transcription (H.-H. Chen et al., 2015). This IRF2BP2 3'UTR translational enhancer functions in part by excluding the binding of a translation inhibitor ELAVL1 (Ragnar O. Vilmundarson et al., 2021). On the other hand, recruitment of the eukaryotic initiation factor 4H (eIF4H) to RNA stem-loop structures to facilitate the RNA helicase function of eIF4A is particularly important to enhance translation of GC-rich RNA sequences that are difficult to unwind for translation (Marintchev et al., 2009; Sun et al., 2012). The IRF2BP2 mRNA has an extremely GC-rich (> 80%) sequence in the 5'UTR spanning 684 nucleotides that is likely difficult to unwind and translate without a translational enhancer. Here, we report that the translational enhancer of the IRF2BP2 3'UTR disrupted by the rs3045215 deletion variant relies on eIF4H to augment translation.

Furthermore, our studies show that IRF2BP2 targets this enhancer region to suppress and regulate its own expression.

2.8. Materials and Methods

IRF2BP2 expression plasmids

Genomic DNA obtained from the Ottawa Heart Genomics Study (Dandona et al., 2010) was genotyped using PCR primers (forward: 5'-CTT CAC CGA ACC CGT CTG-3', reverse: 5'-CTG CTG CCG AAG TCA GAG-3') to identify samples containing either variant of rs7545855. The entire coding sequence of IRF2BP2 containing either variant was then cloned into a pCDEF3 backbone to produce pCDEF3-IRF2BP2-Ser⁷⁸ and pCDEF3-IRF2BP2-Pro⁷⁸ and verified by Sanger sequencing (Applied Biosystems 3730DNA Analyzer, The Ottawa Hospital DNA Sequencing Facility).

RNA folding

The 3' UTR sequence of IRF2BP2 100 nucleotides on either side of the rs3045215 indel variant was subjected to RNA folding using the RNAfold webserver (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>). The minimum free energy algorithm was used to predict RNA secondary structure plots with reliability annotations.

Luciferase Reporter Assays

The IRF2BP2 3'UTR luciferase reporter constructs were previously described (H.-H. Chen et al., 2015). Genomic DNA obtained from the Ottawa Heart Genomics Study was genotyped to identify samples homozygous for either allele of rs7545855. IRF2BP2 cDNA of each allele was then cloned into a pCDEF3 expression plasmid (Addgene plasmid #49212) and verified

by Sanger sequencing (Applied Biosystems 3730DNA Analyzer, The Ottawa Hospital DNA Sequencing Facility).

HEK293 cells were seeded in 12-well plates (5×10^5 cells/ml) and allowed to adhere overnight. The following day, cells were transfected in triplicate using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol with 0.5 ug/ml of either variant of the IRF2BP2 3'UTR luciferase assay. 24 hours after transfection, cells were harvested and the lysate was extracted. Luminescence readings were measured using a Berthold Technologies FB12 Luminometer.

siRNA assay

HEK293 cells were plated and transfected as described above. Cells were co-transfected with 16 nM of control (Santa Cruz Biotechnology sc-37007) or eIF4H siRNA (Santa Cruz Biotechnology sc-89585) according to the manufacturer's protocol. The eIF4H siRNA cocktail consists of 3 pooled siRNA duplexes (sc-89585A, 5'-GCUUUGCUGUAUCUAUCUAtt-3'; sc-89585B, 5'-GCAGAGACCUUGUUGGUAUtt-3'; sc-89585C, 5'-GUAACACCAACACUUAACUtt-3'; sense strands). 24 hours after transfection, cell lysates were extracted and luminescence readings measured as described above.

Immunoblots

HEK293 cells were transfected with 250 ng/ml of control or eIF4H siRNA and protein was extracted 24 hours later in RIPA buffer containing protease inhibitors (Roche – cOmplete Protease Inhibitor Tablets, ThermoFisher). Proteins were size fractionated by tris-glycine extended TGX (4-15%) polyacrylamide gel (Bio-Rad) electrophoresis followed by

electroblotting to polyvinylidene fluoride (PVDF) membranes (0.2 μ m) for 1.5 hours (Thermo Scientific). Proteins were visualized with the following antibodies: anti-eIF4H rabbit polyclonal antibody (Cell Signaling Technology #2444S) used at a dilution of 1:1000 in 5% non-fat milk with TBST (Tris-buffered saline Tween, 20mM Tris, 0.1% Tween 20) overnight at 4°C; anti-IRF2BP2 antibody (Invitrogen #PA5100580) at a dilution of 1:700 in 5% non-fat milk with TBST overnight at 4°C; anti-GAPDH mouse monoclonal antibody (R&D Systems #MAB5718) at a dilution of 1:10,000 in 5% non-fat milk with TBST for 1 hour at room temperature; goat anti-rabbit IgG (Life Technologies #31460) used at a dilution of 1:10,000 in 5% non-fat milk with TBST for 1 hour at room temperature; goat anti-mouse IgG (R&D Systems #HAF007) used at a dilution of 1:10,000 in 5% non-fat milk with TBST for 1 hour at room temperature. Bands were revealed by chemiluminescence (Bio-Rad Clarity Max ECL Western Blot Substrate, #1705062) and were quantified using ImageJ (<https://imagej.net/ij/>).

RNA Electrophoresis Mobility Shift Assay

The RNA electrophoresis mobility shift assay was used to determine whether proteins bind differentially to RNA probes containing the non-deletion and deletion variants of rs3045215, as described previously (R. O. Vilmundarson et al., 2021b). Non-deletion probe: 5'-UAGGCACUUUAUUUAUAACUGGAAUUUGAC-3'; Deletion probe: 5'-UAGGCACUUUGGAAUUUGAC-3' (Integrated DNA Technologies). RNA probes were 5'-end labeled using Gamma P-32 ATP and T4 Polynucleotide Kinase at 37 °C for 1 hour. The reaction was stopped using 0.5M EDTA (pH 8.0) and then the samples were purified using RNase-free G-25 Sephadex spin columns (Roche) and 50,000 CPM were used per well. The following solutions were prepared for non-deletion and deletion probes, separately: 10X

binding buffer, 1 µl; radiolabeled probe (50,000 CPM dilution), 1 µl; 50% glycerol, 1.5 µl; 1M DTT, 0.2 µl; RNasin (Promega), 0.2 µl; Yeast Total RNA (10 mg/mol), 0.2 uL; 5% xylene cyanol, 1 µl; DEPC-treated ddH₂O, 2.9 µl. All components were prepared or purchased RNase-free. Solutions were added to protein extracts, gently vortexed, spun down and incubated at 37°C for 3 minutes. THP1 cells were cultured as described previously (Ragnar O. Vilmundarson et al., 2021) and treated with PBS or LPS (100 ng/ml) prior to cytoplasmic protein extraction in lysis buffer (1% Triton, 25 mM Tris HCl, pH 7.4, 40 mM KCl) according to the method of Fillebeen et al. (Fillebeen et al., 2014). Antibodies for gel shift interference assays were a rabbit anti-human IRF2BP2 peptide-specific antibody (Teng et al., 2010) and a rabbit polyclonal anti-AUF1 antibody (#ab50692, Abcam).

IRF2BP2 3'UTR luciferase reporters

The IRF2BP2 3'UTR luciferase reporter construct was previously described (H.-H. Chen et al., 2015). Briefly, PCR primers containing an XbaI site (underlined) (forward: 5'-CCT CTA GAG CAC ACA TGC AGA AAT GCA GAG TC-3', reverse: 5'-CCT CTA GAC ATA CAG ATA GCT ACC ACA AAT TAG GTC-3') were used to amplify the complete 2,877 nucleotide long fragment of the 3'UTR of human IRF2BP2 with or without the 9-nucleotide deletion of rs3045215. The fragments were cloned into a CMV enhancer-driven pGL3 luciferase vector.

Statistical analysis

Raw luciferase activity units were normalized to empty vector control values and averaged. Two-way ANOVA was used to analyze groups with two or more variables and post-hoc

pairwise comparisons used Tukey's test to correct for multiple comparisons. All statistical tests were carried out using the GraphPad (ver. 9.0) software.

2.9. Results

2.9.1. The 9-nucleotide rs3045215 deletion disrupts a stem-loop structure in the 3'UTR of IRF2BP2

Our previous work established that the rs3045215 deletion variant increased risk of coronary atherosclerosis and calcification and patients who carry two copies of this deletion variant have lower IRF2BP2 protein levels in their circulating white blood cells.

This deletion variant reduces activity of a luciferase reporter bearing the entire 3'UTR sequence of IRF2BP2 but does not affect mRNA stability (H.-H. Chen et al., 2015), indicating that the deletion variant reduces translation of IRF2BP2 mRNA. Complementary sequences in RNA can fold to form various stem-loop structures that sterically hinder the efficiency of translation unless they are recognized by RNA-binding proteins that relieve this hindrance. The RNAfold minimum-free-energy algorithm predicts the 3'UTR RNA of IRF2BP2 forms a stem-loop structure disrupted by the 9-nucleotide deletion of the rs3045215 as in the deletion variant (Figure 2.1).

The eukaryotic translation initiation factor 4H (eIF4H) is known to facilitate translation of mRNAs with complex 5'UTR sequences by recognizing stem-loop structures and promoting RNA unwinding by the eIF4A RNA helicase (Sun et al., 2012). While this function has been ascribed to an interaction of eIF4H with 5'UTR sequences, some initiation factors like eIF3 were found to interact mainly with 3'UTR sequences of highly translated mRNAs (Mestre-Fos et al., 2025). Furthermore, in an unbiased screen of protein-protein interactions, eIF4H was found to physically interact with IRF2BP2 (Wan et al., 2015), although the protein domains that mediate this interaction remain to be elucidated. For these reasons, we chose eIF4H as a candidate to test whether eIF4H may also regulate the

IRF2BP2 translation by interacting with the 3'UTR stem-loop, and whether this regulation is affected by the rs3045215 deletion variant.

2.9.2. eIF4H promotes translation via the IRF2BP2 3'UTR, an effect abolished by the 9-nucleotide deletion of the rs3045215 variant

To test whether eIF4H acts at the 3'UTR to regulate IRF2BP2 translation, HEK293 cells were co-transfected with an eIF4H siRNA or a control siRNA together with a firefly luciferase reporter carrying either rs3045215 allele (non-deletion or 9-nucleotide deletion) of the IRF2BP2 3'UTR. Of note, we did not use a second reporter plasmid to determine differences in transfection efficiencies as this reporter plasmid could also be affected by eIF4H siRNA, rendering it an unreliable control. Luciferase reporter activities were expressed as a fold of the non-deletion luciferase reporter activity at baseline for each experiment. Knockdown of eIF4H was confirmed by immunoblot (Figure 2.2A). In the presence of the eIF4H siRNA, luciferase activity of the non-deletion variant was reduced compared to the control siRNA. In contrast, the luciferase activity of the reporter carrying the deletion variant was not affected by the eIF4H siRNA (Figure 2.2B). Thus, eIF4H promotes translation of a reporter bearing the whole IRF2BP2 3'UTR-containing RNA sequence while this translational enhancement is lost if the 9-nucleotides of the rs3045215 variant are deleted.

Endogenous IRF2BP2 protein expression was also markedly reduced in HEK293 cells 24 hours after eIF4H siRNA transfection (Figure 2.2C), while expression of GAPDH was not affected. These results are consistent with eIF4H having minimal effect on GAPDH

expression, as reported previously (Vaysse et al., 2015), while maintaining high levels of IRF2BP2 translation *in vivo*.

2.9.3. The rs3045215 deletion variant disrupts IRF2BP2-dependent self-regulation

Given that eIF4H is required to mediate the translational enhancer function disrupted by the rs3045215 deletion variant and that eIF4H physically interacts with IRF2BP2 protein, we wanted to know how IRF2BP2 affects eIF4H-mediated translation enhancement. It should be noted that there are two coding variants of IRF2BP2 where a proline residue at position 78 is substituted for a serine residue in the first exon of IRF2BP2. Remarkably, this Ser⁷⁸ coding variant (rs7545855) is co-inherited with the 3'UTR 9-nucleotide deletion variant at rs3045215 (Figure 2.3A). We next tested whether over-expression of either IRF2BP2 protein variant would affect a luciferase reporter bearing the IRF2BP2 3'UTR sequence containing either the deletion or non-deletion variants.

To this end, we generated expression plasmids for each isoform (Figure 2.3B) and confirmed that similar levels of IRF2BP2 expression were achieved with 10, 50 and 100 ng of each expression plasmid (Figure 2.3C); this result suggests that the two isoforms have similar protein stabilities. Co-expression of either the Pro⁷⁸ or Ser⁷⁸ IRF2BP2 isoform markedly suppressed the luciferase activity of the non-deletion-bearing reporter to half the levels observed with empty vector (Figure 2.4A,B). Conversely, neither isoform of IRF2BP2 affected the expression of the 3'UTR luciferase reporter bearing the deletion variant of rs3045215 (Figure 2.4C). These luciferase reporter studies indicate that IRF2BP2 protein, regardless of isoform, regulates its own expression via its 3'UTR, an auto-regulatory function that is lost with the rs3045215 deletion variant.

2.9.4. The rs3045215 deletion variant disrupts IRF2BP2 interaction to its own 3'UTR sequence

Since IRF2BP2 suppresses its own expression through a 3'UTR sequence disrupted by the rs3045215 deletion, we next asked whether IRF2BP2 could form a complex with RNA sequences in the vicinity of the rs3045215 deletion. RNA gel shift using RNA probes of 29 nucleotides (containing the rs3045215 non-deletion variant) or 20 nucleotides (containing the rs3045215 deletion variant) was carried out with cytosolic extracts of THP1 human monocyte cells. Differential binding to these RNA probes was observed, with 3 complexes (C1, C2 and C3) formed by the probe containing the non-deletion (Figure 2.5, lanes 1-7) whereas a single complex was formed by the deletion probe (Figure 2.5, lanes 8-14). LPS treatment of THP1 cells, a condition that suppresses IRF2BP2 expression (H.-H. Chen et al., 2015; Ragnar O. Vilmundarson et al., 2021), was associated with increased formation of complex (C1). To identify the protein associated with this C1 RNA-protein complex, antibodies to different protein candidates, including AUF1 and IRF2BP2, were tested. Addition of the AUF1 antibody had no effect on complex C1 formation, whereas addition of an antibody to IRF2BP2 interfered with the formation of the C1 complex (Figure 2.5, compare lanes 6 and 7), indicating that this effect is specific. These results indicate that IRF2BP2, a factor predominantly found in the nucleus, can also be present in the cytoplasm. Further, IRF2BP2 is part of the C1 complex whose formation is increased by LPS, likely by increasing the cytosolic levels of endogenous IRF2BP2 or by increasing the binding affinity of cytosolic IRF2BP2 to its 3'UTR or an RNA-binding protein.

Taken together, our studies suggest that IRF2BP2 protein levels are regulated by its 3'UTR sequence through an eIF4H-dependent mechanism that is disrupted by 9-nucleotide deletion in the rs3045215 variant. Furthermore, our studies suggest that IRF2BP2 exploits this

mechanism to regulate its own expression under inflammatory conditions; as summarized in a model (Figure 2.6).

2.10. Discussion

IRF2BP2 in macrophages works as a key regulator of innate immunity. IRF2BP2 maintains macrophages in a non-inflammatory state and ablating IRF2BP2 in macrophages augments their inflammatory response (H.-H. Chen et al., 2015). Chronic inflammation is a central driver of coronary artery disease (Almontashiri et al., 2013; H.-H. Chen et al., 2015). Individuals homozygous for a 9-nucleotide deletion variant (rs3045215) in the 3'UTR of IRF2BP2 mRNA have increased risk of developing coronary artery disease. These individuals have reduced IRF2BP2 protein expression in their peripheral blood mononuclear cells (H.-H. Chen et al., 2015), because the deletion variant disrupts a translational enhancer in the 3'UTR required for high levels of IRF2BP2 protein expression (Ragnar O. Vilmundarson et al., 2021). Here, we showed that this deletion variant disrupts a stem-loop structure in the 3' UTR sequences of IRF2BP2 mRNA that is needed to recruit the translation initiation factor eIF4H to maintain IRF2BP2 translation at high levels. This finding adds IRF2BP2 to the growing list of eIF4H-regulated mRNAs such as *c-myc*, *CIAP1*, *BCL-xL* (Vaysse et al., 2015).

While our finding is consistent with the requirement of eIF4H for enhanced translation of IRF2BP2, because we did not measure IRF2BP2 mRNA, we cannot exclude the possibility that eIF4H affects mRNA degradation. eIF4H is targeted by the herpes simplex virus (HSV) virion host shutoff (Vhs) protein (UL41), an endoribonuclease that degrades host cell mRNAs (Sarma et al., 2008). Knockdown of eIF4H prevents this mechanism from degrading

a host mRNA (beta-actin), suggesting that some cellular mRNAs may be degraded by Vhs through the intermediary of eIF4H. While protein levels of IRF2BP2 were reduced after eIF4H knockdown, consistent with impaired translation, we cannot exclude the possibility that the stability of eIF4H-dependent mRNAs like IRF2BP2 is dependent on the presence of eIF4H. This could be addressed in future studies.

Macrophages exposed to the bacterial endotoxin LPS lower their baseline expression of IRF2BP2 to enable their full-blown inflammatory response to fight against infection (H.-H. Chen et al., 2015; Martinez et al., 2006). The eIF4H-responsive stem-loop structure also mediates this LPS-induced downregulation of IRF2BP2 expression. We found that LPS promotes the recruitment of IRF2BP2 protein to these RNA sequences to suppress translation and the 9-nucleotide deletion disrupts this LPS-induced regulation. It is worth noting that our evidence for eIF4H interaction with the stem loop sequence is indirect, based on a differential response of the IRF2BP2 3'UTR-luciferase reporters to siRNA mediated knockdown of eIF4H. Similarly, we do not have direct evidence that IRF2BP2 binds to RNA, but rather is part of a cytoplasmic protein complex that shifts an RNA probe containing the non-deleted sequence at rs3045215 but not an RNA probe with the 9-nucleotide deletion. Also, while our experiments were carried out mostly in HEK293 cells, a similar regulation likely takes place in macrophages.

IRF2BP2 is predominantly found in the nucleus in macrophages, where it regulates the transcription of interferon-responsive genes (H.-H. Chen et al., 2015). Our data here indicate that LPS increases the interaction of cytoplasmic IRF2BP2 to its 3' UTR translational enhancer. Whether this occurs through an increased binding affinity of IRF2BP2

to its UTR sequences or to a protein that interacts with these sequences remains to be determined. Further, this may reflect increased nucleus-cytoplasmic shuttling of IRF2BP2. LPS was reported to drive cytoplasmic translocation of the RNA-binding protein Adenine-thymine (AT)-rich interactive domain 5a (Arid5a) in RAW264.7 macrophages (Higa et al., 2018). Whether LPS-induces IRF2BP2 nuclear export in macrophages is not known.

While IRF2BP2 is a known regulator of transcription, the findings here highlight a new role for IRF2BP2 as an RNA-associated protein that counteracts eIF4H-dependent translation of its own mRNA. Whether IRF2BP2 also affects translation of other eIF4H-dependent mRNAs awaits future studies.

It is worth noting that IRF2BP2 mRNA and protein levels are not always correlated in cells other than macrophages and may reflect translational suppression by cytoplasmic export of IRF2BP2. Previously, we were puzzled by the fact that both cardiac and skeletal muscle express high levels of IRF2BP2 mRNA, but that IRF2BP2 protein levels are much lower in skeletal muscle compared to cardiac muscle (Teng et al., 2011). Unlike in cardiac muscle, IRF2BP2 protein is located predominantly in the cytoplasm of skeletal muscle upon differentiation (Teng et al., 2011), and may account for low IRF2BP2 protein levels, in view of the current finding of translational auto-suppression of IRF2BP2. Although the function of IRF2BP2 in cardiac and skeletal muscle is not fully elucidated, we reported previously that IRF2BP2 is induced by ischemia and may protect against oxidative stress in these tissues (Teng et al., 2010).

The protein isoform of IRF2BP2 encoded by the rs7545855 Ser⁷⁸ polymorphism is co-inherited with the 3'UTR rs3045215 deletion variant. However, we could not identify a functional difference between the Ser⁷⁸ and Pro⁷⁸ isoforms, at least in terms of their suppression of translation via the 3'UTR eIF4H-responsive stem-loop sequences. This was contrary to our expectation given that phosphorylation at Ser⁷⁸, in close proximity to the C4 zinc-finger domain required for homo- or hetero-dimerization with IRF2BP family members (Yeung et al., 2011), has been documented *in vivo* (<https://www.phosphosite.org>), a process that is precluded in the Pro⁷⁸ variant. Thus, the Ser⁷⁸ isoform co-inherited with the rs3045215 deletion variant might just be a “red herring”.

Eukaryotic translation initiation is facilitated by a "closed-loop" structure, where initiation factors (eIFs) connect the 5' cap and 3' poly(A) tail, enabling the recruitment of ribosomal subunits to initiate protein synthesis (Dever, 2002; Jacobson & Favreau, 1983). Regulatory sequences within the 5'UTR and 3'UTR recruit eIFs to control translation efficiency. Mutations within the regulatory elements of UTRs can alter translation efficiency and cause diseases. In patients with multiple myeloma, a C>T mutation in the *c-myc* internal ribosomal entry sequence (IRES) creates a stem-loop structure in the mRNA and increases translation of the *c-myc* protein 4.5 times compared to the wildtype (Cobbold et al., 2010). On the other hand, in patients with Charcot-Marie-Tooth disease, a C>T mutation in the 5' IRES of connexin-32 creates a stem-loop structure that inhibits translation of the protein in Schwann cell (Hudder & Werner, 2000). While these examples are rare mutations occurring at the 5'UTR, to our knowledge, the rs3045215 deletion polymorphism is the first example of a common genetic variant in the 3'UTR that disrupts a stem-loop structure and suppresses translation.

Because IRF2BP2 is ubiquitously expressed and plays a key role in cell differentiation and proliferation pathways in both immune and non-immune cells, changes to its expression could affect many diseases, in addition to coronary artery disease. As such, homozygosity for the rs3045215 deletion allele affecting IRF2BP2 expression should be considered. For example, in acute myeloid leukemia cells, elevated IRF2BP2 expression promotes cancer cell survival and proliferation and suppresses the inflammatory NF κ B pathway (Ellegast et al., 2022). Conversely, in medulloblastoma, elevated activity of IRF2BP2 suppresses PD-L1 expression making tumors more vulnerable to T-cells (Dorand et al., 2016). It would be interesting to learn whether the rs3045215 deletion allele influences outcomes in these two diseases. IRF2BP2 has also been implicated in hepatic inflammation and steatosis (Fang et al., 2020) and individuals homozygous for the rs3045215 deletion allele might be more vulnerable to obesity-associated hepatic steatosis.

The rs3045215 deletion variant is unique to humans and shows remarkable population-specific allele frequency differences. The deletion allele has a frequency of 13% in black Africans and 23% in Europeans but is the predominant allele in East Asians (74%) and Southeast Asians (55%). Given its potential to modulate the response to infectious diseases, this variant has likely undergone selection through generational exposure to different pathogens in different populations.

Taken together, our previous work and the present study propose that the deletion variant keeps macrophages in a chronic inflammatory state due to lower baseline expression of IRF2BP2 from the loss of the enhancing effect of eIF4H. On the other hand, this deletion

variant would also impair LPS-induced auto-suppression of IRF2BP2 expression that is needed to maximize the inflammatory response to fight infection. These two conditions may account for increased risk of coronary artery disease and other macrophage-related chronic diseases (osteoporosis, inflammatory bowel disease) in people who carry 2 copies of the deletion variant.

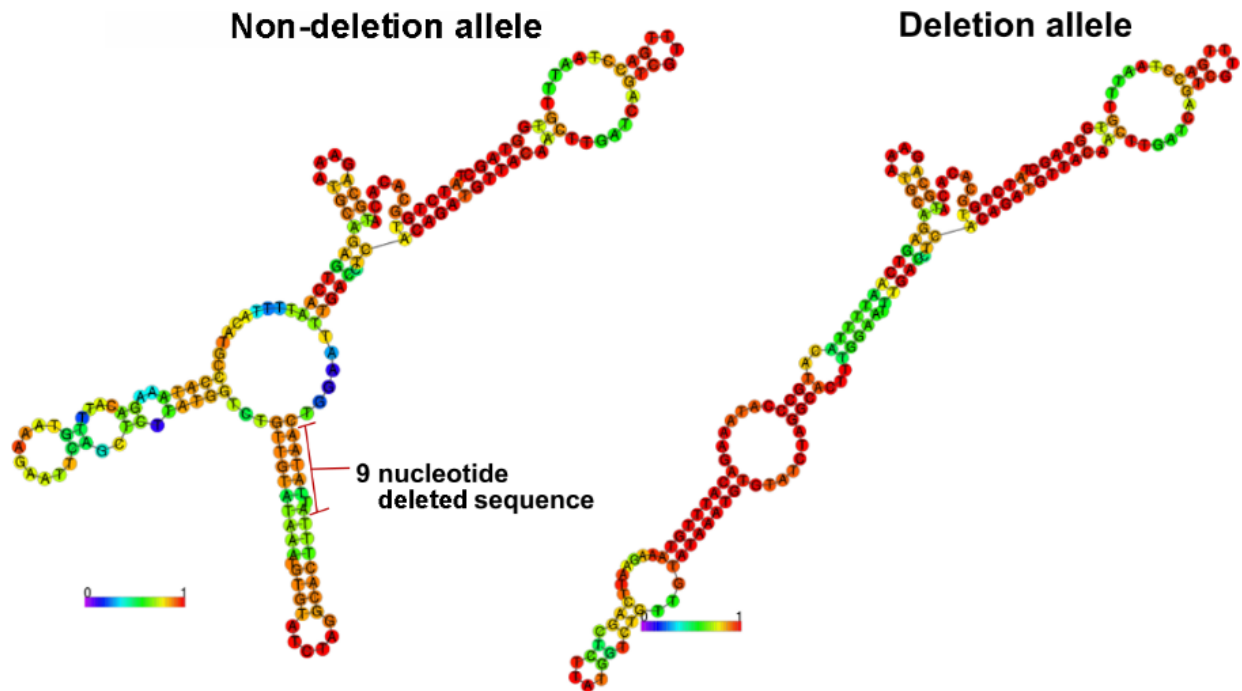


Figure 2.1. Predicted RNA structure of IRF2BP2 3'UTR comparing the deletion (risk) and non-deletion (non-risk) variants (RNAfold Web Server). RNA secondary structure blot based on the minimum free energy and partition function. Scale bar shows probability. For based paired regions, color denotes base-pairing probabilities. For unpaired regions, the color denotes the probability of being unpaired.

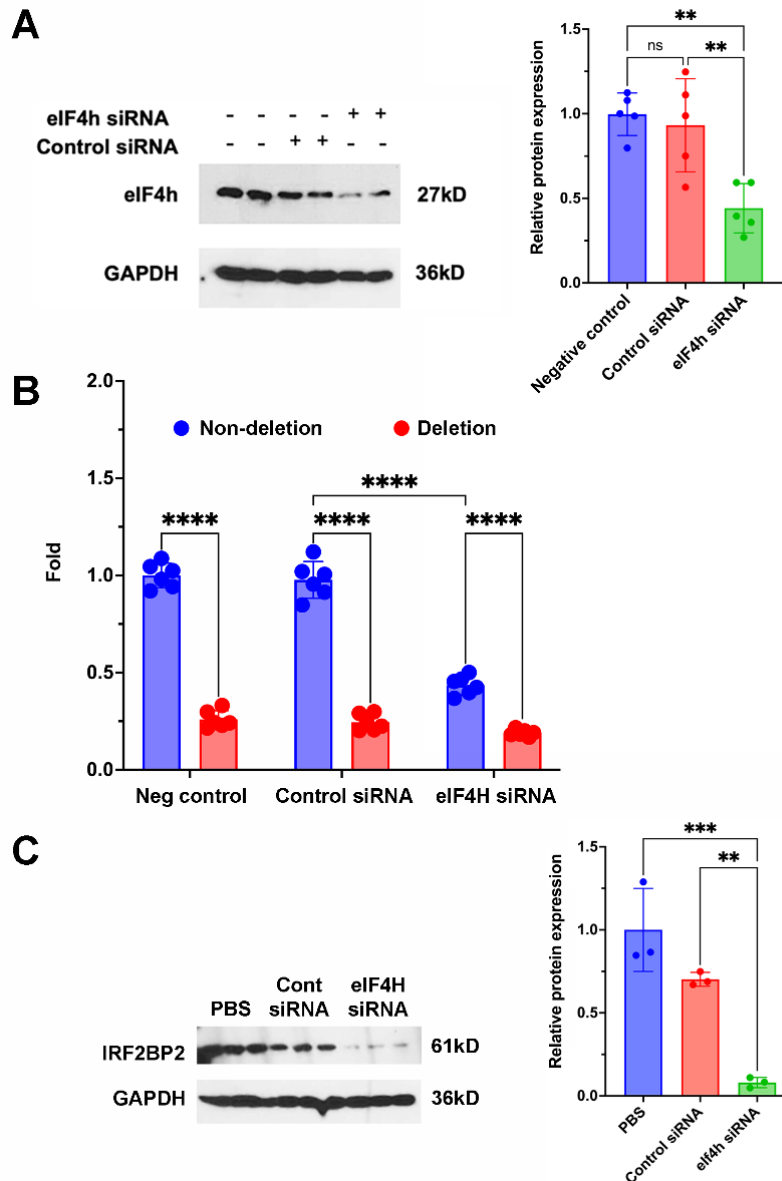


Figure 2.2. eIF4H is required for expression of the IRF2BP2 3'UTR non-deletion variant. (A) eIF4H siRNA lowers eIF4H protein expression in HEK293 cells. **, $p < 0.01$ by t-test, $n=3$. (B) eIF4H siRNA lowers expression of the IRF2BP2 3'UTR luciferase reporter bearing the non-deletion but not the deletion variant. Luciferase reporter activities were expressed as fold of the non-deletion luciferase reporter activity at baseline. HEK293 cells were co-transfected with 250 ng/ml of either IRF2BP2 3'UTR variant luciferase reporter with eIF4H siRNA or control siRNA eIF4H ($n=6$, ****, $p<0.0001$). (C) Endogenous IRF2BP2 protein expression is reduced by eIF4H siRNA. ($n=3$, ***, $p<0.001$, **, $p<0.01$ by t-test). GAPDH was used as a loading control.

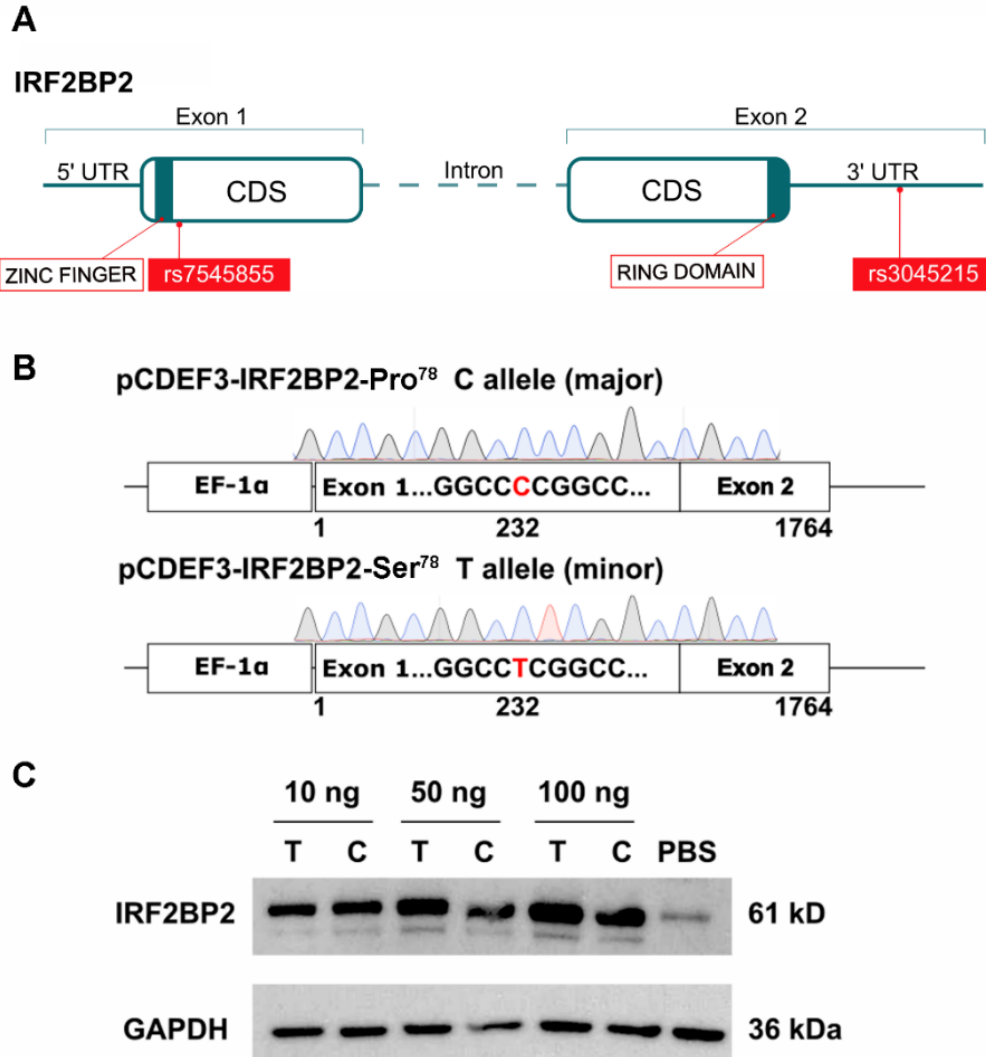


Figure 2.3. IRF2BP2 Ser⁷⁸ variant in exon 1 is co-inherited with the 9-nucleotide deletion (rs3045125) in exon 2. (A) A schematic representation of IRF2BP2. A SNP variant in the first exon located just outside of the zinc finger domain (rs7545855) is co-inherited with a 9-nucleotide deletion variant in the 3'UTR (rs3045215). **(B)** An EF-1 α -driven pCDEF3 expression plasmid carrying the coding sequence of IRF2BP2 with either the Pro⁷⁸ C allele or the Ser⁷⁸ T allele variant was created and confirmed by sequencing (electropherograms overlaid on the diagrams). **(C)** Transient transfection of expression plasmids (ng) to HEK293 cells produced similar levels of expression for each isoform, revealed by immunoblot. GAPDH was used as a loading control. PBS, phosphate-buffered saline, shows endogenous levels of IRF2BP2.

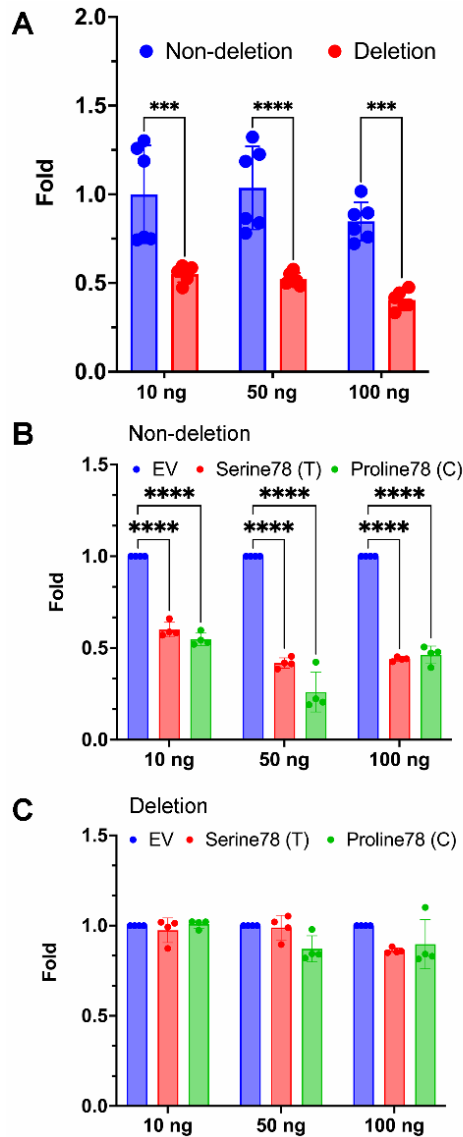


Figure 2.4. Both coding isoforms of IRF2BP2 suppress expression of the IRF2BP2 3'UTR luciferase reporter bearing the non-deletion rs3045215 allele but not the deletion allele. (A) The CMV-driven luciferase reporters carrying the 3'UTR sequence of IRF2BP2 with the deletion (risk; pCMV-Δ-3'UTR-IRF2BP2) allele is less active than the non-deletion (non-risk; pCMV-i-3'UTR-IRF2BP2) allele (n=6, ***, $p<0.0005$, ****, $p<0.0001$). HEK293 were transfected with 250 ng/ml of either the non-deletion (B) or deletion (C) pCMV-3'UTR-IRF2BP2 luciferase reporters with increasing concentrations of empty vector (EV), pCDEF3-IRF2BP2-Pro⁷⁸ or pCDEF3-IRF2BP2-Ser⁷⁸ expression plasmids (10, 50 and 100 ng/ml, n=6, ***, $p<0.0001$). Luciferase reporter activities were expressed as fold of the non-deletion luciferase reporter activity at baseline.

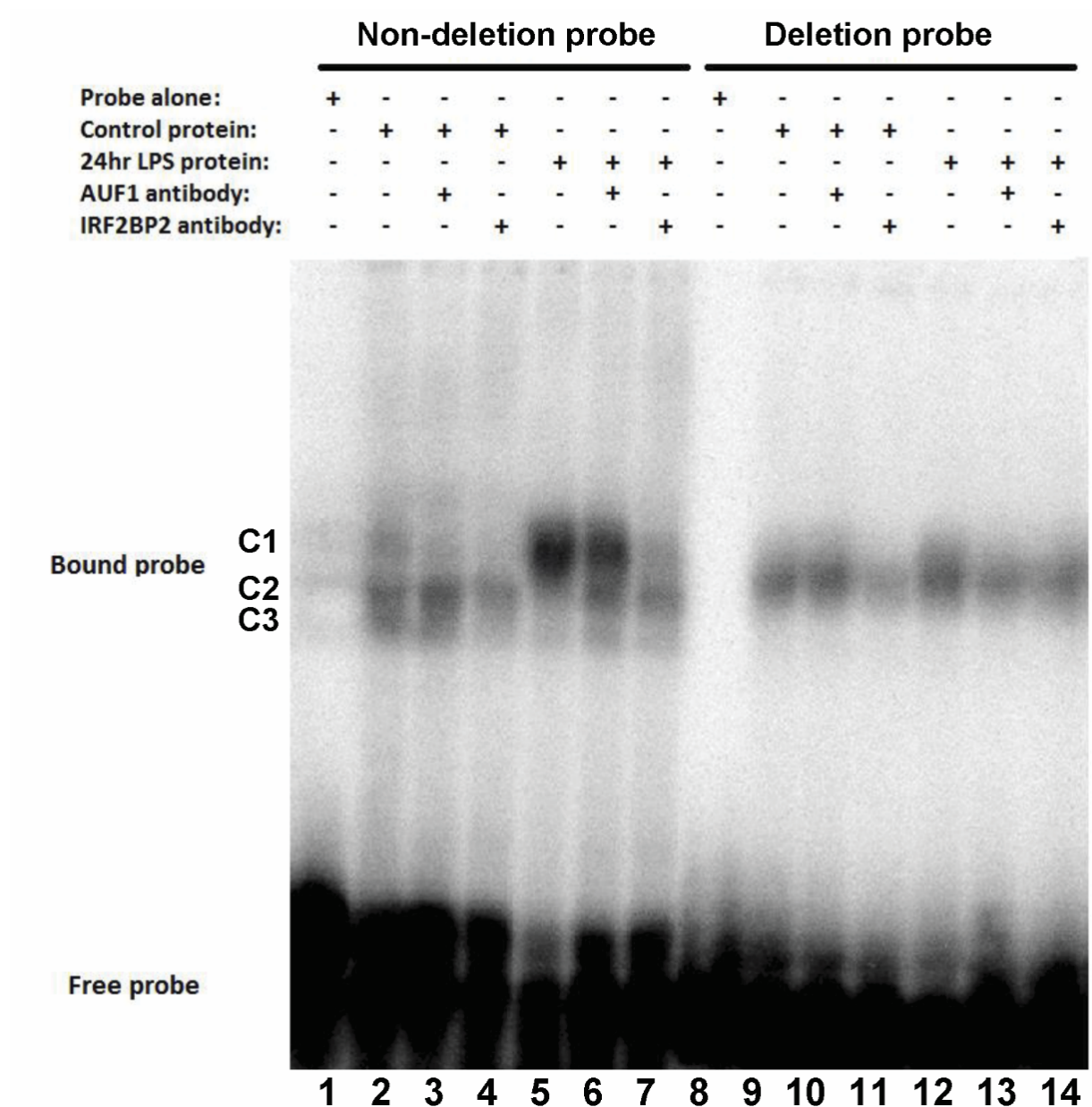


Figure 2.5. RNA gel mobility shift reveals specific binding of IRF2BP2 to rs3045215 non-deletion probe in cytoplasmic extract of LPS-treated THP1 cells. The non-deletion probe produced 3 shifted complexes (C1, C2, and C3) whereas the deletion probe produced a single shifted complex. Complex C1 contains IRF2BP2 protein, as revealed by antibody interference tested with AUF1 and IRF2BP2 antibodies. Free probe (not bound to protein), bound probe (bound to protein).

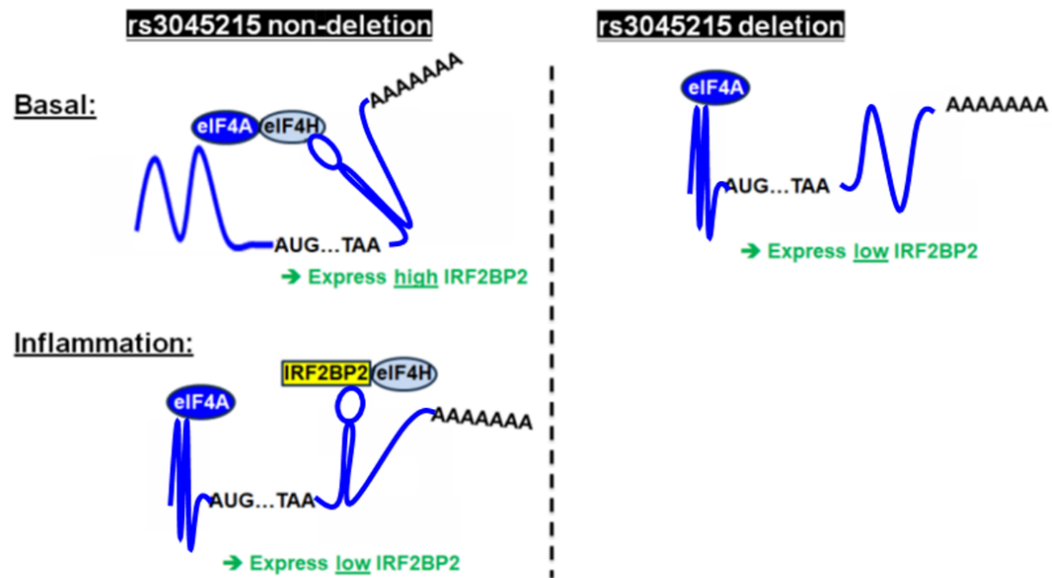


Figure 2.6. Model accounting for low expression of the rs3045215 deletion variant. The reference allele of rs3045215 contains a stem loop structure that interacts with eIF4H to facilitate eIF4A-dependent translation. Inflammation (as occurs with LPS challenge) recruits IRF2BP2 (Pro⁷⁸) to its own 3'UTR displacing eIF4H. Of note, THP1 cells are homozygous for the Pro⁷⁸ variant (Ragnar O. Vilundarson et al., 2021). The deletion variant is a loss of function recessive allele that disrupts the eIF4H-dependent translational enhancer, and IRF2BP2-mediated autoregulation, resulting in low levels of IRF2BP2 protein as seen for the non-deletion variant during inflammation.

3. Chapter II: GDF3 protects mice from LPS-induced QT prolongation

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3.1. Statement of the manuscript

The manuscript “*GDF3 protects mice from LPS-induced QT prolongation*” has been submitted to the Journal of the American Heart Association and is under review.

3.2. Contribution statement

In this chapter, I performed the *in vivo* and *in vitro* work and contributed to nearly all the analysis. JW isolated NRVMs and KK conducted the microarray assay. IH and WL carried out the electrophysiological studies in cardiomyocytes. TT provided essential materials. AFRS and HHC funded the research with extramural grant support. AD, AFRS and HHC wrote the manuscript.

3.3. Acknowledgement

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3.5. Disclosure

None.

3.6. Abstract

Background: Acute infection causes inflammation and delays cardiac repolarization. Using *LysMCre/Irf2bp2^{flx/flx}* mice whose macrophages are prone to inflammation, we examined their cardiac electrophysiological responses to lipopolysaccharides (LPS).

Methods: Surface electrocardiograms (ECGs) were recorded before and after LPS challenge (10 mg/kg, intraperitoneal) in wild type (WT) and *LysMCre/Irf2bp2^{flx/flx}* mice of both sexes. Microarrays compared baseline gene expression in their macrophages. LPS effect was tested on the electrophysiological properties of isolated ventricular myocytes, on TLR4 internalization of fluorescently tagged LPS or transferrin (control), and on TRIF signaling in HL1 cardiomyocytes. Recombinant GDF3, a growth factor made by macrophages, was tested on LPS-induced ECG changes, cytokine production, and NCX1 expression in WT male mice.

Results: LPS prolonged the QT interval as early as 2 hours in WT mice (both sexes) and in male *LysMCre/Irf2bp2^{flx/flx}* mice, whereas female *LysMCre/Irf2bp2^{flx/flx}* mice were protected despite a similar myocardial infiltration of macrophages as in WT mice.

LysMCre/Irf2bp2^{flx/flx} female (but not male) macrophages express more of the growth factor GDF3, as shown by microarrays and ELISA.

Pretreating male WT mice with GDF3 attenuated LPS-induced QT prolongation. In isolated adult ventricular myocytes, LPS had no effect on the transient outward potassium current. Instead, LPS markedly elevated the sodium calcium exchange protein NCX1 accounting for the delayed late repolarization since GDF3 pre-treatment prevented NCX1 upregulation.

While GDF3 did not affect LPS-induced inflammatory cytokines in the circulation, it specifically blocked the TLR4-dependent endocytosis of LPS and activation of the TRIF/IRF3 pathway in mouse HL1 cardiomyocytes, without affecting TLR4-independent endocytosis of transferrin.

Conclusions: GDF3 blocks TLR4-dependent endocytosis and activation of the TRIF pathway and prevents LPS-induced delayed cardiac repolarization by NCX1. GDF3 could be a possible intervention to prevent lethal arrhythmias in endotoxemia.

3.7. Introduction

Prolongation of the QT interval on an ECG is indicative of delays in ventricular repolarization and predisposes patients to life-threatening ventricular arrhythmias (Viskin, 1999). Acquired QT prolongation can be triggered by certain drugs that affect potassium channels, or from inflammation seen in atherosclerosis or acute viral (Lazzerini et al., 2022; Rubin et al., 2021) or bacterial infections (Lazzerini et al., 2020; Liu et al., 2024). A multicenter retrospective study showed that 23% of patients with sepsis, experiencing the acute inflammatory response to bacteria in the blood, developed new onset QTc (QT corrected for heart rate) prolongation that increased their 30-day mortality (Liu et al., 2024). In addition, up to 30% of patients after cardiac surgery on cardiopulmonary bypass developed prolonged QTc (Kertai et al., 2016). The postoperative QTc prolongation may be related to circulating bacterial endotoxins due to stress-related increases in gut permeability (Giron et al., 2021). It should be noted that endotoxemia is found in up to 44% of patients after cardiopulmonary bypass, with 7.7% experiencing levels equivalent to severe sepsis (Klein et al., 2011).

Repolarization of the ventricular myocardium is mediated by several cardiac ion channels, including the late sodium current (I_{NaL}), potassium currents (I_K), L-type calcium current (I_{Ca-L}) and the sodium calcium exchange current (I_{NCX}). Changes in the expression or function of these channels at the cell surface can hasten or delay repolarization (Aromolaran et al., 2018; Scranton et al., 2024; Wang et al., 2012).

Lipopolysaccharide (LPS) endotoxins released by gram-negative bacteria bind

and activate TLR4 receptors on macrophages to trigger severe inflammatory responses: a rapid MyD88 cascade that activates NF κ B and inflammatory cytokine release, and a delayed TRIF (Toll/IL-1 receptor domain-containing adaptor inducing IFN- β) pathway that involves endocytosis of LPS with TLR4 and its coreceptor, CD14, and subsequent phosphorylation and nuclear translocation of IRF3 to activate type 1 interferon (alpha/beta) production (Ciesielska et al., 2021).

Inflammatory cytokines secreted by macrophages act on cardiomyocytes and can downregulate cardiac potassium ion channels (Aromolaran et al., 2018; Kawada et al., 2006; J. Wang et al., 2004). Injection of interleukin-6 (IL-6) to rabbits is sufficient to induce a modest QTc prolongation within 40 minutes (Zhu et al., 2022). These studies indicate that the macrophage MyD88 pathway contributes to delayed cardiac repolarization. On the other hand, the TRIF arm of TLR4 signaling in macrophages is not likely to influence cardiac repolarization since QTc was not affected by interferon alpha treatment of human patients for viral infections (Ding et al., 2021; Simka et al., 2023).

Although TLR4 receptors are present mainly in immune cells of the myeloid lineage, including macrophages, (Ciesielska et al., 2021) they are also found on some non-immune cells including cardiomyocytes (Cowan et al., 2001). LPS can directly affect cardiomyocyte function in the absence of immune cells, causing delayed repolarization and prolonged action potential duration in mouse HL1 cardiomyocytes, (Wondergem et al., 2012) in adult rat cardiomyocytes (Monnerat-Cahli et al., 2014) and in human inducible pluripotent stem cell-derived cardiomyocytes (Wang et al., 2019). A

study using strips of rat ventricular myocardium showed that 24 hour treatment with LPS prolonged action potential duration with a reduction of the transient outward potassium current (I_{to}), the primary current responsible for early cardiac repolarization in rodents. Furthermore, in contrast to macrophages, these direct effects of LPS on cardiomyocytes to delay cardiac repolarization were shown to occur in a TRIF-dependent but MyD88-independent manner (Monnerat-Cahli et al., 2014).

Our previous work has shown that Interferon regulatory factor 2-binding protein 2 (IRF2BP2), a transcriptional corepressor of IRF2, controls the inflammatory phenotype of cultured macrophages (H. H. Chen et al., 2015). Ablation of *Irf2bp2* in the myeloid lineage (*LysMCre/Irf2bp2^{flx/flx}*) abolished the anti-inflammatory response to IL-4 and heightened the pro-inflammatory response to LPS/interferon gamma in bone marrow-derived macrophages (BMDM) isolated from male mice. Even without exposure to LPS or interferon gamma, these macrophages already expressed higher levels of inflammatory markers, such as IL-1 β and IL-6. These male *LysMCre/Irf2bp2^{flx/flx}* mice also had more atherosclerosis when bred to an ApoE^{-/-} background (H. H. Chen et al., 2015) and worse outcomes after ischemic brain injury (Cruz et al., 2017). Given the pro-inflammatory phenotype of *Irf2bp2*-deficient macrophages, we asked whether loss of *Irf2bp2* in macrophages of male and female mice would affect their cardiac electrophysiological responses to LPS challenge.

Contrary to expectations, male *LysMCre/Irf2bp2^{flx/flx}* mice showed no difference in their response to LPS compared to WT mice. Instead, female *LysMCre/Irf2bp2^{flx/flx}*

mice were protected from the detrimental effects of LPS to delay cardiac repolarization. Here, we identified a mechanism whereby selective ablation of *Irf2bp2* in female myeloid cells produces this sex-specific protective effect through the increased production of growth differentiation factor 3 (GDF3).

3.8. Materials and Methods

Animals

C57BL6 mice with myeloid-specific ablation of IRF2BP2 were generated by breeding mice bearing a floxed allele of *Irf2bp2* with LysMCre mice expressing Cre-recombinase in the myeloid lineage, as described previously (H. H. Chen et al., 2015). The animal care and use committee of the University of Ottawa approved all procedures performed in mice. To induce an inflammatory response, mice were given intraperitoneal (IP) injections of lipopolysaccharides (LPS) (Sigma L4524, 10 mg/kg body weight), with 100 µl of PBS used as a control. For GDF3 experiments, mice were given IP injections of recombinant mouse GDF3 protein (rmGDF3, 10 µg/kg in sterile PBS, R&D Systems 9009-GD-010) 12 hours prior to LPS. BSA (10 µg/kg in PBS) was used as a control for rmGDF3.

ECG Studies

Surface electrocardiograms (ECGs, lead II) were performed on 3-5 month old mice under isoflurane anesthesia (1.5%, 1L/min) for 5 minutes, as described previously.(Chen et al., 2004) ECGs were taken at baseline and 4 hours after LPS injection. For GDF3 experiments, ECGs were also taken 12 hours after GDF3 injection. ECGs were analyzed

for heart rate (HR), PR interval, SJ, QT and QRS durations using LabChart ver. 8 (ADInstruments, Inc). The QT interval was measured from the Q wave to the end of the T wave (return to the isoelectric line). The SJ interval was measured from the nadir of the S wave to the peak of the J wave.

ELISA

Blood samples from mice were placed at room temperature for 2 hours, then centrifuged at 1500 rpm for 15 minutes at 4°C to separate serum and plasma. ELISA was used to measure circulating IL-1 β (R&D Systems, M6000B) and GDF3 (MyBioSource, MBS722177) according to the manufacturers' instructions.

Luciferase assay

Hek293 cells were plated at a density of 5×10^5 cells per well in DMEM containing 10% fetal bovine serum, penicillin/streptomycin (100 U/ml, Life Technologies) and Normocin™ (100 μ g/ml, InvivoGen) and left to adhere overnight at 37°C. Medium was then replaced with DMEM without FBS, penicillin/streptomycin or Normocin before transfection with 0.25 μ g/ml of the TGF β -responsive SBE4-Luc reporter (Zawel et al., 1998) (Addgene, #16495) in Lipofectamine 3000 (per the manufacturer's protocol). 4 hours after transfection, medium was changed back to complete medium and rmGDF3 was added to each well. 24 hours after transfection, cell lysate was extracted, and luciferase reporter activity measured with a single luciferase reporter assay, as described previously. (R. O. Vilmundarson et al., 2021b) Luciferase activity was normalized to values for the control (0 ng rmGDF3). Experiments were repeated 3 times, and each

treatment was done in triplicate.

BMDM culture

Femurs and tibias from 5-week-old mice were flushed with DMEM (GE Hyclone) and cells were centrifuged at 1400 g for 5 min, re-suspended and cultured in DMEM containing 20% FBS, 15% L929 cell-conditioned medium, penicillin/streptomycin, l-glutamine on non-tissue culture treated plates (BD Falcon) for 6 days, as described previously (H. H. Chen et al., 2015). Conditioned medium was collected from L929 cells cultured for 7 days, centrifuged at 2000g for 5 min and filtered through a 0.45µm low protein binding filter. Medium was changed on the 4th day.

Microarray analysis.

Total RNA from BMDMs was extracted using the Qiagen RNeasy Mini Kit (74104). RNA purity, integrity, and concentration were determined on an Agilent Bioanalyzer. mRNAs were compared using Affymetrix Mouse Gene 1.0ST expression arrays. cDNA synthesis, fragmentation, biotin labeling and screening of the arrays was carried out by StemCore Laboratories of the Ottawa Hospital Research Institute. Differentially expressed genes between male and female BMDM from controls and *Irf2bp2* knockouts were identified by a Bayesian t-test performed using the Cyber-T analysis package for R (Kayala & Baldi, 2012). A Bayesian estimation of the standard deviation for microarray expression data values was used since it is more accurate than the standard estimation employed by Student's t-test, when there are few replicates ($N < 5$) (Baldi & Long, 2001; Murie et al., 2009). The Benjamini and Hochberg (BH) False Discovery Rate (FDR)

method was used to correct for multiple testing (Benjamini & Hochberg, 1995). The significance threshold for the BH corrected p-values was set at 0.05, equal to an FDR of 5%. All microarray raw intensity values and intra-array differences were normalized through log base 2. Log base 2 transformation is essential as microarray data is heavily skewed with a high level of mean-dependent variance of the standard deviation.

Immunofluorescence

Hearts from WT and *LysMCre/Irf2bp2^{flx/flx}* mice were washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) overnight. Hearts were then immersed in 30% sucrose for one night, and 20% sucrose for two nights. The tissue was then washed in PBS and embedded in Tissue-Tek OCT compound and frozen in liquid nitrogen vapor. For immunofluorescence staining, sections (10 μ m) underwent antigen retrieval in citrate buffer (pH 6), followed by blocking with 10% horse serum. Sections were then stained with anti-mouse CD68 antibody overnight at 4°C. The next day, slides were washed in 1X PBS and stained with an anti-rabbit Alexa Fluor 488 secondary antibody at room temperature for 1 hour. Autofluorescence was reduced using the Vector TrueVIEW Autofluorescence Quenching Kit (ThermoFisher) for 5 minutes, followed by counterstaining for DAPI for 10 minutes at room temperature.

For *in vitro* immunofluorescence assays, HL1 cells (a gift from Dr. Peter Liu) were seeded onto glass coverslips in 6-well tissue culture plates at a density of 5×10^5 cells/well and allowed to adhere for 8 hours. Cells were then treated with recombinant GDF3 (50 or 150 ng/ml) for 12 hours after which cells were treated with fluorescently

labeled LPS (1 µg/ml, LPS from Escherichia coli Serotype 055:B5, Alexa Fluor™ 488 Conjugate, Invitrogen L23351) or fluorescently labeled transferrin (1 µg/ml, Transferrin from Human Serum, Alexa Fluor™ 647 Conjugate, Invitrogen T23366). After washing with PBS, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 10 minutes at room temperature then washed with PBS. Cells were then stained for DAPI (5 µg/ml) and coverslips were mounted with Vectashield Vibrance® Antifade mounting medium onto a glass slide. Images were captured with a Zeiss AXIO Imager.Z1 fluorescent microscope with the ZEN 3.8 application and quantified using color thresholding on ImageJ (<https://imagej.net/ij/>).

Cytokine assay

Inflammatory cytokines in serum were measured using the Proteome Profiler Mouse Cytokine Array, Panel A (R&D Systems, ARY006). Blood samples were obtained via cardiac puncture 4 hours after LPS challenge. Samples were left at room temperature for 30 minutes to clot before centrifugation for 15 minutes at 2000 x g to isolate serum. 100 µl of serum was used for each assay according to the manufacturer's protocol.

Membranes were scanned and integrated density for each spot was measured using ImageJ.

Immunoblot analysis

HL1 cells were seeded (as described above) onto tissue culture plates and treated with rmGDF3 (150 ng/ml) for 12 hours prior to incubation with LPS (1 µg/ml) for 2 hours.

HL1 cells were then lysed in RIPA buffer containing protease inhibitors (Roche -

cOmplete™ Protease Inhibitor Tablets, ThermoFisher). Proteins were size fractionated by tris-glycine extended TGX (4-15%) polyacrylamide gel (Bio-Rad) electrophoresis followed by electroblotting to polyvinylidene fluoride (PVDF) membranes (0.2 um) for 1.5 hours. Proteins were visualized with the following antibodies: anti-phospho IRF3 rabbit polyclonal antibody (1:1000; Invitrogen, #PA5-36775), anti-IRF3 rabbit monoclonal antibody (1:5000; Invitrogen, #MA5-32348), anti-alpha-smooth muscle actin mouse monoclonal antibody (1:5000; Sigma, #A2547). The secondary horseradish peroxidase-conjugated antibodies were used at a dilution of 1:10,000: goat anti-mouse IgG antibody (R&D Systems, #HAF007) and goat anti-rabbit IgG (H+L) antibody (Life Technologies, #31460). Bands were revealed by chemiluminescence (Bio-Rad Clarity Max ECL Western Blot Substrate, #1705062) and quantified using ImageJ.

Ventricular myocardial tissue was excised and flash frozen on dry ice prior to protein extraction in RIPA buffer containing protease inhibitors. Proteins were size fractionated and electroblotted as described above. Proteins were visualized with the following antibodies: anti-NCX1 mouse monoclonal antibody (1:1000 in 5% non-fat milk TBST, Invitrogen, #MA3296), anti-NCX1 mouse monoclonal antibody (1:1000 in 5% non-fat milk TBST, Swant, #R3F1), anti-GAPDH (catalogue). The secondary horseradish peroxidase-conjugated antibodies were used at a dilution of 1:10,000: goat anti-mouse IgG antibody (R&D Systems, #HAF007). Bands were revealed by chemiluminescence (Bio-Rad Clarity Max ECL Western Blot Substrate, #1705062) and quantified using ImageJ.

Isolation of adult mouse ventricular myocytes.

Mice (male, 10-week-old, C57/B6, Charles River) were anesthetized by 5% isoflurane, and hearts were collected after a thoracotomy. Single cardiomyocytes were isolated as we described.(Liang et al., 2010) Hearts were perfused on a Langendorff system with a nominally calcium-free Tyrode solution (in mmol/L): 136 NaCl, 5.4 KCl, 0.5 Na₂HPO₄, 10 HEPES, 1 MgCl₂, and 10 glucose; pH adjusted to 7.40 with NaOH) at 37°C for 10 minutes. Collagenase (1.25 mg/ml, type II, Worthington Biochemical Inc., Catalogue No.: LS004177) and protease (0.028 mg/ml, type XIV, Sigma, Catalogue No.: P5147) were added to calcium-free Tyrode solution for 20-30 minutes. Following this, the hearts were perfused with KB solution (in mmol/L: 100 K-glutamate, 10 K-aspartate, 2.5 KCl, 10 KH₂PO₄, 2 MgSO₄, 5 HEPES, 20 glucose, 20 taurine, 5 creatine, 0.5 EGTA, and 0.1% albumin; pH adjusted to 7.2 with NaOH) for 5 minutes. The lower 1/3 of the left ventricle was dissected, minced using surgical scissors, and gently pipetted with a Pasteur pipette to promote the release of single cardiomyocytes. The minced tissue, suspended in KB solution, was passed through a 100-μm filter to remove any remaining large cell clumps and tissue debris.

Patch-clamp recording of transient outward potassium current (I_{to})

The transient outward potassium current (I_{to}) was recorded from isolated mouse ventricular myocytes using the standard whole-cell patch-clamp technique,(Liang et al., 2020; Liang et al., 2010; Lu et al., 2021; Lu et al., 2020) with an Axopatch 200B amplifier and a Digidata 1550A A/D converter. Cells were placed in a recording chamber and allowed to rest for 15–20 minutes before perfusion with an external bath solution containing (in mmol/L): 130 N-methyl-D-glucamine (NMDG), 5 KCl, 1.0 MgCl₂, 1.0

CaCl₂ 10 HEPES, 10 glucose (pH = 7.2 with HCL), (0.3 mM CdCl₂ added to block Ca²⁺ current). For lipopolysaccharide challenge, LPS dissolved in PBS (10 ng/ml) or PBS alone for the control group was added to the external bath solution. The pipette solution contained (in mmol/L): 98 K-glutamate, 50 KCl, 1.0 MgCl₂, 10 HEPES, 10 EGTA-K⁺ (p^H = 7.2 with KOH). Cells were held at -80 mV, and a series of voltage steps from -80 mV to +60 mV in 10 mV increments was applied to elicit transient outward potassium current.

Statistical Analysis

For between-group comparisons, a 2-tailed unpaired Student's *t* test was used. ANOVA was used to analyze groups with two or more variables. For ELISA assays, a 4-Parameter Logistic Curve (4PL) was used to calculate protein concentrations. All statistical tests, including post-hoc comparisons according to software recommendations, were carried out using the Graphpad (ver. 9.0) software and are presented in Table S1.

3.9. Results

3.9.1. LPS prolongs QT in male wild type mice after 2 hours

LPS is commonly used as a model of sepsis that alters cardiac electrical activity and causes myocardial dysfunction. *In vitro* studies in HL1 cardiomyocytes revealed delayed repolarization and prolonged action potential duration as soon as 20 minutes after incubation with LPS (Wondergem et al., 2012). However, the *in vivo* effect of LPS in rodents to delay cardiac repolarization typically have not been recorded until 24-48 hours after LPS challenge (Esfahani et al., 2021; Monnerat-Cahli et al., 2014). Thus, we first set out to determine how quickly LPS causes a delay in cardiac repolarization measured by surface ECG. QT intervals were obtained at baseline and at several time points after a single injection of LPS (10 mg/kg, i.p.) or PBS (control) to male wild type (WT) mice. This LPS dose was chosen to induce a severe acute inflammatory response in mice without mortality (Poon et al., 2003). QT intervals were not corrected for heart rate, as this correction is irrelevant in mice (Mulla et al., 2018). QT interval was unchanged at 30 minutes but prolonged by 2 hours post LPS injection, and further prolonged at 4 and 6 hours (Figure 3.1A). Delays in ventricular repolarization likely account for QT prolongation since no change in heart rate or QRS duration was observed between PBS control and LPS-treated mice (Figure 3.1B, C, Table S2). Further, while isoflurane was used to anesthetize the mice during each ECG recording, repeated exposure to isoflurane anesthesia had no effect on QT, as shown in the PBS-injected control group. Thus, the timeline for LPS-induced QT prolongation we observed suggests that rapid mechanisms are responsible for reducing repolarizing currents.

In a mouse ECG, the J wave is a prominent positive deflection after the QRS complex (see sample traces in Figure 3.1D), that represents early ventricular repolarization (Boukens et al., 2014). However, the SJ segment was not changed following LPS challenge, suggesting that the early component of repolarization is not affected.

3.9.2. Irf2bp2-deficient macrophages protect female mice from LPS-induced

QT prolongation

Given that ablation of *Irf2bp2* in male BMDMs in culture activates a pro-inflammatory M1 phenotype (H. H. Chen et al., 2015), we asked whether *LysMCre/Irf2bp2^{flx/flx}* mice differed from WT mice in their cardiac electrophysiological properties at baseline and in response to LPS. At baseline, no difference in ECG parameters (heart rate, PR, QRS and QT intervals) was observed (Table S2).

To compare the response of WT and *LysMCre/Irf2bp2^{flx/flx}* mice to LPS challenge (10 mg/kg, i.p.), we chose the 4-hour time point. Although all mice were visibly lethargic 4 hours after LPS challenge, their heart rate, PR, or QRS interval duration were not altered by LPS challenge (Table S2). LPS challenge did cause a significant prolongation of QT intervals in WT male and female mice, as well as *LysMCre/Irf2bp2^{flx/flx}* male mice (Figure 3.1D, E). The extent of QT prolongation was surprisingly similar among these mice, indicating that *Irf2bp2* ablation in macrophages in male mice does not worsen their response to LPS.

In contrast, QT was not significantly prolonged in female *LysMCre/Irf2bp2^{flx/flx}* mice after LPS challenge (Figure 3.1D, E). One of the *LysMCre/Irf2bp2^{flx/flx}* females had a

markedly prolonged QT at baseline and was in fact an outlier by Grubbs' test ($\alpha=0.05$). When the outlier was removed, the mixed effects analysis revealed a significant interaction between sex and genotype ($F_{1,27}=6.220$, $p=0.0191$). A pairwise comparison using Tukey's test corrected for multiple testing showed that the change in QT in female *LysMCre/Irf2bp2^{flx/flx}* mice was significantly smaller than in female WT mice ($p=0.0217$), after excluding the outlier. Thus, ablation of *Irf2bp2* in macrophages has a sex-specific effect, protecting female mice from LPS-induced QT prolongation and delayed repolarization. No change in SJ segment duration was observed after LPS challenge in any mice (Figure 3.1G).

3.9.3. Infiltration of CD68+ macrophages is similar in WT and

***LysMCre/Irf2bp2^{flx/flx}* female mice**

To determine whether the protection from LPS-induced QT prolongation in female *LysMCre/Irf2bp2^{flx/flx}* mice could be explained by fewer macrophages infiltrating the myocardium in response to LPS, the ventricular myocardium of female WT and *LysMCre/Irf2bp2^{flx/flx}* mice at baseline and 4 hours after LPS injection was compared by immunofluorescence for CD68, a macrophage marker. Following LPS challenge, macrophage density was similarly elevated in female WT and *LysMCre/Irf2bp2^{flx/flx}* mice (Figure 3.2A and B), indicating that differences in macrophage infiltration do not account for the protective effect.

3.9.4. GDF3 is upregulated in female *LysMCre Irf2bp2^{flx/flx}* mice

To determine what genetic factors could underlie this sex-specific difference, microarrays compared genome-wide mRNA expression in BMDMs from *LysMCre/Irf2bp2^{flx/flx}* mice to BMDMs from age- and sex-matched WT mice. We identified 377 differentially expressed genes in female *LysMCre/Irf2bp2^{flx/flx}* BMDMs, of which 269 were also differentially expressed in male *LysMCre/Irf2bp2^{flx/flx}* BMDMs at a BH $p < 0.05$. A list of the differentially expressed genes in female *LysMCre/Irf2bp2^{flx/flx}* BMDMs is provided (Table S3) and a complete annotation of the microarrays is provided at ArrayExpress through accession number E-MTAB-14166 (www.ebi.ac.uk/biostudies/arrayexpress).

As reported previously, (H. H. Chen et al., 2015) male *LysMCre/Irf2bp2^{flx/flx}* BMDMs show elevated mRNA expression of several inflammatory cytokines at baseline compared to male WT BMDMs, including IL-1 β (Table 2.1), although ELISA assay did not detect a difference in circulating IL-1 β levels (Figure S1). In contrast, compared to female WT BMDMs, only some of these inflammatory cytokine mRNAs were elevated in female *LysMCre/Irf2bp2^{flx/flx}* BMDMs at baseline. Cxcl9 was selectively elevated in female but not male *LysMCre/Irf2bp2^{flx/flx}* BMDMs. However, given that Cxcl9 does not affect repolarization currents in isolated mouse ventricular myocytes (Scicluna et al., 2015), it is unlikely to account for the protective effect in female *LysMCre/Irf2bp2^{flx/flx}* mice from LPS-induced QT prolongation.

The most plausible candidate is growth differentiation factor 3 (GDF3) that was selectively elevated in female but not male *LysMCre/Irf2bp2^{flx/flx}* BMDM compared to WT controls (Table 2.1). GDF3 is a member of the TGF β family of growth factors that is produced and secreted only by macrophages (Bu et al., 2018; Varga et al., 2016). GDF3 protects the heart from contractile dysfunction in response to LPS challenge (L. Wang et al., 2020) and could account for the sex-specific protective effect of *Irf2bp2* ablation in macrophages. Consistent with our microarray results, female *LysMCre/Irf2bp2^{flx/flx}* mice had higher serum levels of GDF3 measured by ELISA compared to males or WT mice of both sexes (Figure 3.3A).

3.9.5. GDF3 reduces LPS-induced QT prolongation in WT mice

To test whether GDF3 prevents prolongation of the QT interval after LPS challenge, recombinant mouse GDF3 (rmGDF3) was administered to WT male mice 12 hours prior to LPS challenge. To this end, we first verified the biological activity of rmGDF3. Since GDF3 is a member of the TGF β family of ligands and signals through SMAD2/3, different doses of rmGDF3 were tested on a luciferase reporter containing the Smad binding element (SBE4-luc) (Zawel et al., 1998). At 10 ng, GDF3 caused a 10-fold induction of this reporter (Figure 3.3B).

Next, we asked whether rmGDF3 (10 μ g/kg, i.p., as used in a prior study(L. Wang et al., 2020)) could raise circulating GDF3 levels in male mice, where endogenous levels are relatively low. Indeed, circulating levels of GDF3 reached similar levels in male mice 6 hours after rmGDF3 injection as those observed in female *LysMCre/Irf2bp2^{flx/flx}* mice at

baseline. By 12 hours, serum GDF3 levels declined to half of the peak levels observed at 6 hours and returned to nearly baseline levels by 24 hours (Figure 3.3C), indicating a half-life of 11.5 hours. These results suggest that GDF3 is actively produced in female *LysMCre/Irf2bp2^{flx/flx}* mice.

Finally, rmGDF3 or BSA control (10 µg/kg, i.p.) was injected to male WT mice 12 hours prior to LPS challenge. rmGDF3 pre-treatment markedly attenuated QT prolongation measured 4 hours after LPS challenge, compared to controls (Figure 3D, Table S4), without affecting QRS or SJ segment duration (Figure 3.3E, F). Of note, rmGDF3 pre-treatment did not affect any cardiac ECG parameter at baseline (Table S4).

3.9.6. LPS has no effect on potassium currents in ventricular cardiomyocytes

The effects of LPS on cardiac repolarization in mice have typically been attributed to disturbances to the potassium currents, namely I_{to} . However, our ECG data showed that the SJ-segment was not prolonged by LPS, suggesting that I_{to} is not affected in these mice. To verify this assessment, we next looked at the overall potassium currents via patch clamp recording at baseline and 4 hours after LPS (10 ng/ml) incubation in mouse ventricular cardiomyocytes (Figure 3.4A). Indeed, we found no change in the current voltage relationships for I_{to} total (Figure 3.4B), I_{to} fast (Figure 3.4C) and I_{to} slow (Figure 3.4D) following LPS challenge.

3.9.7. GDF3 blocks LPS-induced upregulation of NCX

The effects of LPS on I_K cannot explain the QT prolongation observed *in vivo*. Therefore, we next sought to measure other ion channels that contribute to repolarization. In particular, since transgenic mice with cardiomyocyte-specific overexpression of NCX1 have prolongation of the QT interval (Pott et al., 2012), and since perfusion of rat hearts with LPS increases NCX1 protein levels after just 3 hours (Magi et al., 2015), we focused on NCX1. Expression of NCX1 in the mouse ventricular myocardium was examined by immunoblots and showed a several-fold increase 4 hours after mice were treated with LPS (Figure 3.5). We also detected bands at 70 and 160 kDa, representing the proteolytic fragment and non-reduced form of the exchanger (Saba et al., 1999), which were also increased in mice receiving LPS. This increase was suppressed by pre-treating mice with rmGDF3. Thus, over-expression of Ncx1 is likely to account for the delay in cardiac repolarization induced by LPS.

3.9.8. GDF3 does not affect the LPS-induced activation of circulating inflammatory cytokines in mice

To test whether GDF3 pre-treatment attenuates inflammatory cytokine production *in vivo*, male WT mice were treated with rmGDF3 or BSA (10 μ g/kg) for 12 hours prior to LPS challenge (10 mg/kg) for 4 hours. Serum samples were then obtained via cardiac puncture and cytokine levels analyzed by a dot blot assay (Figure 3.6A and B). The multiplex arrays detected 12 cytokines induced by LPS challenge and their expression levels were not different with or without rmGDF3 pre-treatment. Thus, the protective effects of GDF3 against LPS-induced QTc prolongation is not due to lowered production of these cytokines

by macrophages and monocytes, at least at this time point. Importantly, the array measures cytokines that are predominantly Myd88-dependent (G-CSF, IL-1RA, IL-6, CXCL1, M-CSF, CXCL9, MIP-1 β , MIP-2), TRIF-dependent (BLC, CXCL10, RANTES) or dependent on both (TIMP-1). Thus, 4 hours after LPS challenge, neither the MyD88 pathway nor the TRIF pathways of macrophages and monocytes in vivo appear to be affected by GDF3 pre-treatment. Of note, levels of TNF- α and IL-1 β were below the threshold of detection and might have returned to baseline by 4 hours.

3.9.9. GDF3 blocks LPS-induced phosphorylation of IRF3 in HL1 cells

In rat cardiomyocytes, the TRIF/IRF3 pathway has been implicated in QT prolongation 24 hours after TLR4 (Monnerat-Cahli et al., 2014). This prompted us to ask whether GDF3 might block the TRIF/IRF3 pathway in cardiomyocytes and LPS-induced QT prolongation. LPS challenge for 2 hours activated the TRIF/IRF3 phosphorylation pathway in mouse HL1 cells. Pre-treating HL1 cells with rmGDF3 for 12 hours blocked LPS-induced phosphorylation of IRF3 (Figure 3.6C). Thus, our data support the notion that QT prolongation resulting from LPS/TLR4 signaling via the TRIF/IRF3 pathway can be blocked by GDF3.

3.9.10. GDF3 blocks TLR4 uptake of LPS in cardiomyocytes

Activation of TRIF/IRF3 signaling pathway occurs after endocytosis of LPS with TLR4 (Ciesielska et al., 2021). Thus, we postulated whether the protective effect of GDF3 on LPS-induced QTc prolongation might occur at the level of TLR4 endocytosis, the essential

step of TRIF/IRF3 activation. To test this, fluorescently labeled LPS (1 $\mu\text{g/ml}$) was used to track endocytosis of TLR4 in HL1 cells and neonatal rat cardiomyocytes. LPS was readily taken up by HL1 cells within 2 hours, whereas in cells pretreated with rmGDF3 (50 and 150 ng/ml) for 12 hours, uptake of LPS was inhibited by GDF3 in a dose-dependent manner (Figure 3.6D). In contrast, GDF3 did not prevent uptake of fluorescently labeled transferrin in HL1 cells (1 $\mu\text{g/ml}$, Figure 3.6E). A similar effect of rmGDF3 to block TLR4-mediated LPS endocytosis was observed in neonatal rat cardiomyocytes (Figure 3.6F). Uptake of transferrin involves transferrin receptors and endocytosis machinery that differs from the one used for LPS/TLR4 (Ciesielska et al., 2021; Mayle et al., 2012). Thus, our results suggest that GDF3 selectively blocks TLR4 endocytosis to inhibit TRIF/IRF3 activation in cardiomyocytes.

3.10. Discussion

Inflammation can impair cardiac function by delaying repolarization and prolonging the QTc interval, a condition that predisposes to ventricular arrhythmias and sudden death (Viskin, 1999). While LPS are known to cause QT prolongation (Adu-Amankwaah et al., 2024; Chen et al., 2024; Esfahani et al., 2021; Katare et al., 2020; Q. Liu et al., 2023; Ying et al., 2023) and action potential duration (APD) prolongation (Q. Liu et al., 2023; Wondergem et al., 2012) in murine models, the mechanisms behind this observation are not well understood. Furthermore, while most studies have measured QT prolongation after at least 12 hours of LPS challenge, our data shows that onset of QT prolongation occurs in as little as 2 hours after exposure to LPS. In humans, QT prolongation is often associated with reduced I_{Kr} that is carried through the hERG potassium channel. While I_{Kr} is not a major component of ventricular repolarization in adult mice (Xu et al., 1999), LPS treatment was reported to rapidly (within 20 minutes) reduce the I_{Kr} currents carried by the mouse ERG channels in mouse HL1 cardiomyocytes (Wondergem et al., 2012; Wondergem et al., 2010). The early phase of cardiac repolarization in mice relies predominantly on the transient outward current (I_{to}) carried by Kv4.2/4.3 channels (Niwa & Nerbonne, 2010). However, surface ECG did not reveal an increase in the ST segment 4 hours after LPS challenge and patch-clamp recording of isolated ventricular myocytes also showed no change in I_{to} .

Instead, repolarization of the murine action potential contains a drawn-out phase around -40 mV, and at this membrane potential the Kv4.2/4.3 currents are largely inactive (Knollmann et al., 2007). This plateau phase in murine ventricular cardiomyocytes has been attributed to the activity of the Na^+/Ca^{2+} exchanger (NCX) (duBell et al., 1991; Knollmann et

al., 2003; Schouten & ter Keurs, 1985), which produces an electrogenic current by moving 3 Na⁺ ions into the cell for each Ca²⁺ ion moved out of the cell in the forward state (Ottolia et al., 2013). Indeed, transgenic mice overexpressing NCX in the myocardium have a prolonged QT interval and a concomitant increase in I_{to}. Our immunoblot data shows that mice exposed to LPS for 4 hours experience a rapid increase in the levels of NCX detected in the ventricular myocardium at the time where QT interval is prolonged, consistent with a prior report in rats (Magi et al., 2015). In terms of NCX upregulation, studies in microglia revealed a threefold increase in NCX1 mRNA in response to IFN- γ after 5 hours, associated with an increase in NCX activity measured by radioactive calcium uptake (Nagano et al., 2004). While post-translational modifications might regulate NCX1 function, they would be unlikely to account for the rapid elevation in protein levels we observed. A plausible mechanism for this rapid (4-hour) accumulation of NCX1 protein is an increase in protein stability or a transient reduction in protein degradation pathways, such as the ubiquitin-proteasome system. An intriguing possibility remains that LPS signaling releases the NCX1 mRNA from a state of translational repression due to the presence of a 5' open reading frame and stop codon prior to the true initiation codon, as has been reported for HER2 (Mehta et al., 2006). Finally, our data show that incubation of mouse ventricular myocytes with LPS for 4 hours had no effect on the overall potassium current, contrary to previous reports that examined these currents 24 hours after LPS challenge. Thus, the prolonged depolarization is likely due to the upregulation of NCX1 and the increased NCX current, as was observed in transgenic mice with cardiac-specific over-expression of NCX1 (Pott et al., 2012). A limitation of our study is that we were unable to directly measure the NCX current.

Given that myeloid lineage-specific deletion of *Irf2bp2*, a key regulator of inflammation, confers an inflammatory phenotype to cultured macrophages (H. H. Chen et al., 2015), we expected that these mice would have a worse cardiac electrophysiological response to LPS challenge and show more pronounced delays in cardiac repolarization. Instead, we discovered an unexpected sex-specific protective effect of *Irf2bp2* ablation in macrophages. Our findings are summarized here: 1> *LysMCre/Irf2bp2^{flx/flx}* mice of both sexes have normal QT interval at baseline. 2> Male *LysMCre/Irf2bp2^{flx/flx}* mice did not have a worse QT prolongation after LPS exposure compared to WT mice (both sexes). 3> Female *LysMCre/Irf2bp2^{flx/flx}* were protected from LPS-induced QT prolongation. 4> Female *LysMCre/Irf2bp2^{flx/flx}* mice have increased levels of circulating GDF3. 5> Intraperitoneal injection of recombinant GDF3 protein into WT male mice was sufficient to reduce by half the severity of their LPS-induced QT prolongation at 4 hours.

As for the mechanisms whereby GDF3 confers this cardiac protective effect, we found that systemic administration of GDF3 did not attenuate the inflammatory cytokine storm after LPS challenge, suggesting that the protective effects are not mediated macrophages (neither MyD88-dependent or TRIF-dependent circulating cytokines). Instead, pre-treatment of HL1 cells and neonatal cardiomyocytes with recombinant GDF3 blocked the endocytosis of LPS by TLR4 and the downstream phosphorylation of IRF3. Together, our findings are consistent with GDF3 suppressing LPS-induced QT prolongation by inhibiting TRIF-dependent TLR4 signaling and NCX1 activation in cardiomyocytes.

GDF3 is a signaling molecule in the TGF β superfamily that is critical in early embryonic development, influencing Nodal signaling and left-right patterning (Pelliccia et al., 2017). Several studies identified GDF3 as a macrophage-specific secreted factor in adults, with implications in skeletal muscle regeneration (Patsalos et al., 2018; Varga et al., 2016) and fat metabolism controlled by adipose tissue macrophages (Bu et al., 2018). Acute injection of rmGDF3 was reported to protect mice from LPS-induced cardiac contractile dysfunction (L. Wang et al., 2020). The cardioprotective effects of GDF3 were associated with reduced inflammatory macrophage polarization and infiltration to the myocardium 12 hours after LPS challenge. Further, these authors reported that GDF3 reduced cytokine secretion from cultured macrophages 12 hours after LPS challenge (L. Wang et al., 2020). Using the same treatment regime of rmGDF3 (10 μ g/kg, 12 hours prior to LPS), a GDF3-ascribed protective effect on QT prolongation was evident 4 hours after LPS challenge, without affecting inflammatory cytokine activation. Along these lines, female *LysMCre/Irf2bp2^{flx/flx}* mice have higher levels of circulating endogenous GDF3 and are resistant to LPS-induced QT prolongation, yet they have a similar density of macrophages in their myocardium as in female WT mice that are susceptible to LPS-induced QT prolongation. Thus, while GDF3 may have an autocrine (macrophage-to-macrophage) effect, our data suggest a paracrine effect of GDF3 from macrophages onto cardiomyocytes. It is important to note that GDF3 signals through both the ALK4 (ACVR1B) (Andersson et al., 2007) and ALK7 (ACVR1C) receptors (Andersson et al., 2008), and mice with cardiomyocyte-specific ablation of ALK7 develop severe QT prolongation (Ying et al., 2016). Whether this occurs from NCX upregulation remains to be determined. It is important to note that our published microarray data show that macrophages express high levels of

ALK4 but not ALK7 (H. H. Chen et al., 2015) and suggests that GDF3 might not affect macrophages in the same way as cardiomyocytes.

The mechanism whereby GDF3 prevents QT prolongation is an exciting new area of study. Activation of TLR4 by LPS sets into motion two distinct and sequential signaling cascades: first is the MyD88 pathway, which results in the nuclear translocation of NF- κ B and expression of pro-inflammatory cytokines, and then the TRIF/IRF3 pathway, which is activated subsequent to endocytosis of TLR4 and leads to the expression of type I IFN genes and a number of chemokines (Ciesielska et al., 2021). Based on our cytokine assay at 4 hours, neither pathway appears to be affected by GDF3 in macrophages *in vivo*, although we cannot discount a possible short-term effect on TNF α and IL-1 β that were not detected in the assay. Instead, our data suggest that GDF3 inhibits the endocytosis of LPS and TLR4 into HL1 cells and rat cardiomyocytes. This effect is specific to TLR4, as GDF3 did not block uptake of transferrin in HL1 cells by transferrin receptors targeted by Rab11-dependent endosome recycling (Lindsay & McCaffrey, 2002; Mayle et al., 2012). It is worth noting that Rab5 mediates endocytosis of LPS with its TLR4 receptor (Ciesielska et al., 2021). Whether GDF3 acts as a general inhibitor of Rab5-mediated early endosomal internalization and protein degradation or is specific to LPS/TLR4 remains to be tested.

Is there a therapeutic potential for GDF3? rmGDF3 raises circulating GDF3 levels up to 12 hours after administration, before serum levels return to baseline by 24 hours. While rmGDF3 is readily cleared from the circulation, with a half-life of ~12 hours, it may still be advantageous as a pre-operative intervention to attenuate QT prolongation caused by

endotoxemia that often occurs after cardiopulmonary bypass (Kertai et al., 2016; Klein et al., 2011).

Like all members of the TGF β family of growth factors, GDF3 is only active when cleaved by proprotein convertases in the circulation (Dubois et al., 2001). Of note, the ELISA assay we used cannot distinguish between the un-cleaved inactive form of GDF3 versus the cleaved active form. The rmGDF3 that we used is the cleaved mature form (Ala253-Gly366). Given that ELISA assays showed circulating GDF3 levels after rmGDF3 treatment achieved similar levels as the endogenous GDF3 in female *LysMCre/Irf2bp2^{flx/flx}* mice that are protected from QT prolongation and were sufficient to inhibit LPS-induced QT prolongation in WT mice, these observations suggest that female *LysMCre/Irf2bp2^{flx/flx}* mice constitutively produce elevated levels of a biologically active form of GDF3.

Intriguingly, serum GDF3 is elevated in human patients with sepsis and levels are highest in cases of severe sepsis (L. Wang et al., 2020). However, it is unclear whether these elevated levels of GDF3 represent the biologically active or inactive form. Since only 23% of septicemic patients develop prolongation of the QTc interval (Liu et al., 2024), perhaps patients who are unable to produce enough of the active form are susceptible to QTc prolongation. Along these lines, human plasma GDF3 levels are highly correlated to triglyceride levels (Feringstad et al., 2021) and elevated triglyceride levels have been correlated with endotoxemia (Maatta et al., 2021) and poor outcomes in sepsis (Cetinkaya et al., 2014). Our study shows that while rmGDF3 does not prevent the cytokine storm, it reduces the impact of LPS to delay cardiac repolarization by half. This may reflect an effect

of GDF3 to attenuate the response of cardiac myocytes to inflammatory cytokines, a possibility we have not addressed here. Of note, LPS is known to increase insulin release, creating a hypoglycemic and hypokalemic environment and these systemic effects can contribute to prolongation of the QT interval (Kacheva et al., 2017; Nguyen et al., 2014; Wang et al., 2005). Whether recombinant GDF3 affects LPS-induced insulin release is unknown. Nonetheless, our study points to a rapid and marked upregulation of NCX coincident with the observed QT prolongation by LPS. If recombinant GDF3 can similarly prevent this upregulation in humans, it may have therapeutic application against sepsis-induced QTc prolongation.

Table 2.1 Differentially expressed genes in male and female BMDM of WT and lysMCre/Irf2bp2^{flx/flx} mice

Male					Female				
Gene	KO/WT	WT avg ± SD	KO avg ± SD	BH	Gene	KO/WT	WT avg ± SD	KO avg ± SD	BH
Irf2bp2	0.1	631 ± 25	94 ± 9	0	Irf2bp2	0.2	437 ± 124	83 ± 20	2.47E-08
<i>Female-specific transcripts</i>									
GDF3	0.8	90 ± 22	69 ± 5	2.98E-01	GDF3	1.7	88 ± 11	152 ± 8.6	3.59E-04
Ifi44	0.9	2792 ± 534	2651 ± 616	9.99E-01	Ifi44	0.7	2312 ± 146	1545 ± 442	3.79E-03
Cxcl9	1.0	25 ± 3	25 ± 3	0.99	Cxcl9	1.9	29 ± 4	57 ± 18	6.48E-03
snora73a	1.3	307 ± 26	385 ± 35	3.38E-01	snora73a	0.7	225 ± 18	167 ± 30	3.80E-02
<i>Inflammatory cytokines</i>									
Il1a	0.6	187 ± 6	105 ± 6	8.37E-05	Il1a	1.2	141 ± 39	170 ± 160	9.91E-01
Il1b	3.2	140 ± 12	454 ± 16	5.73E-08	Il1b	2.8	205 ± 61	566 ± 344	6.37E-03
Tnf	1.3	1336 ± 102	1788 ± 103	3.32E-04	Tnf	1.4	1585 ± 185	2275 ± 845	1.29E-01
Ccl2 (MCP-1)	1.8	1104 ± 105	1994 ± 360	6.00E-06	Ccl2 (MCP-1)	1.4	1326 ± 202	1809 ± 468	1.81E-01
Ccl3 (MIP-1α)	1.1	4203 ± 82	4772 ± 56	1.79E-04	Ccl3 (MIP-1α)	1.1	3591 ± 129	3819 ± 232	7.00E-02
Ccl4 (MIP-1β)	1.9	354 ± 44	670 ± 35	5.74E-04	Ccl4 (MIP-1β)	1.8	430 ± 79	789 ± 432	5.87E-01
Ccl7	2.2	553 ± 118	1223 ± 179	5.70E-03	Ccl7	1.7	810 ± 273	1352 ± 473	3.81E-02
Ccl8	2.6	83 ± 23	220 ± 38	6.64E-06	Ccl8	1.8	105 ± 57	184 ± 74	1.37E-01
Ccl12	3.0	52 ± 9	155 ± 20	1.61E-07	Ccl12	1.9	77 ± 20	145 ± 83	2.59E-02

BH, Benjamini-Hochberg p values. Data in grey are not significant (n = 3 mice/sex/genotype).

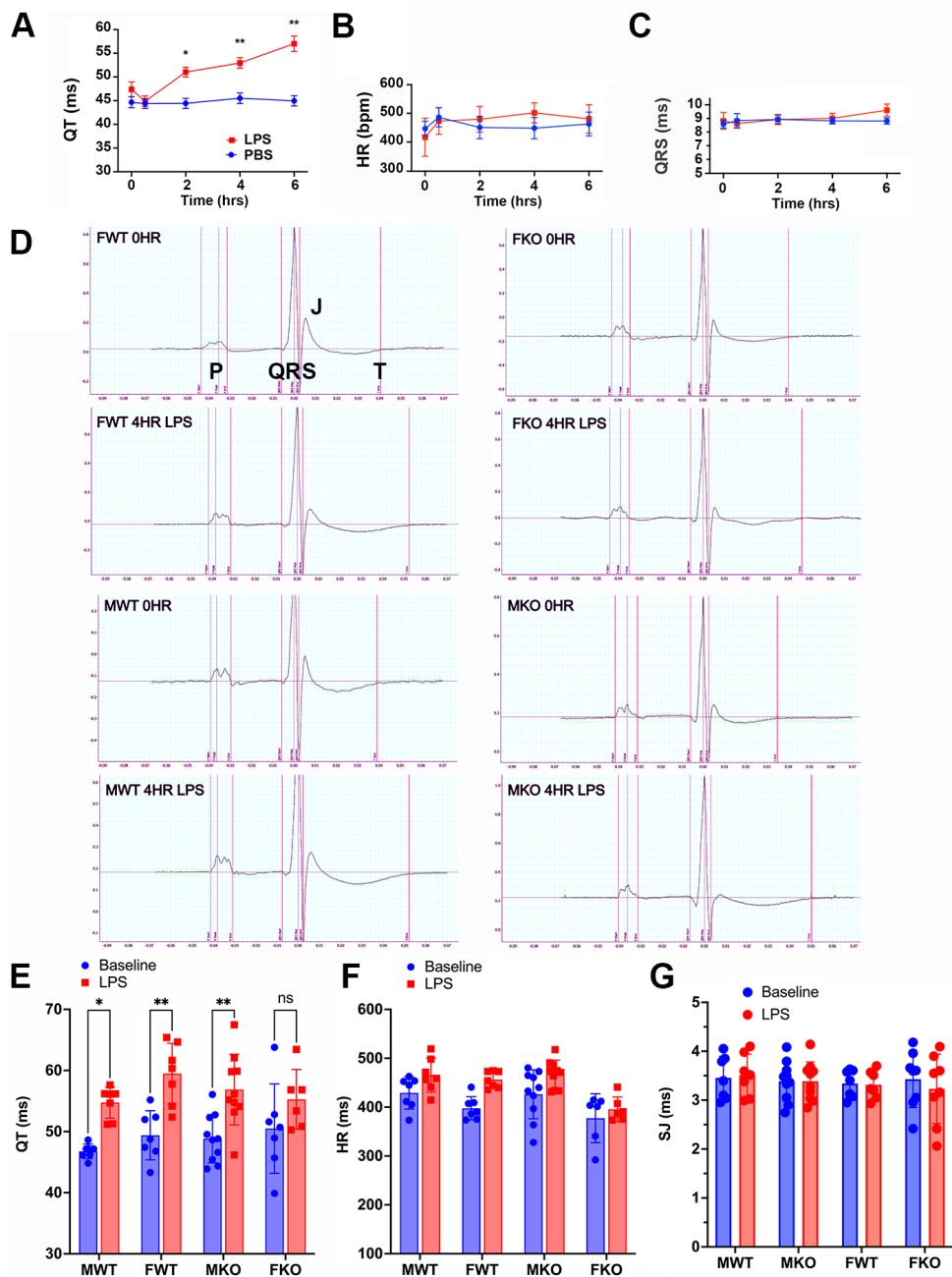


Figure 3.1 Female LysMCre/Irf2bp2^{flx/flx} (KO) mice are protected from QT prolongation following LPS challenge. ECGs were recorded in male WT mice (aged 4-6 months) at t = 0, 0.5, 2, 4 and 6 hours post LPS challenge (10 mg/kg, i.p.) and compared to PBS controls. (A) QT interval, (B) heart rate (HR) and (C) QRS duration were measured and data shown are means $\pm$ SD (n = 5 per group). Repeated measures two-way ANOVA revealed a significant effect of time ($F_{(2,39, 19.12)} = 20.86, p < 0.0001$) and treatment ($F_{1, 8} = 22.58, p < 0.005$) and a significant interaction ($F_{(4,32)} = 19.66, p <$

0.0001). Post-hoc pairwise comparisons to hour 0 were by Sidak's test (*, $p < 0.05$, **, $p < 0.005$). Similarly, male and female WT and *LysMCre/Irf2bp2^{flx/flx}* mice aged 3-5 months were challenged with LPS and ECGs were recorded at baseline or at 4 hours following LPS injection. (D) Sample traces are shown signal-averaged over 5 beats. (E) QT, (F) HR and (G) SJ are presented as means $\pm$ SD (n = 7 per group). Two-way ANOVA revealed a significant effect of LPS treatment on QT ($F_{(1,52)} = 61.39$, $p < 0.0001$), but no effect of genotype ($F_{(3, 52)} = 1.873$, $p = 0.1455$). Post-hoc comparisons to baseline were by Sidak's test (**, $p < 0.01$, ****, $p < 0.0001$)

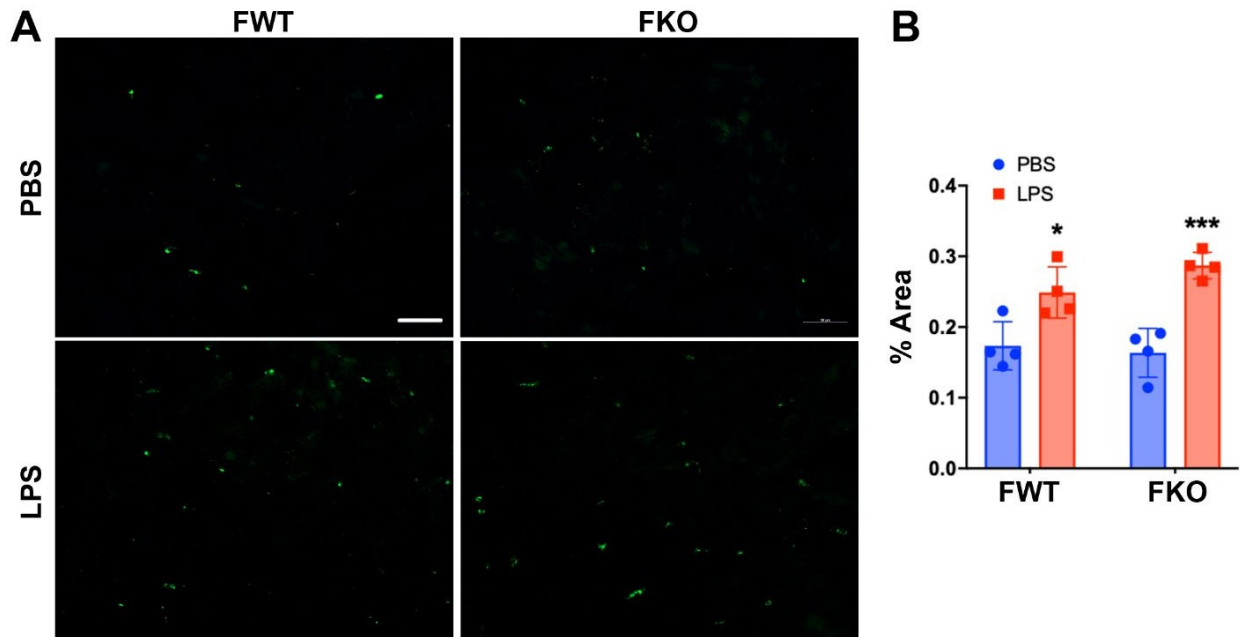


Figure 3.2 Similar infiltration of CD68 cells in the myocardium of female wild type and *LysMCre/Irf2bp2^{flx/flx}* (KO) mice following LPS.

Female mice (aged 3-5 months) were challenged with LPS (10 mg/kg, i.p.) or PBS (100 μ l) and 4 hours after injection, ventricular myocardial tissue was harvested for immunofluorescence staining of CD68+ cells (A) and signals were quantified as percent CD68+ area (B). Values were normalized by arcsine transformation and presented as mean $\pm$ SD (n = 4 per group, scale bar = 100 μ m). Two-way ANOVA revealed a significant effect of LPS treatment ($F_{(1,12)} = 39.35$, $p < 0.0001$), but no effect of genotype ($F_{(1,12)} = 0.7866$, $p = 0.3926$). Post-hoc pairwise comparisons to PBS were by Tukey's test (*, $p < 0.05$, ***, $p < 0.001$).

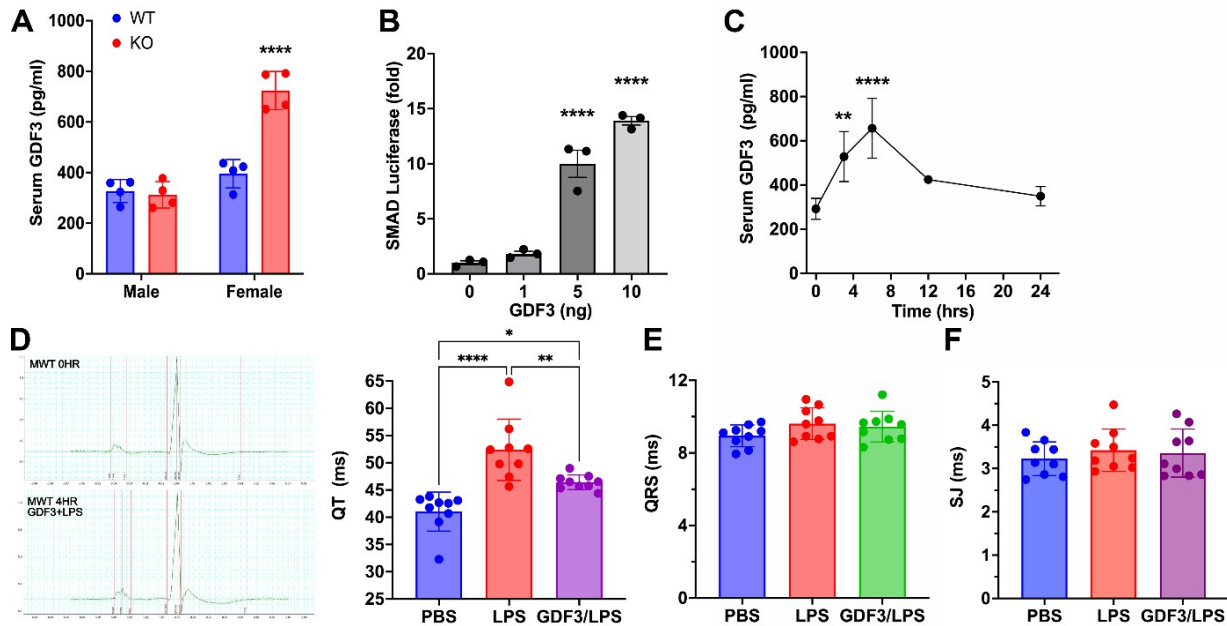


Figure 3.3 Circulating GDF3 is elevated in female KO mice and protects male WT mice from LPS- induced QT prolongation. (A) Serum GDF3 levels in male (M) and female (F) WT and *LysMCre/Irf2bp2^{flx/flx}* (KO) mice (aged 3-6 months) were measured (n = 4). Two-way ANOVA showed a significant effect of sex ($F_{(1,12)} = 67.59, p < 0.0001$) and genotype ($F_{(1,12)} = 28.84, p < 0.001$), and a significant interaction ($F_{(1,12)} = 34.56, p < 0.0001$). Pairwise comparisons to WT using Tukey's test identified significant differences between genotypes (****, $p < 0.0001$). (B) Recombinant mouse GDF3 (rmGDF3) activates a Smad-binding element luciferase reporter in HEK293 cells. One-way ANOVA showed a significant effect of rmGDF3 to activate SMAD luciferase activity ($F_{(3,8)} = 90.32, p < 0.0001$) and pairwise comparisons to negative control using Tukey's test identified significant doses at 5 and 10 ng (****, $p < 0.0001$). (C) IP injection of rmGDF3 (10 μ g/kg) elevates serum GDF3 levels in male mice (aged 3-5 months) as measured by ELISA at 0, 3, 6, 12 and 24 hours after injection. One-way ANOVA showed a significant effect of rmGDF3 to elevate plasma GDF3 ($F_{(4, 15)} = 11.95, p = 0.0001$) and pairwise comparisons to baseline identified significantly elevated values using Dunnett's test (n = 4 mice/time point, **, $p < 0.01$; ****, $p < 0.0001$). (D) Administration of rmGDF3 (10 μ g/kg) to male WT mice 12 hours prior to LPS challenge attenuated QT prolongation 4 hours after LPS challenge. Mice were given BSA (10 μ g/kg) 12 hours

prior to LPS challenge as a control. One-way ANOVA identified a significant effect of treatment ($F_{(2,24)} = 54.37, p < 0.0001$, $n = 9$ per group) and post-hoc pairwise comparisons by Tukey's test identified significant differences between PBS, LPS alone and GDF3/LPS (****, $p < 0.0001$). (E) QRS or (F) SJ segment durations were not affected by LPS or GDF3 administration.

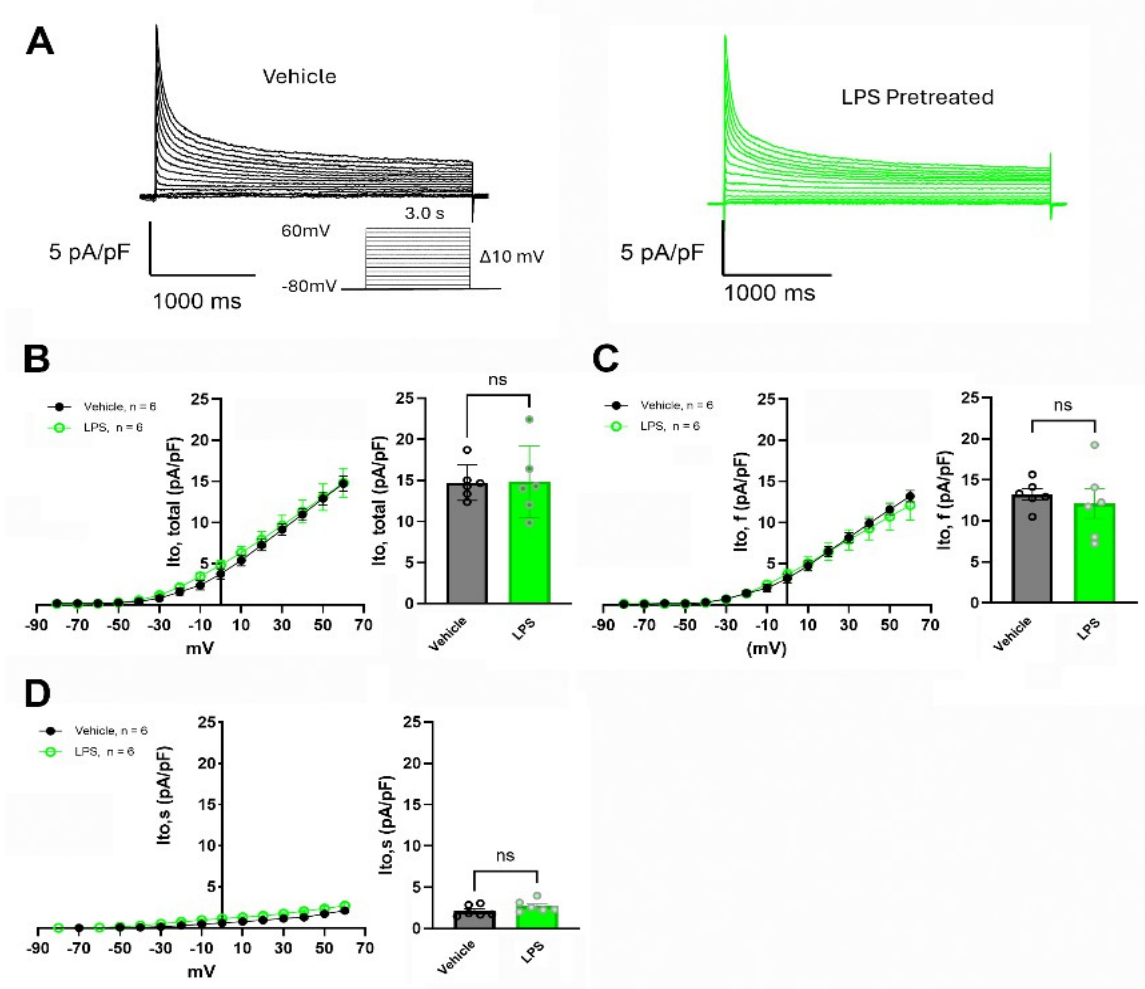


Figure 3.4 LPS has no effect on I_{to} currents in mouse ventricular myocytes.

(A) Representative I_{to} current traces recorded from isolated mouse ventricular myocytes (inset shows the voltage-clamp protocol) without LPS (black traces) or 4 hours after LPS is added (1 μ g/mL) to the external bath solution (green traces). (B-D) Current-voltage (I - V) relationships of total I_{to} , (C) $I_{to, f}$, and (D) $I_{to, s}$ current densities, respectively, in isolated mouse ventricular myocytes without (vehicle, black, $n = 6$) or with LPS added to the external bath solution (1 μ g/mL, green, $n = 5$). Summary data of total I_{to} , $I_{to, f}$, and $I_{to, s}$ current densities at +60 mV in myocytes treated without (vehicle, black, $n = 6$) or with LPS (1 μ g/mL, green, $n = 5$). ns, non-significant by two-tailed Student's t -test.

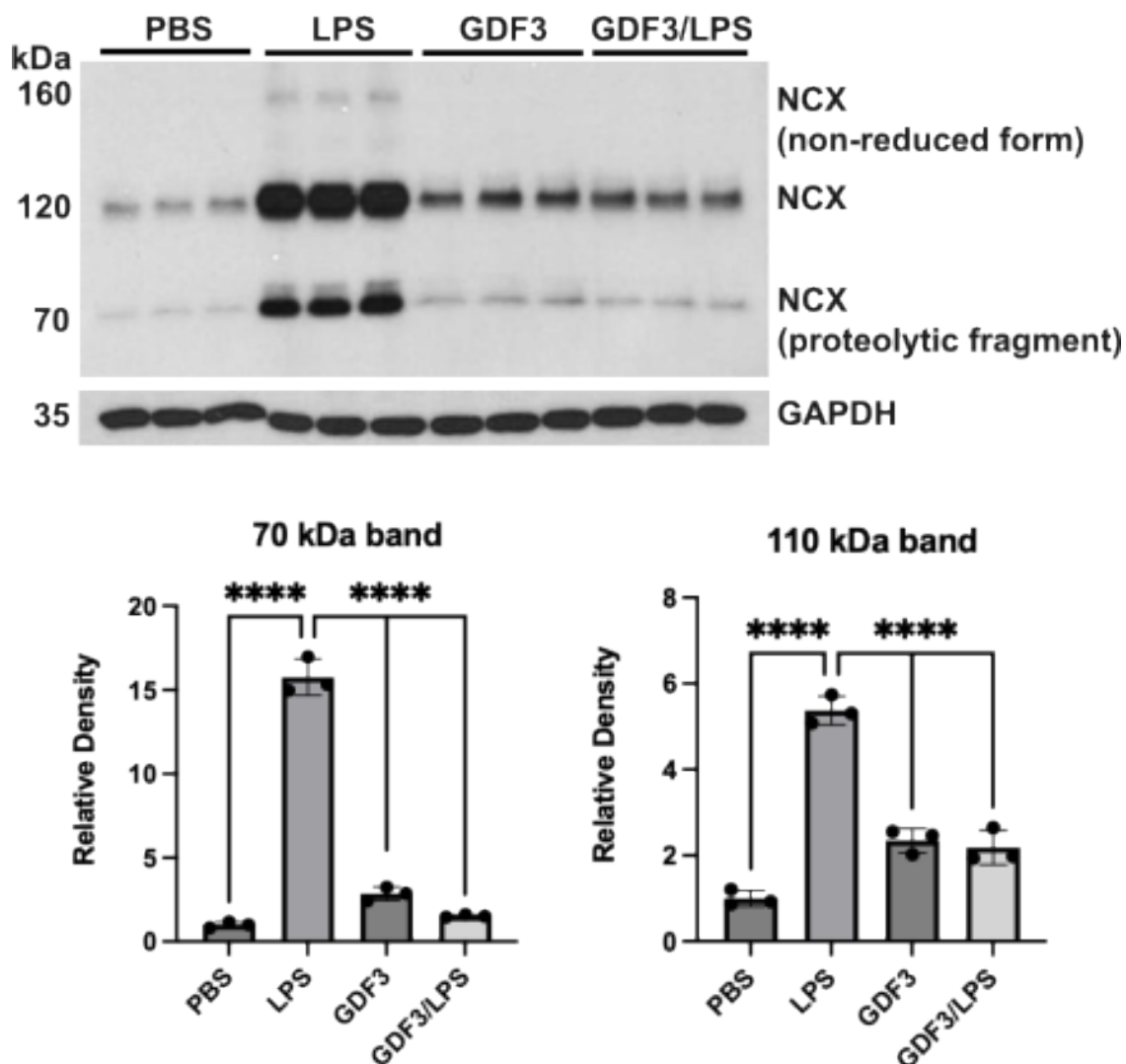


Figure 3.5 LPS activates NCX1 expression in mouse myocardium and rmGDF3 pretreatment blocks this activation. (A) Representative immunoblot of proteins from mouse myocardium reveal a marked upregulation of NCX1 in mice treated with LPS (10 mg/kg) for 4 hours. Mice pretreated with GDF3 12 hours prior to LPS challenge did not upregulate NCX1 (n=3 mice/treatment) after LPS challenge. GAPDH was used as a loading control. (B) Quantitation of immunoblots shows a marked upregulation of NCX1 (Two-way ANOVA, $p < 0.0005$ by Tukey's test).

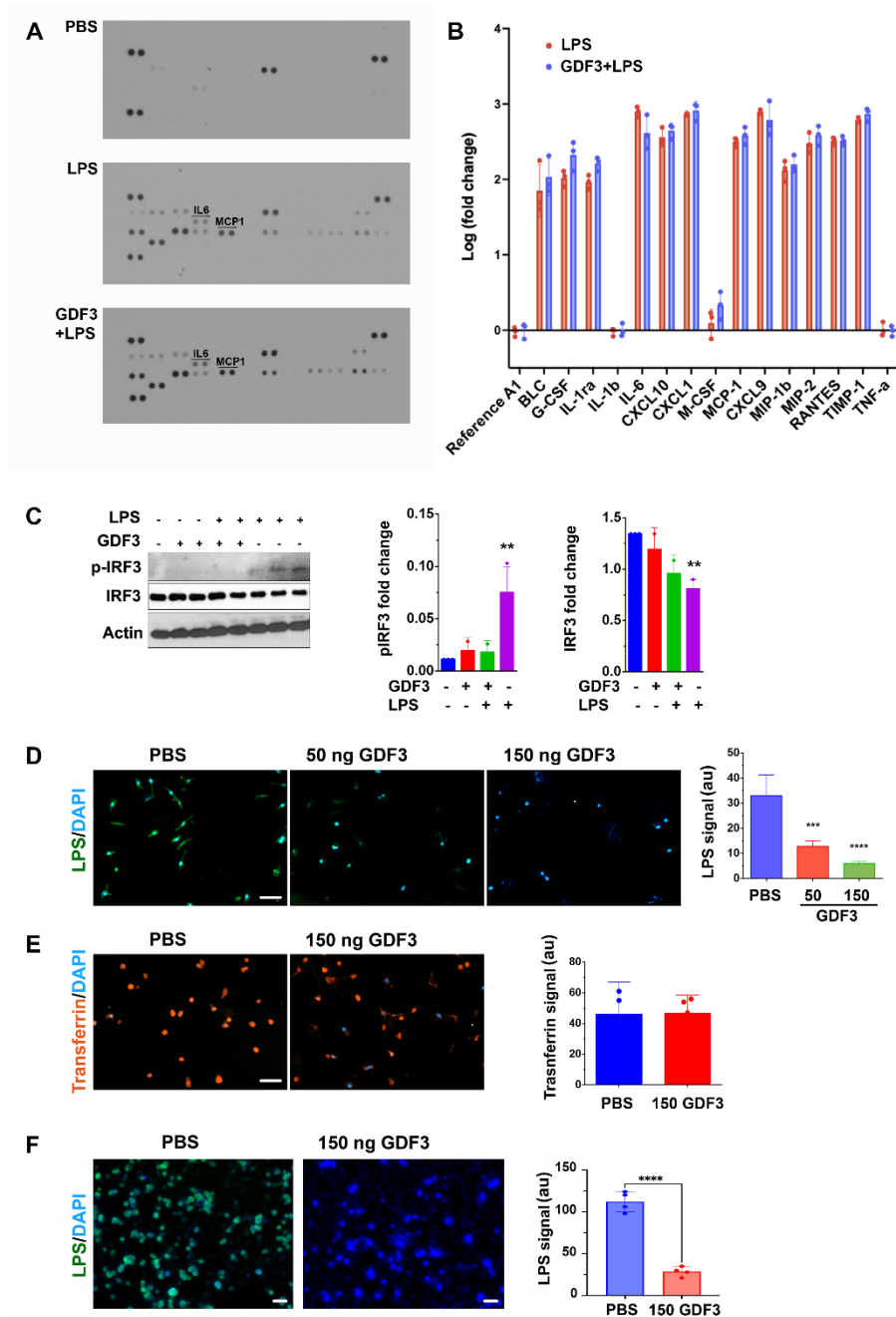


Figure 3.6 GDF3 blocks LPS endocytosis and endocytosis-dependent TRIF signaling in HL1 cells but does not block inflammatory cytokine activation. (A) The LPS-induced surge in circulating inflammatory cytokines that are Myd88-dependent (G-CSF, IL-1RA, IL-6, CXCL1, M-CSF, CXCL9, MIP-1 β , MIP-2), TRIF-dependent (BLC, CXCL10, RANTES) or dependent on both (TIMP-1) myd88- and TRIF was measured by a cytokine dot blot assay of blood from male wild type mice (aged 3 months) pretreated with rmGDF3 (10 μ g/kg) or BSA for 12 hours prior to exposure to LPS (10 mg/kg) for 4

hours. (B) Blots were quantified by densitometry, expressed as log (fold change) $\pm$ SD and data analyzed by t-test ($n = 3$ per group). (C) HL1 cells were treated with rmGDF3 (150 ng/ml) for 12 hours prior to incubation with LPS (1 μ g/ml). phospho-IRF3 and IRF3 were quantified by immunoblot of 30 μ g total protein by densitometry. Fold changes in protein expression were normalized to actin and data are presented as means $\pm$ SD. One-way ANOVA revealed a significant effect of treatment for pIRF3 ($F_{(3,6)} = 10.49, p < 0.01$) and also IRF3 ($F_{(3,6)} = 10.58, p < 0.01$). Post-hoc comparisons to negative control (PBS) were by Dunnet's test (**, $p < 0.01$). (D) HL1 cells were treated with rmGDF3 (50 or 150 ng/ml) for 12 hours prior to incubation with fluorescently labelled LPS (1 μ g/ml) or (E) transferrin (1 μ g/ml) for 2 hours. Nuclei were counterstained with DAPI and cells imaged at 20X and quantified by color thresholding using ImageJ (au, arbitrary units). Scale bars = 100 μ m. Values represent the number of particles that passed the color threshold after background subtraction. One-way ANOVA revealed a significant effect of GDF3 on LPS uptake ($F_{(2,9)} = 31.91, p < 0.0001, n = 4$ per group). Post-hoc comparison to PBS was by Tukey's test (***, $p < 0.001$, ****, $p < 0.000$). (F) rmGDF3 (150 ng/ml) also blocks LPS uptake in neonatal rat ventricular cardiomyocytes. Pairwise comparison by t-test (****, $p < 0.0001, n = 4$). Scale bars = 100 μ m.

4. Discussion

4.1. Summary of findings

4.1.1. General Discussion

IRF2BP2, a member of the IRF2BP family of transcriptional cofactors, is an important regulator of many biological pathways. It has been identified in cell differentiation (Stadhouders et al., 2015), cell survival (M. Koeppel et al., 2009), DNA repair (M. Koeppel et al., 2009) and cancer development (Teng et al., 2010). In macrophages, IRF2BP2 deficiency results in an inflammatory phenotype and increased foam cell formation, increasing the atherosclerotic lesion size in mice (H. H. Chen et al., 2015). A common genetic variant in the 3'UTR of IRF2BP2 that results in a 9-nucleotide deletion (rs3045215) is known to decrease protein expression (H. H. Chen et al., 2015), and men homozygous for the deletion allele are more susceptible to coronary artery calcification (R. O. Vilmundarson et al., 2021a). In this thesis, we demonstrate that expression of IRF2BP2 is enhanced by eukaryotic transcription initiation factor-4H (eIF4H), and that this interaction is dependent on the 9-nucleotide non-deletion sequence in the 3'UTR. As well, we report that IRF2BP2 is capable of autoregulating its own expression via the 3'UTR, and that this capacity for self-regulation is lost in the 9-nucleotide deletion allele.

In mice, we wondered whether IRF2BP2 deficient inflammatory macrophages would impact the electrophysiology of the heart since inflammatory macrophages have been linked to cardiac remodeling and arrhythmias (Xia et al., 2022). Firstly, our studies indicate that mice exposed to LPS experience prolongation of the QT interval after 2 hours, a significantly shorter time frame than has been established in other reports.

Further, while male mice with IRF2BP2-deficient macrophages do not experience an increased prolongation of the QT interval compared to male wild type and female wild type mice, female IRF2BP2-deficient mice are moderately protected from LPS-induced QT prolongation. At the gene expression level, these female mice lacking IRF2BP2 have increased expression of growth differentiation factor 3 (GDF3), coupled with increased circulating levels of the protein. We demonstrate that increasing circulatory levels of GDF3 by i.p. injection of recombinant mouse GDF3 similarly protects mice from LPS-induced QT prolongation. Lastly, we report that exposure to LPS significantly increases the expression of the sodium-calcium exchanger channel (NCX1) after 4 hours and that pre-treatment with rGDF3 12 hours prior to LPS exposure reduces the increase in NCX1.

4.1.2. Regulation of IRF2BP2 by eIF4H and self-regulation

IRF2BP2 is a known regulator of the innate immune system. In macrophages, the non-inflammatory phenotype is dependent upon IRF2BP2 signalling, and ablation of IRF2BP2 in bone marrow-derived macrophages in mice leads to increased foam cell formation and larger atherosclerotic plaque size (H.-H. Chen et al., 2015). In humans, the 3'UTR of IRF2BP2 acts as an important translational enhancer, and disruption of IRF2BP2 translation by the rs3045215 9-nucleotide deletion variant leads to a decrease in protein expression. Clinically, we have shown that this deletion variant leads to an increased risk of coronary artery calcification in men with a minimal burden of atherosclerosis (R. O. Vilmundarson et al., 2021a). Mechanistically, our data shows that the deletion variant disrupts a stem-loop structure in the 3'UTR of IRF2BP2 mRNA that is critical for interaction with the translation initiation factor eIF4H and autoregulation.

Contrary to our expectations, the rs7545855 variant of IRF2BP2, which exchanges a proline for a serine at position 78 in exon 1, does not appear to confer a variation in protein expression or suppression at the 3'UTR.

While initiation of eukaryotic translation canonically focuses on protein interactions at the 5'UTR, many examples of control by protein interaction at the 3'UTR also exist. In general, this model follows the 'closed loop system', whereby sequence-specific interaction of eukaryotic initiation factors (eIFs) at the 5' and 3'UTR are bridged by intermediate proteins (Jackson et al., 2010). This structure then enables the recruitment of ribosomal subunits to initiate protein synthesis (Jacobson & Favreau, 1983). Mutations within the 5'UTR have previously been linked with specific diseases, such as multiple myeloma and Charcot-Marie-Tooth disease, however our data is the first to show a common genetic variant in the 3'UTR that suppresses protein expression. In addition to coronary artery calcification, because IRF2BP2 is found in cell differentiation and proliferation pathways in immune and non-immune cells, the implications behind a variant that affects its expression have the potential to be wide-ranging. Importantly, diseases that have already been linked to variations in IRF2BP2 expression, such as acute myeloid leukemia and medulloblastoma, should be investigated with respect to the rs3045215 minor deletion allele to determine vulnerability.

4.1.3. IRF2BP2 variants in human disease

Because it is found in many immune regulatory pathways and is highly expressed in the bone marrow, IRF2BP2 deficiency has been identified as one of the molecular

causes of common variable immunodeficiency (CVID) (Keller et al., 2016) (Palmroth et al., 2021) (García-Aznar et al., 2023). Studies looking at the functional effects of several IRF2BP2 mutations have found implications for IRF2BP2 deficiency in severe impairment of B-cell differentiation (Endo et al., 2025) (Körholz et al., 2023), immune dysregulation (Udemgba et al., 2025) (Endo et al., 2025), arthritis (Endo et al., 2025) and gastrointestinal disorders (Udemgba et al., 2025). Although mechanistic studies are limited, impaired suppression of nuclear factor of activated t-cells (NFATc), a transcription factor involved in immune cell activation and differentiation, has been implicated (Udemgba et al., 2025). While IRF2BP2 deficiency has been identified in a few families with CVID with autosomal dominance inheritance patterns (Keller et al., 2016) (Baxter et al., 2022), these missense and nonsense mutations have largely been limited to the coding sequence of IRF2BP2. In light of our data demonstrating that the 9-nucleotide deletion in the 3'UTR of IRF2BP2 suppresses translation through loss of an eIF4H-dependent mechanism, future studies looking at common genetic variants in CVID should consider this mechanism of IRF2BP2 regulation.

Somatic, loss-of-function mutations of IRF2BP2 are also found in lymphomagenesis. Several nonsense or frameshift mutations have been found in T-cell lymphomagenesis (Watatani et al., 2019), where loss-of-function of IRF2BP2 leads to enhanced NFAT transcriptional activity and T-cell receptor signaling activation. A mutation which results in the fusion of IRF2BP2 to the retinoic acid receptor RARA (*IRF2BP2-RARA*) was found in a patient with acute promyelocytic leukemia (APL) (Mazharuddin et al., 2018). In integrative genomic analyses of primary mediastinal large

B-cell lymphoma, mutations in IRF2BP2 and other interferon response factors were identified (Mottok et al., 2019) (Chapuy et al., 2019), highlighting the importance of IRF2BP2 in lymphomagenesis. While germline ablation of *Irf2bp2* causes fetal lethality in mice, our work has shown that 2nd and 3rd litters can result in viable *Irf2bp2* null mice that carry *Irf2bp2*-positive myeloid cells in their livers due to microchimerism. Because these mice do not express *Irf2bp2* in their lymphoblasts, lymphoma was a prevalent feature in male and female mice over 6 months (Vilmundarson et al., 2022) (Appendix 5.2). These mice presented with enlarged spleens and livers, with significant lymphocytic infiltration. Of note, *Irf2bp2* deficiency lead to a 3.5 fold increase in PD-L1 expression in their livers, a protein overexpressed in cancer cells (Dorand et al., 2016). Our work therefore provides a model of lymphomagenesis resulting from *Irf2bp2* deficiency in mice.

While many studies have looked at the role of IRF2BP2 in immunoregulatory cells, IRF2BP2 is also known to be enriched in the heart in response to ischemia (Teng et al., 2010). As well, mRNA expression of IRF2BP2 in the lateral wall of the left ventricle is decreased in heart failure compared to the anterior wall and rescued following cardiac resynchronization therapy (Barth et al., 2009). This demonstrates the impact of heart failure on regional changes to the cardiac transcriptome which can be reversed with therapy. Similarly, an increase in IRF2BP2 mRNA in the interventricular septum was found following acute myocardial infarction in mice (Harpster et al., 2006). Together, these studies show that IRF2BP2 undergoes posttranscriptional and posttranslational regulation in response to heart failure and the metabolic status of cardiomyocytes. Our

work here adds more evidence to the players involved in this regulation, suggesting an autoregulatory mechanism that is counterbalanced by eIF4H translational enhancement.

A variant that is found just 3' of IRF2BP2 (rs6672925) confers an increased risk of osteoporosis, and is in perfect linkage disequilibrium with the rs3045215 variant found in the 3'UTR. Patients with osteoporosis have a higher risk of developing coronary heart disease (S. J. Chen et al., 2015), potentially due to the links in vascular mineralization, lipid oxidation and inflammatory signaling (van Dort et al., 2020). IRF2BP2 is a positive upstream regulator of Kruppel-like factor 2 (KLF2), a zinc-finger transcription factor that inhibits osteoclast differentiation and promotes osteoblast differentiation (Kim et al., 2019). Because of this, it is possible that the reduction in IRF2BP2 expression found in individuals carrying the rs3045215 deletion allele impairs osteoblast differentiation, leading to an increased risk of osteoporosis.

4.1.4. The effect of LPS on IRF2BP2

IRF2BP2 regulates the inflammatory phenotype of macrophages, allowing them to respond to the presence of endotoxins in the body. Previous work by our lab has established that, in response to LPS, IRF2BP2 expression is reduced, leading to an increase in expression of inflammatory cytokines, such as IL-1 β , iNOS and TNF α (H.-H. Chen et al., 2015). Suppressing IRF2BP2 allows the body to elicit the immune response required to combat the presence of toxins. In models of sepsis-induced lung injury, the IRF2BP2-NFAT1 axis has been highlighted as a potential therapeutic target to alleviate pulmonary sepsis syndrome (H. F. Li et al., 2020). In this study, the authors demonstrate

that LPS induces the binding of miR-155-5p, a non-coding RNA enriched in immune cells, to the 3'UTR of IRF2BP2. Under normal conditions, IRF2BP2 binds to NFAT1 and represses NFAT1-mediated transcription of inflammatory cytokines (Carneiro et al., 2011). In response to LPS activation of TLR4, miR-155-5p binds the 3'UTR of IRF2BP2, leading to degradation and increased iNOS production. While the miR-155-5p sequence does not target the rs3045215 sequence of the 3'UTR, future studies could look at the effect of this deletion allele on the ability of IRF2BP2 to interact with NFAT1 and the effects on NFAT1-mediated production of inflammatory cytokines.

While previous studies have focused on the presence of IRF2BP2 in the nucleus and its role as a transcriptional regulator, some studies have suggested the presence of IRF2BP2 in the cytosol (Teng et al., 2011). Our findings indicate that IRF2BP2 can also be found in the cytoplasm, where it acts as an RNA-binding protein that is capable of self-regulating expression of its own mRNA. Our study demonstrates that LPS increases the binding of IRF2BP2 to its 3'UTR in the cytoplasm and that this binding relies on the 9-nucleotide sequence found in the 3'UTR.

In contrast to macrophages, in the heart, IRF2BP2 expression is regulated differently. In response to LPS, *Irf2bp2* protein levels are increased in the heart in mouse models of septic cardiomyopathy (T. Li et al., 2020). This study reveals a role for IRF2BP2 as an anti-inflammatory factor that inhibits NF- κ B signaling and the release of inflammatory cytokines. Further, in mice with AAV9-mediated IRF2BP2 overexpression, sepsis-induced cardiac dysfunction was significantly reduced through activation of the

IRF2BP2-AMPK axis. The authors of this study do not specify whether the increase in IRF2BP2 protein is found in the nucleus, cytosol or both, but in light of our data showing that LPS increases IRF2BP2 binding to its own 3'UTR in the cytosol, it would be interesting to see if this mechanism is different in cardiomyocytes.

4.1.5. The effect of LPS on cardiac electrophysiology

Stressors, such as inflammation, can cause remodeling in the heart. These changes can affect electrical conduction or contractility, or they can be structural changes, such as with fibrosis or hypertrophy. With respect to cardiac electrophysiology, inflammation causes prolongation of the action potential duration and QT interval, and is an independent predictor of cardiac sudden death in several inflammatory diseases, such as myocarditis (Ramamurthy et al., 1993) (Ukena et al., 2011), Chagas' disease (Salles et al., 2003) and rheumatoid arthritis (Maradit-Kremers et al., 2005) (Panoulas et al., 2014). Despite the risk that QT prolongation poses to patients, therapies for the condition are limited as the mechanisms are still poorly understood.

In rodents, repolarization relies heavily on the transient outward current (I_{to}) through the Kv4.2/4.3 channels (Niwa & Nerbonne, 2010) and evidence in isolated rat cardiomyocytes demonstrates the impact of LPS on reducing the amplitude of the I_{to} current as well as Kv4.3 mRNA levels 24 hours after exposure (Monnerat-Cahli et al., 2014). Despite this evidence, our electrophysiological data showed no change in I_{to} after 4 hours of LPS incubation compared to control. While most studies have focused on a time frame for QT prolongation of 24 hours or more after LPS exposure (Monnerat-Cahli

et al., 2014) (Wondergem et al., 2012) (Wang et al., 2019), our data suggests that onset of QT prolongation in mice occurs as soon as 2 hours after LPS injection. This is notable in that quicker, posttranslational response mechanisms previously not considered may be responsible for changes to cardiac conduction channels involved in QT prolongation. While I_{to} is primarily responsible for the early phase of repolarization (Niwa & Nerbonne, 2010), the drawn-out phase of the mouse action potential occurs around -40 mV, at which point the Kv4.2/4.3 channels are largely inactive (Knollmann et al., 2007). Instead, our results reveal an increase in the sodium-calcium exchanger (NCX1) protein in the myocardium 4 hours after LPS exposure, consistent with previous results shown in rats (Magi et al., 2015). In support of this evidence, transgenic mice overexpressing NCX1 in the myocardium display an increase in the QT interval and a compensatory increase in Kv current (Pott et al., 2012). Whether this increase in protein results in changes to the NCX1 current will need to be addressed.

4.1.6. GDF3 protects mice from LPS-induced QT prolongation

Growth differentiation factor 3 is a member of the TGF β family of signaling molecules that is involved in left/right patterning during embryogenesis (Pelliccia et al., 2017), skeletal muscle regeneration in adults (Varga et al., 2016) and fat metabolism in adipose tissue (Bu et al., 2018). GDF3 signals through the Alk7 receptor and leads to the phosphorylation of Smad2/3, signal transduction molecules that regulate gene expression involved in cell differentiation, proliferation and apoptosis (Brown et al., 2007) (Souchelnytskyi et al., 1997). Importantly, it has also been previously shown to mitigate the negative effects of LPS on cardiac function (ejection fraction, fractional shortening,

left ventricular internal dimension at end systolic (LVID)), increasing survival. The authors of this study also show that serum GDF3 levels are increased in patients with sepsis and correlate with mortality (L. Wang et al., 2020). Our data also shows an increase in circulating GDF3 levels 4 hours after exposure to LPS in mice, although QT was still prolonged at this time. Raising circulating GDF3 levels 12 hours prior to LPS exposure was required to prevent severe LPS-induced QT prolongation. Contrary to the report from Wang et al., (L. Wang et al., 2020), we did not find an effect of GDF3 pre-treatment on cytokine release in mice 4 hours after LPS injection. Instead, our results demonstrate that GDF3 suppresses the severity of LPS-induced QT prolongation in mice by blocking the increase in expression of NCX1 in cardiomyocytes. Similarly, female *LysMCre Irf2bp2^{flx/flx}* mice with increased levels of circulating Gdf3 are protected from severe LPS-induced QT prolongation despite similar levels of infiltration macrophages in the myocardium.

On a molecular level, our *in vitro* study suggests that GDF3 acts by specifically blocking the uptake of LPS and TLR4 in cardiomyocytes. Activin A, another member of the TGF β family of signaling molecules, inhibits the expression of TLR4 on LPS-stimulated mouse peritoneal macrophages *in vivo* and *in vitro*, and in RAW264.7 macrophages (Li et al., 2013). Signaling of activin A also occurs via Smad2/3 (A. Q. Lu et al., 2017) and exposure to LPS increases mRNA levels of Smad2/3 in peritoneal macrophages (Li et al., 2013), and increases phosphorylation of the Smad linker region (Afroz et al., 2020), suggesting a feedback loop that further augments activin A signaling. Recently, it has been shown that, in addition to Alk7, GDF3 has a high affinity for

multiple type II receptors, such as ACVR2A and B and BMPR2, making it analogous to activin A signaling (Kotikalapudi et al., 2025).

4.2. Limitations

4.2.1. Interaction between eIF4H and IRF2BP2

Our work highlights the importance of the interaction with eIF4H in translational enhancement of IRF2BP2. However, while our data demonstrates the necessity for eIF4H to interact with the 3'UTR of IRF2BP2, we were not able to show direct binding between eIF4H and IRF2BP2. While an unbiased screen of protein-protein interactions shows a physical interaction between eIF4H and IRF2BP2 (Wan et al., 2015), our attempts to show a direct binding of eIF4H and IRF2BP2 mRNA binding at the 3'UTR by RNA-protein pull-down assay were unsuccessful. To test this interaction, biotinylated RNA probes of the IRF2BP2 3'UTR containing the rs3045215 deletion and non-deletion sequences were created (Table 3.1) and tested against Hek293 protein lysate.

RNA probe	Sequence (sense)
RS3045215 non-deletion probe	GCACACAUGCAGAAAUGCAGAGUCAAUUUUACAUGCCCA UAAAGACAUUUGUAAAGAAUUCAGCUCUUAUGGUCUGU UGUAUAAAUGUGUAUCUAGGCACUUUAUUAUAACUGGA AUUUGACCUCACAGAUGUUACAACUUGAUCAGUCGUUU GACCUGAAUUUGUGGUAGCUAUCUGU
RS3045215 deletion probe	GCACACAUGCAGAAAUGCAGAGUCAAUUUUACAUGCCCA UAAAGACAUUUGUAAAGAAUUCAGCUCUUAUGGUCUGU UGUAUAAAUGUGUAUCUAGGCACUUUGGAAUUUGACC UCACAGAUGUUACAACUUGAUCAGUCGUUUGACCUGAAU UUGUGGUAGCUAUCUGU

Table 3.1. RNA probes used in IRF2BP2 3'UTR eIF4H RNA pull-down assay

The ineffectiveness of the assay may be attributed to an incomplete folding of the secondary structure of the 3'UTR probes, which could be required for eIF4H binding. While a stem-loop structure was predicted to be formed by this sequence, the folding of the biotinylated probes could not be confirmed. Further, it is possible that other translational enhancers form a complex with eIF4H and IRF2BP2 that mask the eIF4H antibody epitope, which would inhibit band detection by immunoblot. However, by showing the significant reduction in IRF2BP2 translation with eIF4H siRNA knockdown, both in the 3'UTR luciferase assays and with endogenous IRF2BP2 protein, we believe that this study is consistent with the requirement of eIF4H for enhanced translation of IRF2BP2.

4.2.2. Direct measurement of NCX current

While our data shows an upregulation of the sodium-calcium exchanger channel in the ventricular myocardium after 4 hours of LPS, a limitation of our study is that we were unable to directly measure the NCX current in murine ventricular cardiomyocytes to show an electrophysiological effect of LPS. Because the NCX channel produces a bidirectional current, the manner in which an increase in NCX channel protein impacts the action potential duration is not always clear. The net direction of ion transport through the NCX channel is dependent on intra- and extracellular ionic conditions, membrane potential, making the prediction of the effects of channel upregulation difficult. Further, the importance of calcium levels in excitation-contraction coupling and the dependence of calcium stores on NCX activity may further complicate our interpretations. However, upregulation of NCX in the ventricular myocardium has been documented in heart failure

(Flesch et al., 1996) (Studer et al., 1994), which can present alongside QT prolongation in mouse models using transverse aortic constriction (M. Boulaksil, 2010), leading to many studies that examine the impacts of NCX upregulation in models of heart failure. One major finding in mice with inducible overexpression of NCX1 was a dramatic prolongation of APD, despite no changes in resting membrane potential, action potential amplitude and I_{Ca} (Wang et al., 2009). Similarly, another study looking at transgenic mouse myocytes overexpressing the NCX channel found an increase in action potential duration at 90% repolarization compared to wild type myocytes, which the authors suggest is due to an increase in the forward NCX current during the $[Ca^{2+}]_i$ transient (Yao et al., 1998). Importantly, the results of this study showed that APD50 was reduced, consistent with a compensatory increase in early repolarization. Finally, an *in vivo* study looking at the effects of cardiomyocyte-directed overexpression of NCX in transgenic mice found a prolongation of the QT interval compared to wild type mice and an absence of reduced K_V , similar to our findings (Pott et al., 2012). Therefore, while future studies should directly determine the impact of LPS on the NCX current, given the breadth of existing data that links an upregulation of NCX with an increase in APD and QT, we feel confident drawing a link between the LPS-mediated upregulation of NCX and QT prolongation in our study.

4.2.3. The effect of eIF4H on IRF2BP2 mRNA stability

Another limitation of this study is that we did not measure IRF2BP2 mRNA levels after eIF4H knockdown. eIF4H is targeted by the herpes simplex virus (HSV) virion host shutoff (Vhs) protein (UL41), an endoribonuclease that degrades host cell mRNAs

(Sarma et al., 2008). Knockdown of eIF4H prevents this mechanism from degrading host mRNAs, suggesting that some cellular mRNAs may be degraded through the intermediary of eIF4H. While protein levels of IRF2BP2 were reduced after eIF4H knockdown, consistent with impaired translation, we cannot exclude the possibility that the stability of eIF4H-dependent mRNAs like IRF2BP2 are dependent on the presence of eIF4H.

4.3. Future Directions

4.3.1. Co-inheritance of rs3045215 with other variants

Expression of IRF2BP2 is critical for the inflammatory response of the innate immune system and understanding the specific mechanisms underlying the regulation of IRF2BP2 will be important in contributing to future therapies. Genetic variants of IRF2BP2 have been linked with inflammatory diseases such as COVID (Keller et al., 2016) (Baxter et al., 2022) and coronary artery calcification (Ragnar O. Vilmundarson et al., 2021) and the work in this thesis sheds new light on how the rs3045215 deletion variant found in the 3'UTR of IRF2BP2 disrupts a secondary structure required for interaction with eIF4H, reducing its translation efficiency. As well, we show that in response to endotoxins, binding of cytoplasmic IRF2BP2 to its own 3'UTR is increased, leading to suppression of its own expression under inflammatory conditions. Because rs3045215 is co-inherited with several other genetic variants, including rs6672925 which is linked to osteoporosis (Morris et al., 2019b), it will be important to better elucidate the functional consequences of each variant and how they contribute to coronary artery calcification. By

generating mouse lines that separate these variants, we can track progression of coronary artery disease and better understand the effects of each variant independently.

4.3.2. Upregulation of NCX in response to LPS

We revealed that short term exposure of cardiomyocytes to LPS blocks the uptake of TLR4 and increases the level of NCX1 protein in the myocardium, leading to prolongation of the QT interval. However, the mechanism behind this should be further explored. NCX is known to be upregulated in heart failure in response to an increase in CaMKII (Xu et al., 2012), which activates cytoplasmic polyadenylation element binding proteins (CPEBs) that enhance the translation of certain mRNAs via the 3'UTR (Wu et al., 1998) (Atkins et al., 2004). The cardiac NCX mRNA contains many CPEs and determining whether LPS induces the targeting of these regulatory domains by CPEBs will help us elucidate the pathways involved in this inflammatory response. This can be done by analyzing the expression and phosphorylation of CPEB1, 3 and 4, present in cardiomyocytes (Theis et al., 2003) (Riechert et al., 2021) through RT-PCR and immunoblot of cardiac tissue harvested from LPS-treated mice. As well, because uptake of TLR4 is mediated by GTPase Rab5-positive endosomes (Ciesielska et al., 2021), it will be interesting to see whether GDF3 specifically blocks the action of Rab5. This can be done by transfecting GFP-coupled Rab5 constructs and treating them with rGDF3 to determine its effect on the formation of early enlarged endosomes.

4.3.3. The correlation between GDF3 and severity of QT prolongation

In patients with sepsis, serum GDF3 levels are elevated and correlate with the severity of sepsis (L. Wang et al., 2020). However, since 23% of septicemic patients go on to develop QT prolongation, this begs the question as to whether their circulating levels of GDF3 are not raised to levels that would offer protection against QT prolongation. While most studies measure the total level of circulating GDF3 in serum, it is important to remember the GDF3 exists as a biologically inactive and active form that is cleaved at the processing site between residues 249-252, generating a mature GDF3 protein that is 114 amino acids long (McPherron & Lee, 1993). Whether the study looking at serum GDF3 in humans was able to discern between the biologically active or inactive form is unknown. Importantly, our studies using recombinant GDF3 were done with the biologically active form of the protein. As well, recent evidence suggests that the effects of GDF3 on inflammation may change with age, promoting an inflammatory phenotype in adipose tissue macrophages in older mice, but not younger ones (Jang et al., 2026). Future studies could look at the correlation between the active form of GDF3 in patients with sepsis, stratified by age, and the extent of QT prolongation to determine whether there is a link.

4.3.4. Sex-specific differences in IRF2BP2 deficiency

Many aspects of human health and disease have variations in age and sex, but these sex-specific differences are often not explored. Understanding the interplay between sex and protein expression is important for basic science, translational research, and drug development. Here, we identify two key sex differences resulting from

IRF2BP2 deficiency and knockout, highlighting the need for more research to include biological sex as a variable.

Our data on the functional effects of the 9-nucleotide deletion allele in the 3'UTR of IRF2BP2 demonstrates its importance in expression of IRF2BP2 and proposes a potential regulatory mechanism. A recent report from our lab revealed that the rs3045215 deletion allele affects calcification and is associated with an increased risk of coronary artery calcification in men with a minimal burden of coronary artery disease and two copies of the allele (R. O. Vilmundarson et al., 2021c). This effect was not seen in women homozygous for the deletion allele, even after controlling for age and smoking status, suggesting that IRF2BP2 deficiency does not affect calcification in women in the same manner. Part of this finding may relate to the smaller sample size of female patients, or potentially the amplitude of the effect of rs3045215 in women being smaller. Future studies looking at what factors might be involved in this sex-specific risk will be important in our understanding of the disease progression.

A second sex-specific difference revealed by our study is the protection from severe LPS-induced QT prolongation observed in female IRF2BP2 knockout mice. In this case, we were able to demonstrate that female mice have higher expression of GDF3 at the mRNA and protein level and we were able to confirm this protective mechanism with our *in vivo* studies. It has long been observed that there are age and sex-specific differences found in cardiovascular disease (Rajendran et al., 2023). With respect to TGF β , oestrogen is involved in the signaling pathways of several members. Interestingly, estrogen

receptors ERalpha and ERbeta form a protein complex with Smad and ubiquitin ligase, enhancing the degradation of Smad in an estrogen-dependent manner in MCF-7 and MDA-MB-231 breast cancer cells (Ito et al., 2010). However, our data establishes an increase in GDF3 gene expression along with circulating protein in female *LysMCre* *Irf2bp2^{flx/flx}* mice, suggesting a sex-specific transcriptional mechanism is at play.

5. Conclusion

Cardiovascular disease is the leading cause of mortality worldwide and in addition to lifestyle and environmental factors, genetics are an important risk factor that can predispose patients to an increased risk of CVDs such as atherosclerosis. A better understanding of the innate immune response and the mechanisms governing inflammatory signaling provide key insights into genes that are correlated with an increased risk of atherosclerosis. In this study, we examined the regulation of interferon regulatory factor 2-binding protein 2 (IRF2BP2) and how disruption of its expression is tied to an increased risk of coronary artery disease. We provide evidence for a novel regulatory mechanism for IRF2BP2 via its 3'UTR, which relies on an interaction with eIF4H, as well as an autoregulatory capacity for IRF2BP2, again via the 3'UTR. Finally, we demonstrate the importance of a 9-nucleotide variant in the 3'UTR of IRF2BP2 that is correlated with an increased risk of coronary artery disease in men and highlight the mechanisms underlying reduced expression of this variant.

We also demonstrate a protective effect of IRF2BP2 knockout for reducing the severity of sepsis-mediated QT prolongation in female mice. Our *in vivo* studies showed that these female *LysMCre Irf2bp2^{flx/flx}* mice had elevated expression of GDF3, both at the mRNA and protein level, and that increasing the levels of circulating GDF3 in male wild type mice through injections of recombinant GDF3 was sufficient to similarly protect them from severe LPS-induced QT prolongation. Our study also confirms that QT prolongation sets in as quickly as 2 hours after exposure to LPS, highlighting a new short-term effect of LPS on the cardiac conduction system. Finally, we demonstrate that LPS increases the expression of NCX in the mouse myocardium, an effect which is suppressed by elevated levels of GDF3 prior to LPS

exposure. We also clarify that LPS and GDF3 act on the TRIF-dependent arm of TLR4 signaling. Together, these findings provide valuable insight into potential mechanisms that may be targeted for clinical management of sepsis and its effects on cardiac electrophysiology.

6. References

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7. Appendix

7.1. Co-authored paper

Title: *IRF2BP2 3'UTR Polymorphism Increases Coronary Artery Calcification in Men*



IRF2BP2 3'UTR Polymorphism Increases Coronary Artery Calcification in Men

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Interferon regulatory factor 2 binding protein 2 (IRF2BP2) suppresses the innate inflammatory response of macrophages. A 9-nucleotide deletion (rs3045215) in the 3' untranslated region (3'-UTR) of human IRF2BP2 mRNA confers risk of coronary artery disease (CAD) in the Ottawa Heart Genomics Study (OHGS). Here, we sought to identify regulatory mechanisms that may contribute to this risk. We tested how lipopolysaccharides (LPS) affects IRF2BP2 expression in human THP-1 macrophages and primary aortic smooth muscle cells (HAoSMC) genotyped for the deletion allele. Both cell types are implicated in coronary atherosclerosis. We also examined how the deletion affects interaction with RNA binding proteins (RBPs) to regulate IRF2BP2 expression. LPS altered allele-specific binding of RBPs in RNA gel shift assays with the THP-1 macrophage protein extracts. The RBP ELAVL1 suppressed the expression of a luciferase reporter carrying the 3'UTR of IRF2BP2 with the deletion allele. Other RBPs AUF1 or KHSRP did not confer such allele specific regulation. Since it is co-inherited with a risk variant for osteoporosis, a condition tied to arterial calcification, we examined the association of the deletion allele with coronary artery calcification in individuals who had undergone computed tomography angiography in the OHGS. In 323 individuals with a minimal burden of atherosclerosis (<30% coronary stenosis) and 138 CAD cases (>50% stenosis), Mendelian randomization revealed that the rs3045215 deletion allele significantly increased coronary artery calcification in men with minimal coronary stenosis. Thus, not only does the rs3045215 deletion allele predict atherosclerosis, but it also predisposes to early-onset calcification in men.

Keywords: coronary artery calcification, ELAVL1/HuR, IRF2BP2, atherosclerosis, genetic variant

INTRODUCTION

Inflammatory macrophages play a central role in atherosclerosis (1) and are also emerging as an important component of osteoporosis (2). Atherosclerosis and osteoporosis are two conditions that predispose to coronary artery calcification (3, 4). When stimulated with agents such as bacterial lipopolysaccharides (LPS) or viral infection, macrophages acquire an inflammatory M1 phenotype that is thought to hasten the progression of these aging-related diseases. Macrophages

respond to LPS by activating the production of type I (5) and type II interferons (6). Induction of interferon regulatory factor 1 (IRF1) plays a key role in the activation of the inflammatory response to LPS (7) by binding to *cis*-regulatory DNA sequences of interferon-responsive genes. Under basal conditions, these interferon-responsive genes are suppressed by competitive binding of the related factor IRF2 that is constitutively expressed (8). IRF2 owes its repressor function to its interaction with IRF2 binding protein 2 (IRF2BP2) (9) that recruits the corepressors NCOR1 (10) and VGLL4 (11).

Ablation of *Irf2bp2* in the myeloid lineage by mating mice with a floxed *Irf2bp2* allele to *LysMCre* mice causes macrophages to acquire an inflammatory phenotype (12). Macrophages lacking *Irf2bp2* worsened atherosclerosis in murine models due in part to impaired macrophage cholesterol export. *Irf2bp2*-deficient macrophages have a greater propensity to become laden with triglycerides and cholesterol droplets and to form inflammatory foam cells that are characteristic of atherosclerotic lesions. *Irf2bp2* is required for macrophages to achieve the M2 anti-inflammatory phenotype, because *Irf2bp2*-deficient macrophages fail to activate the M2 program and hyper-activate inflammatory genes when exposed to LPS (12). In the brain of *LysMCre/Irf2bp2^{fllox}* mice, microglia also display an inflammatory phenotype that delays functional recovery from ischemic brain injury (13, 14) and leads to anxiety-like behaviors in newborn mice (15).

In human macrophages derived from circulating monocytes, IRF2BP2 mRNA levels are reduced in pro-inflammatory M1 macrophages stimulated with LPS compared to anti-inflammatory M2 macrophages (16). Similarly, mouse bone marrow-derived macrophages stimulated with LPS downregulate the *Irf2bp2* protein within 8 h (12). We identified a 9-nucleotide deletion variant (rs3045215) in the 3' untranslated region (3'UTR) of human IRF2BP2 mRNA that associates with reduced protein levels of IRF2BP2 in peripheral blood mononuclear cells (12). Since loss of *Irf2bp2* in mouse macrophages worsened atherosclerosis, we asked whether this deletion variant associated with coronary artery disease (CAD) in a subset of the OHGS consisting of 1,066 cases of CAD and 1,011 controls. In the OHGS, under a recessive model, having two copies of the deletion allele increased the odds of CAD compared to no or one copy of the deletion allele when adjusting for known risk factors (12).

To determine whether the deletion was sufficient to confer differential expression to the IRF2BP2 mRNA, we constructed luciferase reporters with the entire 3'UTR of IRF2BP2 differing only in the presence or absence of the risk (deletion) allele. The luciferase reporter bearing the deletion risk allele was significantly less active than the reporter without the deletion (12). This difference was not accompanied by a difference in mRNA stability, suggesting the deletion affects the translation efficiency of IRF2BP2, although the exact mechanism underlying this effect was not known (12).

The deletion variant is in a region of the 3'UTR of IRF2BP2 that is highly conserved between species and occurs only in humans among primates. We asked whether the deletion risk allele might disrupt binding or regulation by RNA-binding proteins (RBPs). RBPs are essential for post-transcriptional

regulation of mRNAs. RBPs bind to RNA through specific domains and many such domains are well-characterized, such as K homology domains and zinc finger domains, but many previously uncharacterized RBPs with no obvious RNA-binding domains have been uncovered through methods such as interactome capture (17, 18). Since the sequence deleted by the rs3045215 variant is AU rich (AUUUAACU), RBPs that bind preferentially to AU-rich elements (AREs) were considered, including KHSRP (K homology type splicing regulatory protein), AUF1 (AU-Rich Element RNA Binding Protein 1) and ELAVL1 (Embryonic lethal abnormal visual protein-like 1). KHSRP binds preferentially to AREs in the 3'UTR of Interleukin-8 mRNA leading to mRNA degradation (19, 20). AUF1 regulates IL10 expression as part of the NF- κ B pathway (21). When knocked out in mice, absence of AUF1 leads to large-scale induction of pro-inflammatory pathways within macrophages, suggesting an important inhibitory function for this RBP (22). ELAVL1, also known as HuR, binds to mRNAs in response to innate immune activation with LPS and together with a partner RBP called TTP regulates mRNA stability and translation (23). Of interest, ELAVL1 has emerged as an important target of a macrophage-specific long non-coding RNA that contributes to the process of atherosclerosis (24).

More recently, a single nucleotide polymorphism (SNP), rs6672925, that lies just 3' of the IRF2BP2 gene was found to associate with osteoporosis in two large genome-wide association studies (GWAS) (25, 26). The rs6672925 osteoporosis risk variant is in perfect linkage disequilibrium (LD) with the rs3045215 CAD risk deletion variant (27), meaning the 2 alleles are co-inherited. Osteoporosis is associated with elevated risk of coronary artery disease (3) and predicts the occurrence of advanced coronary artery calcification (4). Since osteoporosis and atherosclerosis share similar disease mechanisms, we asked whether the rs3045215 deletion may be the functional allele accounting for these macrophage-mediated diseases (2, 28). In addition to macrophages, smooth muscle cells also participate in the process of atherosclerosis (29) and coronary calcification (30). Intriguingly, the rs6672925 variant was also found to affect IRF2BP2 mRNA levels in the aortic root, suggesting expression in aortic smooth muscle cells may be affected by the linked rs3045215 deletion allele (31).

Here, we show that the IRF2BP2 3'UTR rs3045215 deletion variant affects IRF2BP2 protein levels in human aortic smooth muscle cells (HAoSMC) and mRNA translation modulated by the RBP ELAVL1. Remarkably, rs3045215 increases coronary artery calcification in men with a minimal burden of atherosclerosis, suggesting that the variant not only contributes to osteoporosis (through its linkage to rs6672925), but also contributes to arterial calcification independent of atherosclerosis.

MATERIALS AND METHODS

Cell Culture—THP-1 and HAoSMC

THP-1 cells, an immortalized human monocyte cell line, were grown in suspension in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and

penicillin-streptomycin (Pen-Strep, Gibco) at a density of 1×10^6 /ml. For macrophage differentiation, THP-1 cells were plated at 2×10^5 cells/ml in PMA-containing medium (phorbol 12-myristate 13-acetate at 1×10^{-7} M) for 72 h and then switched to PMA-free medium, rested for 24–48 h prior to use. THP-1 macrophages were found to be HNR for the rs3045215 variant after genotyping with the BsrI/PCR method (12) with DNA isolated by the HotSHOT method (32).

Adult primary HAoSMC (Cell Applications) from healthy human donors were grown in SmGm-2 medium (Lonza, Smooth Muscle Growth Medium-2) with 5% FBS and SingleQuots supplements (Lonza, human epidermal growth factor, human fibroblastic growth factor, insulin, and gentamicin/amphotericin-B). Cells were split at 80% confluence using Trypsin-EDTA (Gibco, 0.05%, containing phenol red) and medium was renewed every 2 days. DNA was extracted using the HotSHOT method (32). HAoSMC rs3045215 genotypes were as follows: homozygous non-risk (non-deletion), HNR = 1,441, 1,473 and 3,003; heterozygous, HET = 1,596 and 2,228. No homozygous risk (deleted) HR HAoSMC was identified by genotyping.

Luciferase Reporter Assays With RBP Co-transfection

The empty control mammalian expression plasmid pReceiver-M02 (#EX-NEG-M02-B), and plasmid vectors for AUF1 (#EX-Z0257-M02-B), ELAVL1 (#EX-Q0365-M02-B), and KHSRP (#EX-Z9878-M02-B) were purchased from Genecopoeia. These vectors were co-transfected with non-risk (rs3045215 non-deletion allele) and risk (rs3045215 deletion allele) luciferase reporters in HEK293 cells using the Lipofectamine 3000 kit (Invitrogen). The luciferase reporters were described previously (12). HEK293 cells were plated at 1×10^6 /ml, 1 ml per well, in 12-well-plates and allowed to adhere overnight. The next day, cells were transfected with 1 μ g of luciferase reporter (either non-deletion or deleted) in triplicate together with the empty pEZ vector control or with 10, 50, or 100 ng of each RBP vector (either AUF1, KHSRP, or ELAVL1). Each experiment was repeated 5–7 times. Overexpression of each vector in HEK293 cells was confirmed by immunoblot. HEK293 cells were grown in 6-well-plates and maintained in DMEM (low glucose, Gibco) medium with 10% FBS and Pen-Strep.

LPS Treatment of THP-1 Macrophages and HAoSMC

THP-1 macrophages were treated with LPS (either 10 or 100 ng/ml) to activate the M1 pro-inflammatory state. For immunoblots, protein was extracted at baseline and 4, 8, 24, 48, and 72 h after LPS treatment. The cells were in LPS-containing medium for the entire time until they were harvested (except for baseline cells which were not treated). Baseline controls were harvested at the earliest LPS treatment time point. For cytoplasmic lysis buffer (1% Triton, 25 mM Tris-HCL pH 7.4, 40 mM KCL) and M-PER (Mammalian Protein Extraction Reagent, Thermo Fisher Scientific) whole-cell extracts were acquired at baseline, 4, 8, and 24 h and used in RNA-gel shift experiments.

HAoSMC were treated with LPS (10 ng/ml) for 8, 24, or 48 h prior to RIPA buffer extraction and lysates were used for immunoblots. Cells were seeded to be 50% confluent at the time of LPS treatment. As with THP-1 macrophages, except for baseline controls, HAoSMC were in LPS-medium for the entire duration. Medium was not changed after start of LPS treatment. For RNA gel shifts, cytoplasmic and M-PER whole-cell extracts were obtained at baseline and 24 h after LPS, based on the THP-1 and HAoSMC immunoblot results.

Immunoblot Analysis

Proteins were size fractionated by SDS polyacrylamide gel electrophoresis followed by electroblotting to PVDF membranes. Proteins were visualized with the following antibodies: a rabbit peptide-specific antibody to IRF2BP2, described previously (11), anti-ELAVL1 mouse monoclonal (Abcam, #ab136542), anti-KHSRP mouse polyclonal (Abnova Corporation, #H00008570-A01), anti-AUF1 rabbit polyclonal (Abcam, #ab50692), anti-alpha-smooth muscle actin mouse monoclonal (Sigma, #A2547), anti-GAPDH mouse monoclonal (Santa Cruz Biotechnology, #sc-59540), and anti- β -actin mouse monoclonal (Sigma, #A2228). Primary antibodies were used at 1/5,000 dilution. The secondary horseradish peroxidase-conjugated antibodies used at a 1/10,000 dilution, goat anti-mouse IgG antibody (R&D Systems, #HAF007) and goat anti-rabbit IgG (H+L) antibody (Life Technologies, #31460), were revealed by chemiluminescence using the SuperSignal West Dura substrate (Thermo Fisher Scientific). Bands were quantified using the ImageJ software (33) with each lane signal normalized to loading control prior to fold conversion. Fold change compared to average of baseline values.

Electrophoresis Mobility Shift Assay

An RNA EMSA method was used to determine binding differences between risk and non-risk RNA probes. RNA probes (Non-deletion probe, 5'-UAGGCACUUUAUUAUACUGGAAUUGAC-3'; Deleted probe, 5'-UAGGCACUUUGGAAUUGAC-3') were synthesized by Integrated DNA Technologies. The non-deletion (non-risk) probe included the 9-nucleotide deletion sequence plus 10 bases of flanking sequence on either side. The deleted probe contained only the flanking sequence (20 nucleotides total). Probes containing either the non-deletion or deletion allele of rs3045215 were 5'-end labeled using [32 P]-ATP and T4 polynucleotide kinase at 37°C for 1 h, as described previously (34). The reaction was stopped using 0.5 M EDTA (pH 8.0) and then the samples were purified using RNase-free G-25 Sephadex spin columns (Roche). The following cocktails were prepared for non-deletion and deleted probes separately: 10X binding buffer, 1 μ L; Radiolabeled probe (50,000 CPM dilution), 1 μ L; 50% glycerol, 1.5 μ L; 1 M DTT, 0.2 μ L; RNasin (Promega), 0.2 μ L; Yeast Total RNA (10 mg/ml), 0.2 μ L; 5% xylene cyanol, 1 μ L; DEPC-treated ddH₂O, 2.9 μ L. All components were prepared or purchased RNase-free. Binding buffer composition can be found in the Yakhnin et al. (35) reference under Csra binding buffer 10X. Cocktails were added last to tubes containing protein extracts, gently vortexed, spun down and incubated at 37°C for

30 min. For DNA gel shifts, a double-stranded NF- κ B-specific oligonucleotide probe (sense 5'-AGAGGGGACTTTCCGAGG-3') was synthesized by IDT Technologies. DNA EMSA were carried out as described previously (36). Samples were run on a 7% poly-acrylamide gel for 3 h at 200 volts in a cold room. Gels were dried before being placed in a cassette with a phosphor screen overnight at room temperature and the image was captured the next day using a Storm phosphor imaging system (GE Healthcare Lifesciences). Quantification for EMSA experiments was performed using ImageQuant software (V2.0, Cytiva Lifesciences, formerly GE Healthcare Lifesciences). The probe alone lane, where no shifted complex was present, was used to subtract background values from all lanes.

Genotyping Rs3045215 in OHGS CT Angiography Samples

The OHGS cohort consists of 3,273 individuals of mixed European ancestry who have undergone coronary angiography and are defined as early onset cases of coronary artery disease (CAD, with >50% stenosis in a major coronary artery, before 55 years in males and 65 years in females) and 1,066 asymptomatic elderly controls (>65 years of age for males, >70 years of age for females) recruited from the Ottawa region who have undergone genome-wide genotyping of SNPs (37, 38). The OHGS excluded individuals with diabetes. For the subset of individuals who were recruited from CT angiography for which coronary calcium Agatston scores are available, the age restrictions were relaxed by 10 years to include 323 controls who displayed minimal coronary stenosis (<30% stenosis in a major coronary artery) and 138 CAD cases (>50% stenosis in a major coronary). The study (protocol number 2009857) was approved by the Ottawa Health Science Network Research Ethics Board (OHSN-REB), conformed to the principles outlined in the Declaration of Helsinki and all participants gave written informed consent to participate in the study. Because the rs3045215 variant is not detected on Affymetrix arrays used to genotype the OHGS cohort, DNA samples were genotyped using a BsrI restriction enzyme digest of a PCR amplicon of the rs304515 sequence, as described previously (12). Genotypes did not deviate from Hardy-Weinberg equilibrium.

Statistical Analysis

All statistical analyses were carried out using the R statistical computing software (<https://www.R-project.org>). For all transfection experiments, luciferase reporter activity levels were normalized as log₂ fold change relative to the non-deletion construct baseline average. To determine if co-transfection produced a dose-dependent effect and whether there was an interaction between dose and vector type (non-deletion/deletion luciferase reporters) a two-way analysis of variance (ANOVA) was performed for each co-transfection experiment. Dose, luciferase reporter type plus a dose:vector interaction variable were included in each ANOVA. $P \leq 0.05$ were considered significant. Tukey's *post-hoc* analysis was performed when there was significant interaction between vector type and dose.

To determine the association of the rs3045215 deletion with coronary artery calcification a linear regression model was

used. The rs3045215 variant was coded as counts of the minor (deletion) allele, i.e., HNR = 0, HET = 1, and HR = 2. The analysis was split by sex and CAD case status with smoking status and age included in the linear model. Agatston scores were normalized using log₂ after adding 1 to address zero values in the data. Linear regression was performed using the *lm* function in R and data was plotted using the data visualization package *ggplot2* (39).

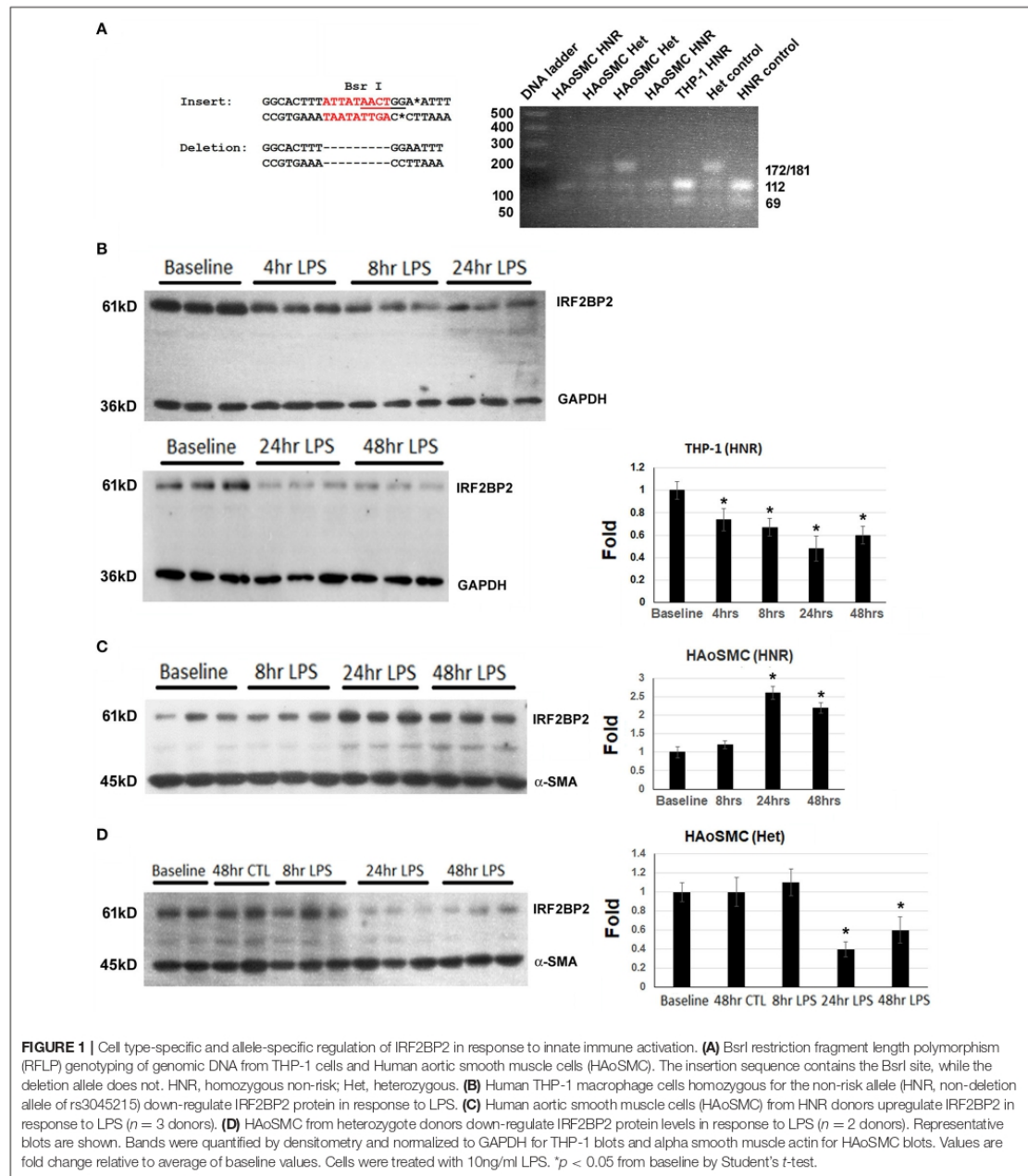
RESULTS

LPS Modulation of IRF2BP2 Expression Is Cell and Allele-Specific

THP-1 macrophages were genotyped and found to be homozygous for the non-risk (HNR) (i.e., 2 copies of the non-deletion) allele of rs3045215 (Figure 1A). THP-1 macrophages showed a rapid time-dependent reduction in IRF2BP2 protein levels in response to LPS (Figure 1B). Primary human aortic smooth muscle cells (HAoSMC) from 5 donors were also genotyped and 3 were found to be HNR and 2 were heterozygotes (HET, carrying 1 copy of the non-deletion allele and 1 copy of the 9-nucleotide deletion allele). We were unable to detect HAoSMC from any donor homozygous for the risk allele, due to the low minor allele frequency. In contrast to THP-1 cells, HAoSMC from HNR donors showed a delayed increase in IRF2BP2 levels in response to LPS challenge (Figure 1C). Surprisingly, HAoSMC from HET donors showed a delayed reduction in IRF2BP2 expression with LPS treatment (Figure 1D).

LPS Alters Allele-Specific and Cell-Specific Binding of RBPs

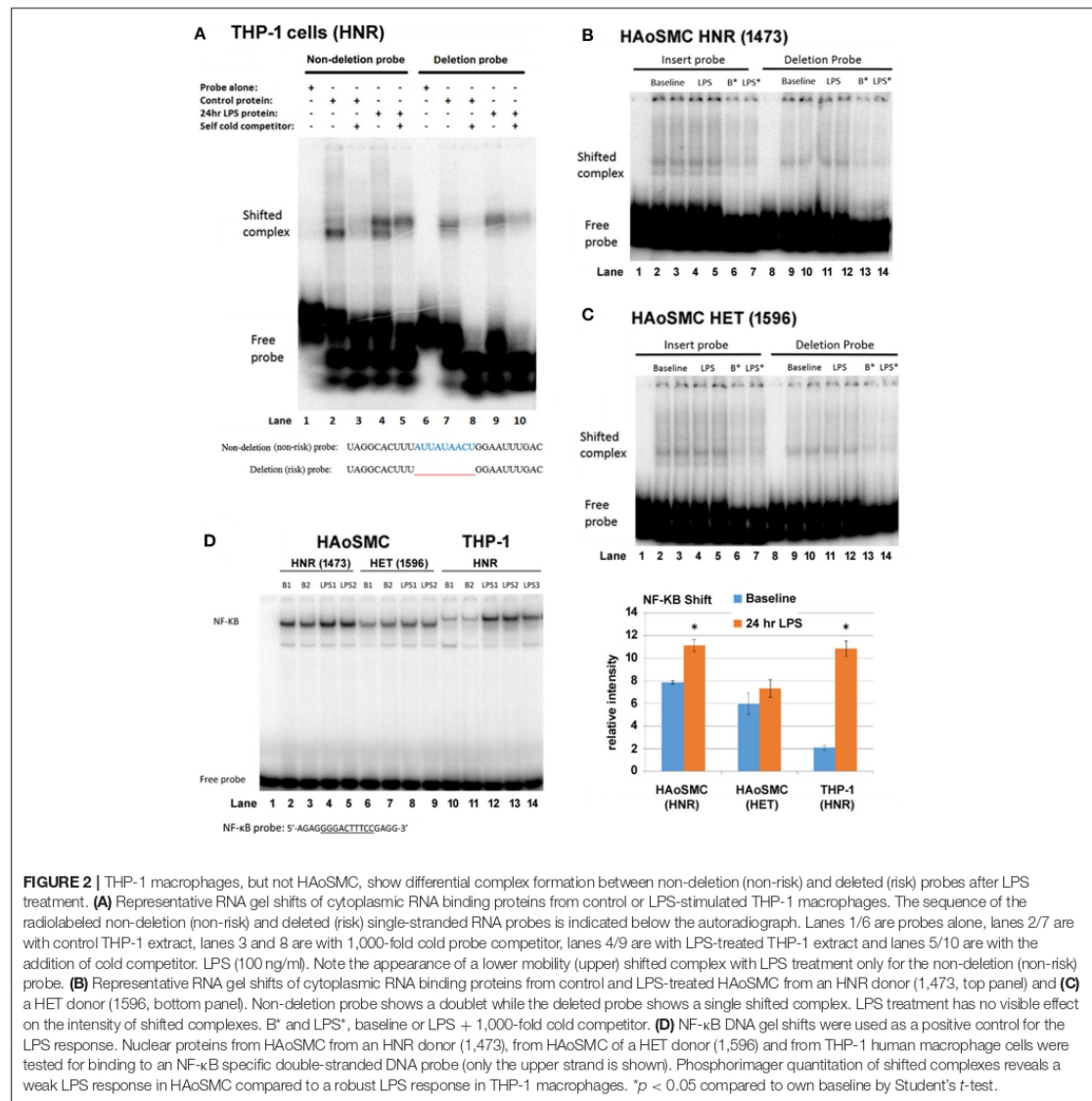
We asked whether the region of the 3'UTR of IRF2BP2 containing the deletion showed any allele-specific binding of RBPs that could account for allele-specific regulation of IRF2BP2 protein levels. RNA gel shifts were carried out using protein extracts from THP-1 macrophages tested on radiolabeled RNA probes carrying either the non-deletion or deletion alleles. The effect of LPS on RBP binding to these two alleles was also compared. The radiolabeled RNA probe of the non-deletion allele revealed 2 shifted complexes, a robust lower fast mobility complex and a weaker higher slower mobility complex, using cytosolic proteins from untreated THP-1 macrophages (Figure 2A, lane 2). In contrast, the RNA probe carrying the deletion allele revealed a single shifted complex (Figure 2A, lane 7). Competition with 1000-fold excess of non-radiolabeled cold RNA probes showed all these complexes were specific (Figure 2A, lanes 3 and 8). LPS-treatment changed the distribution of the shifted complexes with the non-deletion probe: the slower mobility upper complex became predominant (Figure 2A, lane 4). On the other hand, LPS treatment increased binding to the single shifted complex formed with the deletion probe (Figure 2A, lane 9). These RNA gel shift results reveal an allele-specific effect on RBP interaction with the IRF2BP2 mRNA.



Moreover, the effect of LPS on RBP binding to IRF2BP2 mRNA is lost with the deletion (risk) allele.

Next, we tested whether an allele-specific effect on RNA binding in response to LPS treatment could be observed in cytosolic extracts of HAoSMC from donors HNR and HET for

rs3045215 (Figures 2B,C). As in THP-1 macrophages, HAoSMC of either genotype showed allele-specific differences in RNA binding complexes, with 2 complexes formed with the non-deletion probe and a single complex formed with the deletion probe. The lower mobility complex (whose binding increases in



THP-1 cells with LPS treatment) was more abundant relative to the higher mobility complex in HAoSMC at baseline. However, HAoSMC did not show increased binding of this complex in response to LPS treatment.

Concerned that HAoSMC might not respond to LPS treatment to the same extent as THP-1 macrophages, nuclear protein extracts were tested for changes in NF-κB binding in DNA gel shift assays. Indeed, in contrast to THP-1 macrophages that showed a robust increase in NF-κB binding in response to LPS treatment (Figure 2D, compare lanes 10/11 to 12–14),

HAoSMC for a HNR or HET donors showed a much smaller increase in binding to the NF-κB probe (Figure 2D, compare lanes 2/3 to 4/5, and lanes 6/7 to 8/9). Importantly, while these cells are of human origin, the size of the shifted complexes is clearly different between HAoSMC and THP-1 macrophages, suggesting different isoforms of NF-κB are expressed in these 2 cell types. Also, baseline levels of the NF-κB complex were much more abundant in untreated HAoSMC than in THP-1 macrophages. Thus, primary HAoSMC may already be LPS “primed” compared to THP-1 macrophages.

ELAVL1 Elicits Allele-Specific Suppression of Protein Expression

Given that the deletion allele affects RBP interaction with the 3'UTR of IRF2BP2 mRNA, and that luciferase reporters carrying the entire 3'UTR of human IRF2BP2 but differing only in the presence or absence of the rs3045215 deletion showed different expression, we sought to identify RBPs involved in suppressing the expression of IRF2BP2 deletion variant.

HEK293 cells were co-transfected with either of these two IRF2BP2-luciferase reporters (diagram, **Figure 3A**) together with expression plasmids for various RBPs. Since the deleted sequence contains an AU-rich element, we focused on RBPs that target AU-rich sequences, including KHSRP, AUF1, and ELAVL1. Immunoblot showed similar levels of over-expression of each RBP was achieved (**Figure 3B**). Luciferase activities were reported as fold of baseline for the non-deletion reporter (**Figures 3C–F**).

As we reported previously (12), the luciferase reporter bearing the 3'UTR deletion allele had an intrinsically lower luciferase reporter activity (**Figure 3C**). In cells co-transfected with the empty expression plasmid, two-way ANOVA revealed a significant effect of the luciferase reporter vector [$F_{(1, 32)} = 1.367$, $p = 0.0011$], but no dose effect of the empty expression plasmid [$F_{(3, 32)} = 0.471$, $p = 0.7043$] nor an interaction between expression plasmid dose and the reporter plasmid [$F_{(3, 32)} = 0.276$, $p = 0.8421$].

ELAVL1 expression dose-dependently suppressed both luciferase reporters [$F_{(3, 48)} = 32.8$, $p < 1.12 \times 10^{-11}$; **Figure 3D**]. Importantly, the effect was much more prevalent for the reporter carrying the 3'UTR deletion allele; interaction between the luciferase reporter plasmid and the ELAVL1 expression plasmid dose [$F_{(3, 48)} = 4.322$, $p = 0.0089$]. The difference between luciferase reporters remained highly significant [$F_{(1, 48)} = 64.2$, $p < 2.10 \times 10^{-10}$]. Thus, ELAVL1 may contribute to selective suppression of the IRF2BP2 deletion allele.

In contrast, the KHSRP expression plasmid had no suppressive effect on either luciferase reporter (**Figure 3E**), indicating that KHSRP is unlikely to directly regulate IRF2BP2 expression; no effect of KHSRP dose [$F_{(3, 32)} = 0.340$, $p = 0.797$] or any interaction between KHSRP plasmid dose or the reporter plasmids [$F_{(3, 32)} = 0.154$, $p = 0.927$] was observed. Surprisingly, with the over-expression of KHSRP, the difference between the two luciferase reporters was no longer significant [$F_{(1, 32)} = 2.379$, $p = 0.133$], suggesting that over-expression of KHSRP may overwhelm the endogenous mechanism conferring the allele-specific difference in IRF2BP2 expression.

AUF1 indiscriminately suppressed both luciferase reporters in dose-dependent manner [$F_{(3, 48)} = 8.48$, $p = 0.00013$; **Figure 3F**]; no interaction between AUF1 expression plasmid dose and the reporters was observed [$F_{(3, 48)} = 0.090$, $p = 0.965$]. The difference between luciferase reporters remained significant with AUF1 over-expression [$F_{(1, 48)} = 16.98$, $p = 0.00015$]. Thus, AUF1 downregulates IRF2BP2 expression, and this effect is independent of the deletion allele.

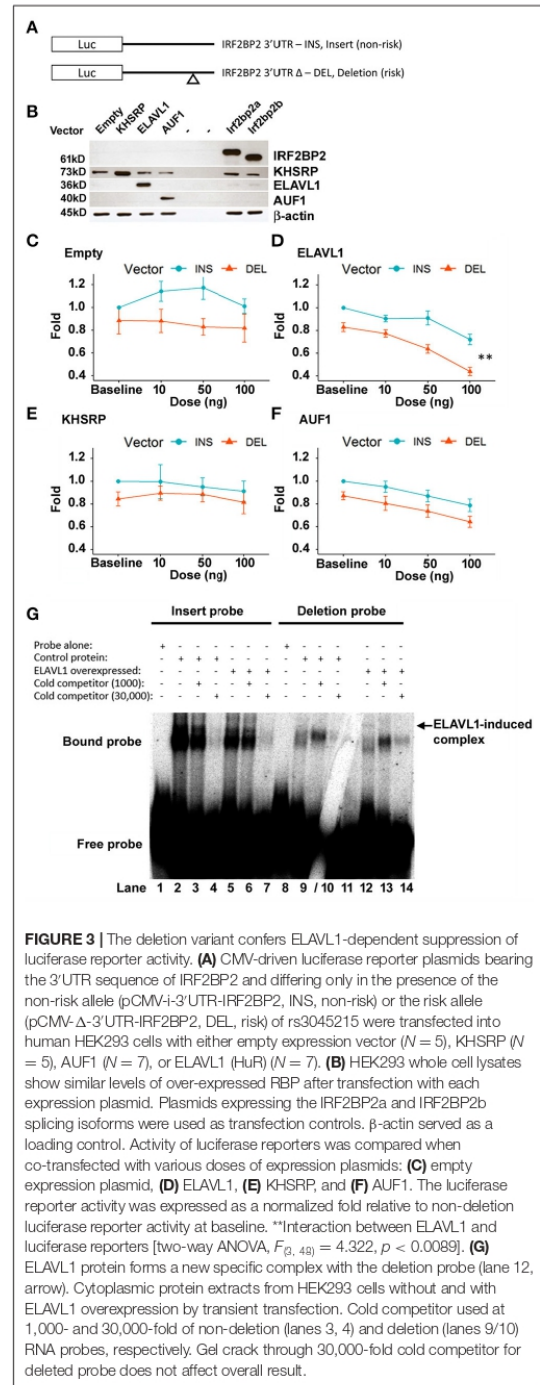


FIGURE 3 | The deletion variant confers ELAVL1-dependent suppression of luciferase reporter activity. **(A)** CMV-driven luciferase reporter plasmids bearing the 3'UTR sequence of IRF2BP2 and differing only in the presence of the non-risk allele (pCMV-4-3'UTR-IRF2BP2, INS, non-risk) or the risk allele (pCMV- Δ -3'UTR-IRF2BP2, DEL, risk) of rs3045215 were transfected into human HEK293 cells with either empty expression vector ($N = 5$), KHSRP ($N = 5$), AUF1 ($N = 7$), or ELAVL1 (hHuR) ($N = 7$). **(B)** HEK293 whole cell lysates show similar levels of over-expressed RBP after transfection with each expression plasmid. Plasmids expressing the IRF2BP2a and IRF2BP2b splicing isoforms were used as transfection controls. β -actin served as a loading control. Activity of luciferase reporters was compared when co-transfected with various doses of expression plasmids: **(C)** empty expression plasmid, **(D)** ELAVL1, **(E)** KHSRP, and **(F)** AUF1. The luciferase reporter activity was expressed as a normalized fold relative to non-deletion luciferase reporter activity at baseline. **Interaction between ELAVL1 and luciferase reporters [two-way ANOVA, $F_{(3, 48)} = 4.322$, $p < 0.0089$]. **(G)** ELAVL1 protein forms a new specific complex with the deletion probe (lane 12, arrow). Cytoplasmic protein extracts from HEK293 cells without and with ELAVL1 overexpression by transient transfection. Cold competitor used at 1,000- and 30,000-fold of non-deletion (lanes 3, 4) and deletion (lanes 9/10) RNA probes, respectively. Gel crack through 30,000-fold cold competitor for deleted probe does not affect overall result.

TABLE 1 | Clinical characteristics of OHGS CT angiography samples with Agatston scores genotyped for rs3045215.

Variable	Controls (N = 323)	Cases (N = 138)	p
Agatston score, HU	0.00 [0.00, 82.50]	344.00 [121.00, 601.75]	<0.001
Age, years	58.63 (11.05)	62.03 (8.26)	0.001
Male sex, N (%)	168 (52.0)	89 (64.5)	0.018
BMI, kg/m ²	28.35 (5.30)	28.53 (4.94)	0.732
Smoking, N (%)	177 (54.8)	100 (72.5)	0.001
HTN, N (%)	112 (34.7)	76 (55.1)	<0.001
Total-C, mmol/L	5.69 (1.16)	6.15 (1.19)	0.001
LDL-C, mmol/L	3.57 (0.97)	3.96 (0.85)	0.001
HDL-C, mmol/L	1.37 (0.37)	1.28 (0.35)	0.026
TG, mmol/L	1.40 [0.95, 2.03]	1.65 [1.19, 2.34]	0.004
Statins, N (%)	121 (37.5)	79 (57.2)	<0.001
rs3045215 (%)			0.044
0	164 (50.8)	59 (42.8)	
1	138 (42.7)	61 (44.2)	
2	21 (6.5)	18 (13.0)	

Agatston score and triglycerides have non-normal distribution and are therefore given as median with interquartile range (IQR). All other continuous variables are given as mean $\pm$ SD. Cholesterol and triglyceride measurements are baseline values. Abbreviations: SD, standard deviation; HU, Hounsfield units; HTN, anti-hypertensive medication; Total-C, total-cholesterol; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; TG, triglycerides. rs3045215 genotypes are counts of the minor (deletion) allele. Categorical variables were tested with the Chi-square test, continuous variables with the Student's t-test when normally distributed and the Wilcoxon test when not normally distributed.

ELAVL1 Produces a New Specific Complex With the Deletion Allele RNA Probe

We next asked whether the differential effect of ELAVL1 would be reflected in an RNA gel shift assay using the non-deletion and deleted RNA probes. Overexpression of ELAVL1 in HEK293 cells did not change the RNA complexes formed with the non-deletion probe compared to non-transfected cells (Figure 3G, lanes 2 and 5), but induced the formation of a new complex with the deletion RNA probe (Figure 3G, compare lane 8–12, arrow). This new complex was completely competed by 1,000-fold excess of cold probe, demonstrating this binding is specific to the deletion allele (Figure 3G, lane 13). Of note, while the 9-nucleotide deletion removes an AU-rich sequence, the remaining flanking sequence of the deletion RNA probe still contains AU-rich sequences that could bind to ELAVL1.

IRF2BP2 Deletion (Risk) Allele Associates With Coronary Artery Calcification in Men

To determine whether the deletion allele of rs3045215 contributes to coronary artery calcification, we examined the distribution of this variant in individuals of the OHGS (37) who had undergone CT angiography to assess the degree of coronary artery stenosis and coronary artery calcification, quantified by Agatston scores (40). According to the criteria used in the OHGS, cases with coronary artery atherosclerosis were defined as having >50% stenosis in any major coronary artery and controls as having <30% stenosis. As shown in Table 1, we

genotyped rs3045215 in 138 CAD cases and 323 controls and found an association of the minor allele with atherosclerosis ($p = 0.044$), consistent with our prior report (12).

We next examined the association of the deletion allele with coronary artery calcification. Crude analysis revealed a significant association of the deletion allele with coronary artery calcification, measured as elevated Agatston scores (Table 2, $p = 2.17 \times 10^{-4}$). When split by sex, the association of the deletion allele remained significant only in males ($p = 0.00331$). We next carried out regression analysis, considering CAD status, age at consent, sex, and smoking history as covariates in all CT samples together. The deletion allele still associated with the Agatston score ($p = 0.0448$), but CAD status ($p < 2 \times 10^{-16}$), sex ($p < 1.06 \times 10^{-7}$), age at consent ($p = 2 \times 10^{-16}$), and smoking ($p = 0.016$) also contributed significant effects on Agatston scores.

We also examined whether the association of the deletion allele with calcification was affected by the degree of atherosclerosis, by repeating the regression analysis in CAD cases separately from controls (i.e., Mendelian randomization controlling for the effect of CAD). Unexpectedly, the deletion allele of rs3045215 showed a robust association with Agatston score in controls ($p = 0.0106$), but not in CAD cases (Table 2). Further, we tested whether the effect was sex-dependent by repeating the regression analysis separately in male and female controls. The deletion allele showed a strong association with coronary calcification in males with a minimal burden of atherosclerosis ($p = 0.00934$). The distribution of the rs3045215 genotypes among individuals with minimal coronary disease revealed a strong effect on calcification (with an Agatston score of 254) in men homozygous for the deletion allele, compared to men carrying one or no copy of the deletion (with Agatston scores of 110 and 113) (Figure 4).

DISCUSSION

Here, we examined the effect of the rs3045215 deletion allele in the 3'UTR of IRF2BP2 on the modulation of IRF2BP2 protein expression by LPS in HAoSMC. We found that HAoSMC that carry 1 copy of the deletion allele down-regulate IRF2BP2 when stimulated with LPS. In contrast, HAoSMC that carry 2 copies of the non-deletion allele upregulate IRF2BP2 with LPS treatment. Thus, the rs3045215 deletion confers a differential response to LPS stimulation in HAoSMCs and may increase their inflammatory response. We also found that human THP-1 macrophages that carry 2 copies of the non-deletion allele downregulate IRF2BP2 with LPS treatment, further revealing a cell type-specific regulation of IRF2BP2 expression by LPS. RNA probes containing the deletion and non-deletion alleles displayed differential binding to RBPs in protein extracts from THP-1 macrophages and HAoSMC. Among candidate RBPs tested, the AU-rich element binding protein ELAVL1 specifically suppressed expression of a luciferase reporter bearing the deletion allele in the 3'UTR of IRF2BP2. RNA gel shift revealed this protein makes a specific complex with the deletion but not the non-deletion RNA probe. Lastly, we found that men homozygous for the rs3045215 deletion allele with a minimal

TABLE 2 | Regression analysis of rs3045215 minor allele effect on coronary artery calcification.

Analysis		Variable	Beta	SE	p	Risk allele $p \leq 0.05$
Crude		rs3045215	1.03	0.277	2.17e-04	*
Crude, split by sex	Males	rs3045215	1.06	0.359	0.00331	*
	Females	rs3045215	0.741	0.422	0.0803	
Risk factor adjusted, including CAD in model		rs3045215	0.418	0.208	0.0448	*
		CAD	4.01	0.298	<2e-16	
		sex	1.47	0.271	1.06e-07	
		age	0.129	0.0131	<2e-16	
		smoking	0.659	0.272	0.016	
Risk factor adjusted, split by CAD case/control	Controls	rs3045215	0.704	0.274	0.0106	*
		Sex	1.613	0.339	2.90e-06	
		Age	0.134	0.0153	<2e-16	
		Smoking	0.849	0.335	0.0117	
	Cases	rs3045215	-0.124	0.288	0.666	
		sex	0.915	0.430	0.0354	
		age	0.0961	0.0258	2.95e-04	
		smoking	-0.0725	0.448	0.872	
Controls only, split by sex	Males	rs3045215	0.984	0.374	0.00934	*
		age	0.155	0.0219	3.99e-11	
		smoking	0.805	0.473	0.0911	
	Females	rs3045215	0.382	0.403	0.344	
		age	0.113	0.0222	1.09e-06	
		smoking	0.758	0.493	0.126	

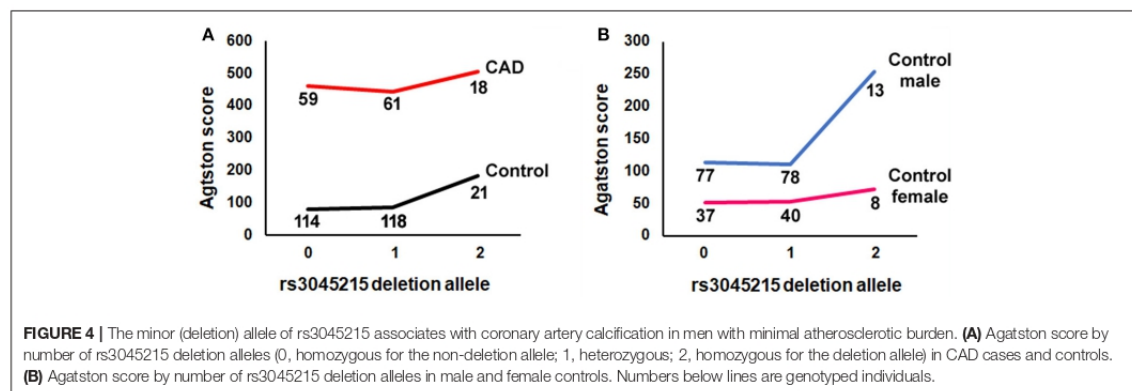


FIGURE 4 | The minor (deletion) allele of rs3045215 associates with coronary artery calcification in men with minimal atherosclerotic burden. **(A)** Agatston score by number of rs3045215 deletion alleles (0, homozygous for the non-deletion allele; 1, heterozygous; 2, homozygous for the deletion allele) in CAD cases and controls. **(B)** Agatston score by number of rs3045215 deletion alleles in male and female controls. Numbers below lines are genotyped individuals.

burden of coronary atherosclerosis are susceptible to coronary artery calcification.

IRF2BP2 expression is suppressed in THP-1 macrophages stimulated with LPS, and this augments their innate inflammatory response (12). THP-1 macrophages are homozygous for the non-deletion allele. In contrast, we found HAoSMC homozygous for the non-deletion allele upregulate IRF2BP2 when stimulated with LPS. A similar LPS-induced elevation in IRF2BP2 in the heart of mice has been reported in a study of sepsis-induced cardiomyopathy (41). In this study, IRF2BP2 was found to protect the heart from LPS-induced inflammation and cardiomyopathy. HAoSMC carrying one copy

of the rs3045215 deletion allele displayed reduced IRF2BP2 expression in response to LPS. Individuals carrying the deletion allele of rs3045215 may have more inflammatory vascular smooth muscle cells in the presence of atherosclerosis, accounting for the associated increased CAD risk (12). Our observation is consistent with a study of eQTL (expressed quantitative trait loci) SNPs that found lower expression of IRF2BP2 in the human aortic root in individuals who carry the rs6672925 allele (31) that is co-inherited with the rs3045215 deletion allele.

Our previous study found an association of the rs3045215 deletion allele with coronary artery disease in individuals who underwent standard coronary angiography for early onset

coronary artery disease and asymptomatic population controls (12). Here, using a CT angiography subgroup of the OHGS that we had not genotyped previously, we were able to replicate the association of rs3045215 deletion allele with coronary artery disease. Importantly, this CT angiography cohort provided coronary artery calcification scores that enabled us to determine whether the rs3045215 deletion allele affects calcification. Considering recent reports that a nearby co-inherited SNP was tied to osteoporosis and the link between osteoporosis and vascular calcification, here we found that the minor deletion allele of rs3045215 showed a more robust association with coronary calcification in men with <30% stenosis of any major coronary artery. It is also important to note that a single copy of the deletion allele did not elevate the calcium score above the level in carriers of the non-deletion ancestral allele (see **Figure 4**). This result is also consistent with our prior report on the association with CAD being more robust in a recessive model than in an additive model (12). While in cultured HAoSMC a single copy of the deletion allele was sufficient to alter the expression of IRF2BP2 in response to LPS, *in vivo*, two copies of the deletion allele may be required to manifest the calcification phenotype.

The lack of association of the rs3045215 deletion allele with coronary calcification in CAD cases is perhaps not surprising given that atherosclerosis is the major driver of calcification in these individuals. However, the finding that men with two copies of the deletion allele have minimal burden of atherosclerosis yet moderate calcification (Agatston score of 254) would significantly increase their risk for cardiovascular events compared to men with one or no copies (Agatston scores of 110 and 113, respectively) (42). Moreover, this result suggests that calcification associated with the rs3045215 deletion may precede atherosclerosis.

Our study has several limitations. First, we do not know the consequence of the deletion allele on the response of macrophages to LPS, since our studies used THP-1 macrophages homozygous for the non-risk allele of rs3045215. Second, our study used a limited number of HAoSMC primary cultures, 3 from homozygous non-risk donors and 2 from heterozygous donors. While all 3 homozygous non-risk donor HAoSMC showed increased IRF2BP2 levels after LPS treatment (in contrast to THP-1 macrophages), heterozygous cells showed either no response or reduced IRF2BP2 levels after LPS. Third, we have not identified the RNA-binding protein that targets the rs3045215 deleted sequence. While this protein may be related to the ELAVL1 partner protein TTP (ZFP36), there is a prohibitive number of RNA-binding protein candidates to test (including ELAVL2, ELAVL3, ELAVL4, ZFP36, ZFP36L1, ZFP36L2, and among others) (43). While the 9 nucleotide rs3045215 deletion might disrupt a micro-RNA binding target or a putative alternative polyadenylation site, these are unlikely mechanisms. First, while the rs3045215 deletion lies immediately adjacent to a conserved target of the miR-17-93 cluster of micro-RNAs (<http://www.targetscan.org/>) it does not actually disrupt the consensus sequence and is unlikely to affect regulation by this mechanism. Second, IRF2BP2 is one of many genes that select proximal alternative polyadenylation sites to shorten their mRNAs upon immune activation,

reducing the available micro-RNA targets in their mRNAs (44). However, the rs3045215 deletion does not coincide with any documented alternative polyadenylation signal (https://exonapps.wistar.org/PolyA_DB/v3/). What our study has established is that the deletion variant affects how ELAVL1 modulates the IRF2BP2 3'UTR to control protein expression. Moreover, our study identified a novel association of the rs3045215 deletion with coronary artery calcification in men with a minimal burden of atherosclerosis. This finding will need to be confirmed in other geographically distinct independent CT angiography cohorts.

A previous Mendelian randomization analysis revisited the contribution of genetic variants at 23 loci contributing to atherosclerosis to ascertain whether these variants also predispose to coronary artery calcification beyond their effect on atherosclerosis (45). Of 23 loci showing genome-wide significant association with coronary atherosclerosis, only two remained genome-wide significant for coronary artery calcification when allele frequencies were compared between individuals with atherosclerosis with minimal calcification to individuals with calcified atherosclerosis. One was the well-known locus at 9p21.3 and the other was at the PHACTR1 locus. Thus, these loci contribute not only to atherosclerosis, but also to the process of coronary artery calcification beyond that which associates with atherosclerosis. The mechanisms whereby these loci contribute to calcification remain unknown.

Single gene mutations are known to cause arterial calcification independent of atherosclerosis. For example, mutations in the ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) gene cause generalized arterial calcification of infancy (46), due to a failure to produce pyrophosphate required to inhibit hydroxyapatite crystal growth in the vessel wall. It is intriguing that heterozygous carriers of ENPP1 mutations are prone to osteoporosis (47). On the other hand, loss of ABCC6, the transporter that carries ATP outside the cell required for pyrophosphate generation causes pseudoxanthoma elasticum, a condition associated with peripheral artery calcification (48, 49) that does not appear to predispose to osteoporosis (50). Thus, mechanisms that cause arterial calcification may not always contribute to osteoporosis. Similar to what we have reported here, a genetic variant near the gene encoding the matrix GLA protein (MGP) that also inhibits hydroxyapatite crystal growth has been tied to osteoporosis (25, 26) and is in linkage disequilibrium with a genetic variant in the MGP 5'-UTR tied to arterial calcification independent of atherosclerosis (51). Over 500 loci show genome-wide significant association with osteoporosis (25). It will be interesting to determine whether any of these osteoporosis risk alleles increase coronary artery calcification in CT angiography cohorts randomized by the degree of atherosclerotic burden, as we have done here. Some of these osteoporosis risk variants may contribute to coronary artery calcification either with or without atherosclerosis.

Recent studies have shown that IRF2BP2 activates KLF2 and plays a central role in bone formation by suppressing osteoclasts and promoting osteoblast differentiation (52). IRF2BP2 also

works as a corepressor of NFAT1 (53) and this action may limit the NFAT1-dependent activation of osteoclasts. Thus, reduced expression of IRF2BP2 tied to the rs3045215 deletion may explain why individuals who carry the co-inherited SNP rs6672925 have increased susceptibility to osteoporosis. Here, we found that ELAVL1 plays an important part in mediating differential expression of IRF2BP2 by suppressing mRNAs carrying the rs3045215 deletion allele. ELAVL1 knockdown increases osteogenic differentiation and the mRNA levels of genes controlling the extracellular matrix (54). ELAVL1 also protects against non-alcoholic fatty liver disease (NAFLD) (55), a condition that associates with increased risk of coronary artery calcification (56). NAFLD is made worse by ablation of *Irf2bp2* in the mouse (57). Together with recent work tying ELAVL1 in mediating the pro-atherosclerosis effect of a macrophage-specific lncRNA (24), these studies point to an interesting parallel that may contribute to arterial calcification mediated through ELAVL1 modulation of IRF2BP2.

DATA AVAILABILITY STATEMENT

All data are available in the main text or the supplemental material. Only summary patient data and genotypes are provided

to protect the confidentiality of study participants. Data are available upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ottawa Health Science Network Research Ethics Board (OHSN-REB). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RV and AD obtained and analyzed the data. RV, AD, FS, H-HC, and AS wrote the manuscript. H-HC and AS obtained research funding. All authors contributed to the article and approved the submitted version.

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7.2. Co-authored paper

Title: Savior Siblings Might Rescue Fetal Lethality, but not Adult Lymphoma in *Irf2bp2*-Null Mice



Savior Siblings Might Rescue Fetal Lethality But Not Adult Lymphoma in *Irf2bp2*-Null Mice

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Interferon regulatory factor 2 binding protein 2 (*Irf2bp2*), a co-repressor of *Irf2*, is required for fetal hepatic erythropoiesis through the expansion of erythromyeloid progenitors. Mice with germline ablation of the entire *Irf2bp2* transcript produced no viable *Irf2bp2*-null pups in first litters. In subsequent litters, fewer than 1/3 of the expected *Irf2bp2*-null pups were born and half survived to adulthood. As in humans with somatic mutations in *IRF2BP2*, adult *Irf2bp2*-null mice developed lymphoma. Transcriptome profiling of liver, heart, and skeletal muscle from *Irf2bp2*-null adult mice revealed a predominant upregulation of interferon-responsive genes. Of interest, hematopoietic stem cell-enriched transcription factors (*Etv6*, *Fli1*, *Ikzf1*, and *Runx1*) were also elevated in *Irf2bp2*-null livers. Intriguingly, *Irf2bp2*-positive myeloid (but not lymphoid) cells were detected in the livers of adult *Irf2bp2*-null mice. In female *Irf2bp2*-null mice, these cells carried a Y chromosome while in male *Irf2bp2*-null livers, no cells with Barr bodies (inactivated X chromosomes) were detected, indicating that *Irf2bp2*-positive erythromyeloid cells might be acquired only from male siblings of prior litters by transmaternal microchimerism. These cells likely rescue the deficit in fetal erythropoiesis, but not adult-onset lymphomagenesis, caused by *Irf2bp2* ablation.

Keywords: germline deletion, myeloid cells, microchimerism, adult lymphoma, transcriptome profiling

INTRODUCTION

Interferon-responsive genes are activated by upregulation of interferon regulatory factor 1 (IRF1) that binds to interferon-responsive *cis*-regulatory DNA sequences. Under basal conditions these genes are maintained in a repressed state by competitive binding of the constitutively expressed related factor IRF2 (1). IRF2 owes its repressor function to its interaction with IRF2BP2 (2) that recruits the corepressor NCOR1 (3). IRF2BP2 is one of 3 members of the IRF2BP family of structurally related proteins distinguished by an amino-terminal zinc finger motif and a C-terminal ring finger domain (2). IRF2BP2 also acts as a corepressor of the nuclear factor of activated T cells (NFATC2) (4). On the other hand, we and others have also observed a transactivation function for this protein. We reported that IRF2BP2 acts

as a co-activator of the TEAD/VGLL4 complex to promote gene expression in skeletal and cardiac muscle cells (5). More recently, IRF2BP2 was also reported to co-activate transcription regulated by the glucocorticoid and androgen steroid hormone receptors (6). Human IRF2BP2 genetic polymorphisms that reduce IRF2BP2 expression are tied to coronary atherosclerosis (7) and coronary artery calcification (8). Selective ablation of Irf2bp2 in macrophages triggers an inflammatory response and worsens atherosclerosis in mice (7).

Mice with global ablation of Irf2bp2 by a gene trap mutation that inserts a splice acceptor after the first exon die during fetal development (3). The lethal phenotype was tied to deficient fetal erythropoiesis (3), a process that relies on the expansion of an erythromyeloid lineage in the fetal liver (9). These Irf2bp2-deficient mice produce a truncated Irf2bp2 chimeric mRNA that contains the zinc finger motif encoded by the first exon fused to a β -galactosidase/neomycin transcript. The zinc finger motif of Irf2bp2 mediates protein-protein interaction with other Irf2bp family members, whereas the nuclear localization signal of Irf2bp2 is encoded by the second exon (10). Thus, it was unclear to what extent the lethal phenotype of the gene trap mutant was due to loss of functional Irf2bp2 or aberrant function of the chimeric protein.

In humans, rare germline autosomal dominant mutations in IRF2BP2 have been linked to a familial form of common variable immune deficiency disorder (11, 12). On the other hand, somatic mutations of IRF2BP2 are associated with tumors of the lymphoid lineage, suggesting a role of IRF2BP2 loss-of-function in lymphomagenesis. For example, mutations that cause fusions of the IRF2BP2 transcript with the retinoic acid receptor RARA are found in human promyelocytic leukemia (13, 14). Single nucleotide substitutions in the coding sequence of IRF2BP2 mRNA are often detected in patients with primary mediastinal large B cell lymphoma (15, 16) and in T cell lymphoma (17). To date, a mouse model replicating the lymphomagenic phenotype observed in humans with loss-of-function mutations in Irf2bp2 has not been reported.

Lethal anemias can be rescued with transplantation of human leukocyte antigen (HLA)-matched bone marrow from a sibling. If none exists, some parents resort to preimplantation genetic diagnosis of human zygotes after *in vitro* fertilization to select embryos for implantation that are free of the genetic defect causing the anemia yet are HLA-compatible with their child affected by the lethal anemia. After birth, these “savior” siblings provide their bone marrow for transplantation to their affected sibling to replace the defective hematopoietic stem cells (18). Here, we describe a natural process of transmaternal microchimerism in mice where a lethal deficit in fetal erythropoiesis due to loss of Irf2bp2 appears to be rescued by erythromyeloid stem cells that are retained in the mother from male “savior” siblings of prior litters.

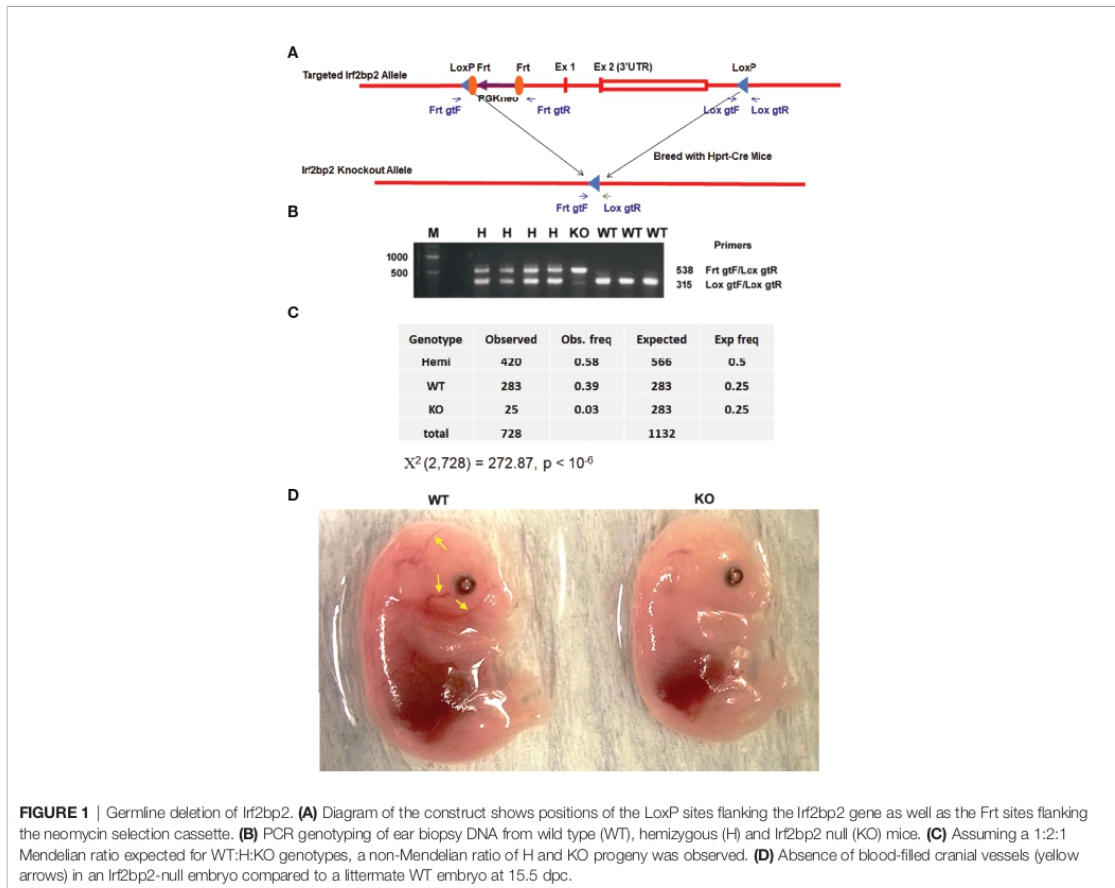
RESULTS

We generated mice with loxP sites that bracket the entire Irf2bp2 gene (~6,000 base pairs, including exon 1, exon 2 and the intron) (7). These mice were mated to Hprt1-Cre mice (19) to obtain

germline deletion of Irf2bp2 (**Figures 1A, B**). A non-Mendelian ratio of Irf2bp2 null and hemizygous progeny was observed (**Figure 1C**). Over a 9-year period, the occurrence of newborn Irf2bp2-null mice was initially thought to represent rare cases that survived fetal lethality, but further examination revealed that Irf2bp2-null mice only occurred in 2nd and 3rd litters of multiparous dams. No Irf2bp2 null mouse was found in first litters (168) and only a small number of Irf2bp2 null mice were born from 2nd or 3rd litters (25 in 62 litters, ~1/3 of expected) of multiparous dams. Thus, most Irf2bp2 null mice die during development. Half of Irf2bp2 null mice born from 2nd and 3rd litters died within the first few days after birth while the other half survived up to 1 year. To determine why Irf2bp2 null mice die during development, a series of embryos from 9.5 to 18.5 days post-coitum (dpc) was examined in first litters of pregnant dams. Viable null embryos were detected up to 15.5 dpc, but not at 16.5 or 18.5 dpc. Of the only 2 Irf2bp2-null embryos at 15.5 dpc, one was degenerating and the other had a marked reduction in blood-filled vessels (**Figure 1D**, compare littermate WT mouse, yellow arrows), in line with the requirement for Irf2bp2 in fetal liver erythropoiesis (3).

Since viable adult Irf2bp2-null mice have not been obtained previously (3), we carried out RNA profiling of adult liver, heart, and skeletal muscle to reveal shared and tissue-specific Irf2bp2-dependent gene programs. Northern blot analysis of total RNA isolated from 3 male Irf2bp2-null and 3 male age-matched wild type mice confirmed loss of Irf2bp2 mRNA in these tissues (**Figures 2A, B**). Two major transcripts were detected with the 3 kilobase (kb) cDNA probe encompassing the entire 3' untranslated sequence of the mouse Irf2bp2 mRNA, one at ~5 kb representing the full length cDNA and another at ~3 kb representing the use of a proximal alternative polyadenylation signal (see the alternative polyadenylation database, <http://tools.genxpro.net/apadb/> for details). Note that liver expresses predominantly the shorter transcript, whereas heart and skeletal muscle express both and the longer transcript is ~6-10 times less abundant than the shorter transcript. An aliquot of these RNA samples was used to probe cDNA microarrays to identify differentially expressed genes (**Figures 2C–H** and **Supplementary Table S1**, ArrayExpress accession E-MTAB-11558).

In all 3 tissues, volcano plots (**Figures 2C–E**) revealed that a preponderance of genes was upregulated in Irf2bp2 null mice, reflecting the co-repressor function of Irf2bp2. Venn diagrams also compared the distribution of genes differentially expressed between Irf2bp2 null and wild type mice in each of the 3 tissues (**Figures 2F–H**, **Supplementary Table S1**). In keeping with the repressor function of Irf2bp2, pathway analysis of transcription regulation indicated that more transcription factors are activated (**Figure 2I**) than inhibited (**Figure 2J**) by Irf2bp2 ablation. Nearly all activated genes (**Supplementary Table S1**) were interferon responsive (<http://www.interferome.org/>). Consistent with loss of Irf2 repression function, increased Irf1 and Irf3 activity was among the transcription mechanisms activated by Irf2bp2 ablation in all 3 tissues (**Figure 2I**). It should be noted that we did not observe a change in the expression of other



Irf2bp-family members (Irf2bp1 and Irf2bp1), in contrast to what was reported for the gene-trap mutant mice (3).

Other transcription regulatory mechanisms activated as a consequence of Irf2bp2 ablation in all 3 tissues (Figure 2I) were Spi1 (aka hematopoietic transcription factor PU.1), the NFkB subunits Nfkb1, Nfkb2, RelA, and the RelA-interacting protein myotrophin MTPN (20), the Class II Major Histocompatibility Complex Transactivator Ciita, the nuclear factor of activated T cells Nfat5, the basic helix-loop-helix (bHLH) and PAS-domain proteins Arnt2 and Sim1. Contrary to the reported co-activator function for Irf2bp2 with the glucocorticoid receptor (6), the classic glucocorticoid-inducible metallothionein genes (21) Mt1 and Mt2 were upregulated in all 3 Irf2bp2-null tissues (Supplemental Table S1), suggesting instead a co-repressor function of Irf2bp2 with the glucocorticoid receptor.

Transcription factors activated in both liver and muscle include Irf8, Jun, Egr1, Cebpa, Foxl2, the hypoxia-inducible factor Hif1a, and Runx1, a key regulator in hematological malignancies (22). Transcription factors activated in both liver and heart include Irf5 and Irf7, the chromatin remodeling factors

Smarca4 and Hmgb1, the signal transducers of activated T cells STAT1 and STAT4, the TGF beta receptor signaling transcription factors Smad3 and Smad4, Ets2, ATF2 (a Jun partner), and the bHLH-PAS protein EPAS1 (aka, Hypoxia-inducible factor 2a). All these factors are likely co-repressed by Irf2bp2. Surprisingly, in the Irf2bp2-null liver, many transcription factors of hematopoietic stem cells (23), including Etv6, Fli1, Ikzf1, and Runx1 were elevated, perhaps reflecting a compensatory mechanism to promote hematopoiesis in the absence of Irf2bp2.

Among transcription factors whose activity was reduced in all 3 tissues by Irf2bp2 ablation were the repressors Sirt1 and Gfi1. Between liver and muscle, activity of the E3 ubiquitin ligase Cbl was reduced, as were Trim24, Bcl6, Nkx2-3, Taf4, Ikzf2 and Prdm16 in liver and heart. Intriguingly, many of the factors that show reduced activity in Irf2bp2 null mice are known to be transcription repressors themselves.

Lymphoma was a prevalent phenotype of adult Irf2bp2-null mice over the age of 6 months in both male and female mice; upon sacrifice, mice displayed an enlarged spleen (Figure 3A) and liver (Figure 3B). Histological examination revealed

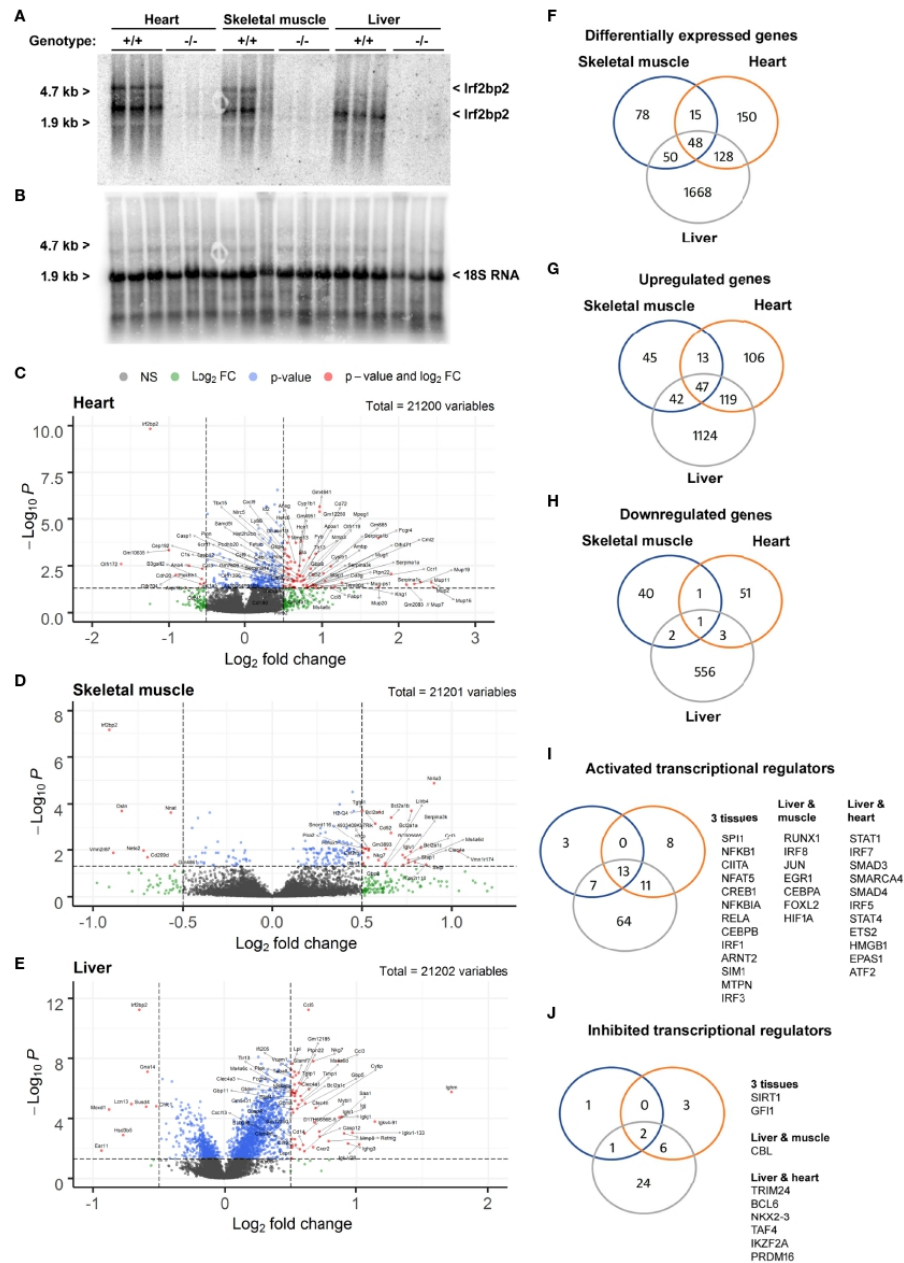


FIGURE 2 | Differentially expressed genes in heart, skeletal muscle, and liver from Irf2bp2-null adult mice. **(A)** Northern blot with total RNA from heart, skeletal muscle and liver of 3 wild type (WT) and 3 Irf2bp2 null (KO) male mice probed with the 3'UTR of Irf2bp2 confirms loss of Irf2bp2. **(B)** Northern blot was stripped and re-probed with 18S RNA to control for loading. **(C–E)** Volcano plots show preponderance of upregulated genes in Irf2bp2-null heart, muscle, and liver tissues. **(F–J)** Venn diagrams show overlap of: **(F)** differentially expressed genes, **(G)** upregulated and **(H)** down-regulated genes in 3 tissues. Ingenuity[®] pathway analysis identified **(I)** activated and **(J)** inhibited transcription factors common to different Irf2bp2-deficient tissues. Also see **Supplementary Figures 1–3** for heatmaps and supplemental Tables 1–7 for gene lists.

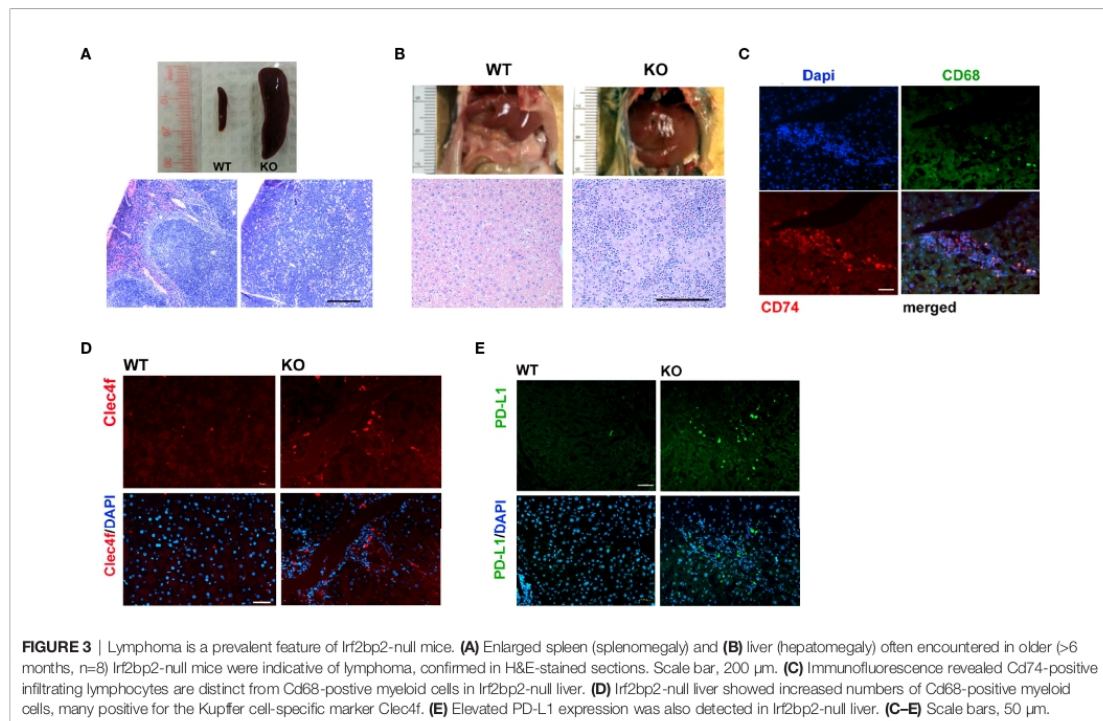


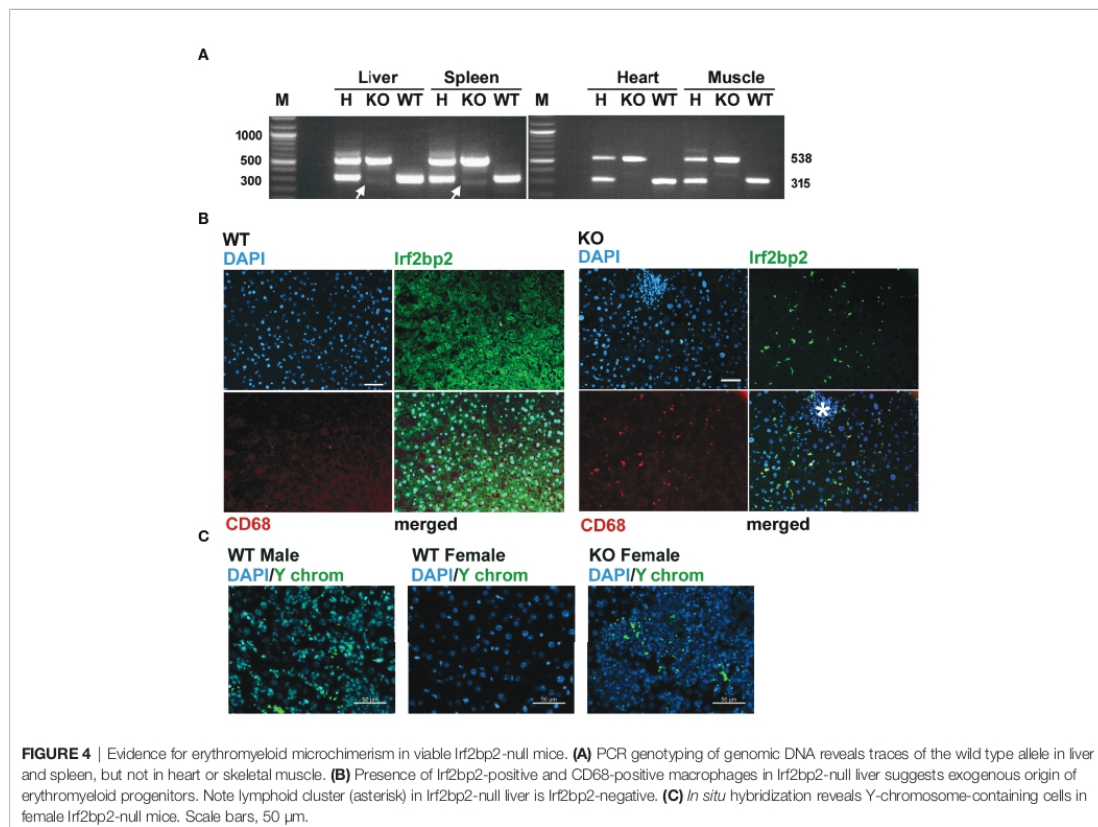
FIGURE 3 | Lymphoma is a prevalent feature of Irf2bp2-null mice. **(A)** Enlarged spleen (splenomegaly) and **(B)** liver (hepatomegaly) often encountered in older (>6 months, n=8) Irf2bp2-null mice were indicative of lymphoma, confirmed in H&E-stained sections. Scale bar, 200 μm. **(C)** Immunofluorescence revealed Cd74-positive infiltrating lymphocytes are distinct from Cd68-positive myeloid cells in Irf2bp2-null liver. **(D)** Irf2bp2-null liver showed increased numbers of Cd68-positive myeloid cells, many positive for the Kupfer cell-specific marker Clec4f. **(E)** Elevated PD-L1 expression was also detected in Irf2bp2-null liver. **(C–E)** Scale bars, 50 μm.

disorganized cytoarchitecture in the spleen, with a complete displacement of red pulp (venous sinus with red blood cells) by lymphocyte-rich white pulp, consistent with lymphoma (**Figure 3A**). The livers of Irf2bp2 null mice also showed extensive lymphocytic infiltration (**Figure 3B**) and this was associated with upregulation of many lymphocyte-specific genes (including Cd74, Lsp1, Bcl2a1a, Bcl2a1c, Bcl2a1d, etc.) (**Supplementary Table S1**). Immunofluorescence using Cd74 antibody confirmed lymphoid infiltration in the liver tissue (**Figure 3C**). Lymphocytic infiltration was even detected within the ventricular myocardium (**Supplementary Figure 6**). We also observed elevated expression of myeloid-specific genes Cd68 and Clec4f in Irf2bp2 null livers (**Supplementary Table S1**), and immunofluorescence confirmed a higher number of Cd68 and Clec4f-positive Kupfer cells (**Figure 3D**).

Our microarray data revealed a 3.5-fold increase in PD-L1 (Cd274) expression in Irf2bp2-deficient livers and 1.7-fold increase in the Irf2bp2-deficient heart (**Supplementary Table S1**). PD-L1 expression is known to be suppressed by Irf2bp2 (24, 25). PD-L1 encodes a ligand for PD-1 (aka Pdc1), a receptor on T lymphocytes that suppresses cancer growth (26). PD-L1 inhibits PD-1 function and is permissive to cancer growth. A marked increase in PD-L1-immunopositive cells was detected in Irf2bp2 null livers (**Figure 3E**), consistent with our array data. Elevated PD-L1 expression is documented in B cell lymphoma (27), and elevated PD-L1 expression likely facilitates lymphoma proliferation in these tissues.

Since Irf2bp2 null embryos are detected in the first litter but none survive to term, this result indicates germline ablation of Irf2bp2 is lethal during fetal development. An important question is why a few Irf2bp2-null mice from the 2nd and 3rd litters survive postnatally. PCR genotyping had revealed trace amounts of the wild type allele in genomic DNA from ear biopsies of these Irf2bp2-null mice (**Figure 1B**). Initially, we thought this might be a contaminant since the northern blot (**Figure 2A**) and microarray data (**Supplementary Table S1**) clearly showed loss of Irf2bp2 mRNA in the 3 tissues. However, further comparison of the microarray data revealed that the residual Irf2bp2 signal in the liver of Irf2bp2 null mice was higher than the signal in the heart or skeletal muscle for the same mice. Intrigued by these apparent contradictory data, we carried out further PCR genotyping of DNA isolated from these 3 tissues in Irf2bp2 null mice. While all tissues revealed the floxed Irf2bp2 PCR product, trace levels of the wild type Irf2bp2 allele were detected in DNA from the liver and spleen, but not in skeletal muscle or heart of Irf2bp2-null mice (**Figure 4A**).

Immunofluorescence confirmed the presence of a few Irf2bp2-positive cells in the liver of Irf2bp2-null mice compared to all cells being Irf2bp2-positive in wild type mice (**Figure 4B**). Importantly, these Irf2bp2-positive cells detected in Irf2bp2 null livers were all Cd68-positive myeloid cells (**Figure 4B**). It is also noteworthy that the cluster of lymphocytes (infiltrating lymphoma, **Figure 4B**, asterisk) in the Irf2bp2 null liver was Irf2bp2 negative. The presence of



Irf2bp2-positive myeloid cells in the liver of Irf2bp2-null mice suggests that these cells are of exogenous origin.

Microchimerism is a condition whereby embryos acquire from their mother or from prior siblings, exogenous progenitor cells that persist into adulthood (28). If the Irf2bp2-positive myeloid cells in Irf2bp2 null livers were of maternal origin, we should have detected female-specific immune transcripts in the RNA profiles of the 3 male Irf2bp2-null mice: the X-chromosome inactivation transcript *Xist*, *Rsa2* and *Oas3* (29). However, none of these transcripts was significantly elevated in male Irf2bp2 null livers compared to WT (*Xist*, BH = 0.987, KO/WT = 1.01; *Oas3*, BH = 0.191, KO/WT = 1.256; *Rsa2*, BH = 0.0874, KO/WT = 1.59). It's important to point out that the microarrays detected elevated expression of the myeloid-specific transcripts *CD68* and *Clec4f* in the livers of Irf2bp2 null mice (**Supplementary Table S1**) and immunofluorescence confirmed that these markers were only detected in cells that are also Irf2bp2-positive (**Figure 4B**). Therefore, if these Irf2bp2-positive cells were of maternal origin, the *Xist* RNA should also have been detected. In line with this observation, we were also unable to detect Barr bodies (inactivated X chromosomes) in the nuclei of myeloid cells in the livers of Irf2bp2-deficient male mice, while Barr bodies were readily detectable in female livers

(**Supplementary Figure S7**). Thus, given that maternal myeloid-specific transcripts and inactivated X chromosomes were not detected in male Irf2bp2 null livers, the Irf2bp2-positive myeloid cells are unlikely to be of maternal origin, but rather these are likely erythromyeloid progenitor cells left behind from male siblings of prior litters. Indeed, *in situ* hybridization confirmed the presence of cells bearing the male Y chromosome in liver tissue of 3 female Irf2bp2-null mice (**Figure 4C**).

DISCUSSION

Here, we showed that germline ablation of Irf2bp2 causes fetal lethality in mice, since no viable Irf2bp2 null mice were detected in 1st litters. The presence of a few viable Irf2bp2-null mice in 2nd and 3rd litters carrying Irf2bp2-positive myeloid cells in their livers suggests that these mice are microchimeric, acquiring cells from siblings of prior litters that likely rescue Irf2bp2-deficient mice from fetal lethality. However, since Irf2bp2-positive lymphoblasts are not detected in Irf2bp2-null mice, these chimeric mice are not protected from adult-onset lymphoma caused by loss of Irf2bp2.

In humans, microchimerism (the contribution of exogenously acquired stem cells to the developing fetus) is well known to persist into adulthood (28). The effect of microchimerism can be beneficial in some instances. For example, hemophilia A patients lacking blood clotting factor viii develop alloantibodies to this protein after repeated transfusions. However, rare cases exist where hemophilia A patients do not develop these antibodies because they acquire maternal cells that produce factor viii and induce tolerance (30). While microchimerism may induce tolerance to exogenous factor viii, hemophilia A is not considered life-threatening. Since viable Irf2bp2-null chimeric mice were only found in 2nd and 3rd litters, and because we were unable to detect maternal transcripts or inactivated X chromosomes (Barr bodies) but did find Y chromosome in all female KO livers tested, we propose that sibling microchimerism may account for these cells.

Sibling chimerism has been reported in human dizygotic twins to account for chimeric ABO blood groups (31). Moreover, transmaternal microchimerism as we describe here has been documented in humans where male stem cells transferred from older brothers to their younger sisters were detected in umbilical cord blood samples (32). Remarkably, half of the daughters with older brothers were microchimeric with male cells. However, we could find no report on microchimerism rescuing fetal lethality in humans or mice. To our knowledge, our finding of sibling microchimerism in sequential litters of Irf2bp2 null mice would be the first example of microchimerism rescuing fetal lethality.

Lethality from targeted truncation of Irf2bp2 has been ascribed to a deficit in fetal erythropoiesis, a process that takes place largely in the liver (3). Our observation of empty cranial vessels lacking erythrocytes in an Irf2bp2-null fetus is consistent with this mechanism. The proliferation of erythromyeloid cells in the fetal liver is critical for erythropoiesis during late fetal development (9). The presence of Y-chromosome-containing cells in the livers of viable female Irf2bp2-null mice suggests these cells may be critical to rescue fetal erythropoiesis. The absence of Barr bodies in similar cells of male mice suggests a preferential transfer of male progenitor cells. Unfortunately, because antigen retrieval for Irf2bp2 immunofluorescence was incompatible with *in situ* hybridization of the Y chromosome probe, this technical limitation prevented us from showing that Y-chromosome positive cells were also Irf2bp2-positive. However, given that the number of Y-chromosome-positive and Irf2bp2-positive cells is similar in female Irf2bp2-null mice and that Y-chromosome containing cells were not detected in wild type female livers, these observations suggest they are the same cells. About one third of the expected number of Irf2bp2-null mice was detected in 2nd and 3rd litters, suggesting that transmaternal microchimerism may not always occur, as documented in humans (32), or that sibling-derived myeloid stem cells may not always be acquired in sufficient numbers to rescue Irf2bp2-null mice. Definitive studies to address the frequency and numbers of cells transferred by microchimerism could be addressed with the Irf2bp2-deficient mice generated by the gene trap expressing a β -galactosidase reporter (3), but these

studies are beyond the scope of the present manuscript. Chimeric Irf2bp2-null mice that survived to adulthood had a relatively normal hematocrit suggesting their myeloid chimerism may have been adequate to support erythropoiesis through adulthood.

We previously discovered Irf2bp2 as a positive coactivator of the transcription cofactor Vgll4 (5). A recent study in zebrafish reported that Vgll4b is part of a Hif1a/Irf2bp2 complex essential for erythropoiesis (33). Paradoxically, transcription factors enriched in hematopoietic stem cells including Etv6, Flil, Runx1, and Spi1 were upregulated in tissues of viable Irf2bp2-deficient adult mice, as were several genes tied to erythropoiesis including the oncostatin M receptor. Whether this reflects a compensatory activation due to loss of Irf2bp2 remains unclear. Alternatively, the presence of Irf2bp2-positive chimeric myeloid cells might contribute to upregulation of these erythropoietic factors. We reported that Irf2bp2 protein levels are elevated in response to ischemia in skeletal and cardiac muscle (5), and this would be consistent with stimulated erythropoiesis in chimeric Irf2bp2-null mice.

Those 47 genes that were upregulated in the heart, skeletal muscle and liver tissues, suggest a common Irf2bp2-dependent regulatory mechanism for these genes in these 3 tissues. Conversely, many other genes showed tissue-specific changes in their expression with Irf2bp2 ablation suggesting Irf2bp2 affects transcription differently in different tissues. For example, Irf2bp2 promotes expression of anti-inflammatory genes in macrophages (7). Irf2bp2 is down regulated by lipopolysaccharides (LPS) and ablation of Irf2bp2 in macrophages activates inflammation, impairs macrophage cholesterol efflux and worsens atherosclerosis (7). Ablation of Irf2bp2 in microglia hinders recovery from focal ischemic brain injury (34, 35) and blocks the anxiety-reducing effect of enhanced perinatal maternal care (36). In the heart, Irf2bp2 functions differently. LPS elevates cardiac Irf2bp2 protein levels and Irf2bp2 overexpression in the heart protects against LPS-induced contractile dysfunction (37). In addition, Irf2bp2 protects the heart from hypertrophic stimuli (38) likely by repression of the hypertrophic transcription factor NFAT1 (aka Nfatc2) (4). It is noteworthy that our array data confirmed that Nfatc2-dependent gene expression was significantly activated in the heart of Irf2bp2 null mice.

The presence of lymphoma in Irf2bp2-deficient/chimeric mice was noteworthy because it suggests that loss of Irf2bp2 facilitates this process. It is important to point out that the erythromyeloid lineage does not give rise to lymphocytes (9), so that lymphoid progenitors in chimeric Irf2bp2-null mice should remain Irf2bp2-deficient. Diverse viral pathogens are tied to lymphomagenesis including Epstein Barr virus in Burkitt's lymphoma, Kaposi's sarcoma virus, among others. Lymphoma in mice can arise from spontaneous activation of endogenous retroviral sequences (39). Adult T-cell leukemia/lymphoma in humans is caused by the HTLV-1 (human T-cell leukemia virus) retrovirus and is often tied to mutations in Irf2bp2 (40). Whether lymphomagenesis is a direct consequence of Irf2bp2 loss of function or whether Irf2bp2 deficiency increases susceptibility

to virus-induced tumors remains to be determined. Our mouse model should enable studies of these mechanisms of lymphomagenesis.

Our finding suggesting that sibling microchimerism in mice can rescue the fetal lethality caused by failure of adequate hepatic erythropoiesis due to *Irf2bp2* ablation is potentially clinically important. Given that sibling microchimerism has also been described in humans (31, 32), it will be important to test whether human carriers of autosomal dominant mutations in *IRF2BP2* that develop common variable immunodeficiency disorder (11, 12) carry otherwise healthy myeloid cells from older healthy siblings that overcome fetal lethality.

METHODS

Generation and Genotyping of *Irf2bp2*-Null Mice

All procedures in mice were approved by the University of Ottawa Animal Care and Use Committee in accordance with the guidelines of the Canadian Council on Animal Care. C57B6-*Irf2bp2*-floxed mice were produced at the University of Connecticut transgenic core facility and mated to *Hprt1*-Cre mice (19) to enable germline deletion of the entire *Irf2bp2* gene (see **Figure 1A**). PCR genotyping used primers to *Irf2bp2* sequence flanking the *LoxP*/Frt site 5' to the neomycin cassette, and to 3' sequences flanking the 3' *loxP* site, FRT gene target forward (FrtgtF), 5'-TAACCTTGACTCTTGACAGC-3', *LoxP* gene target reverse (LoxgtR), 5'-CCCTTCATGTAAAGTATTCTCACTAGG-3', and *LoxP* gene target Forward (LoxgtF), 5'-GTGCTCCTTAAGTGTGCAG-3'. Hemizygous mice were interbred for several consecutive litters and progeny were genotyped by PCR of genomic DNA.

Timed Pregnancies

To obtain fetuses, timed matings were carried out by intercrossing *Irf2bp2*^{+/-} mice and designating 0.5 dpc (days post coitum) to the day of identification of a vaginal plug. Fetuses were harvested from the first litter of a single pregnant female sacrificed by CO₂ euthanasia at each time point at 11.5, 13.5, 15.5, 16.5 and 18.5 dpc. Fetuses were separated from the placenta and yolk sac, and tails were removed for genotyping.

RNA Isolation

Total RNA was isolated from WT and *Irf2bp2*^{-/-} mouse heart, skeletal muscle, and liver (n=3 males per genotype) using the RNeasy Midi Kit (#75142, Qiagen) for Northern blot and microarray analysis. RNA concentrations and A260/230 purity ratios were measured with the NanodropTM 2000 (Model #ND-2000, Thermo Scientific).

Northern Blot

Total RNA (15 µg/lane) was fractionated on a 3-(N-morpholino)-propanesulfonic acid (MOPS) buffered formaldehyde agarose gel at 80V for 3 hours, as we recently described (41). The gel was washed 4 times in diH₂O, soaked for

15 minutes in water containing 50 µL ethidium bromide (10 mg/mL), then washed again 6 times in diH₂O and photographed under UV light to visualize the 18S and 28S RNAs. RNA was transferred to nylon membrane (GeneScreen Plus Hybridization Transfer Membrane, #NEF988, Perkin Elmer) by overnight capillary transfer in 10X SSPE solution (1.5 M NaCl, 100 mM NaH₂PO₄, 100 mM Na₂EDTA). RNA was immobilized by UV crosslinking and membrane was prehybridized in ExpressHybTM Hybridization Solution (#636832, Clontech) at 65°C for 1 hour.

The *Irf2bp2* 3'UTR cDNA probe was released by *Sall*/*NotI* digestion (clone ID #6476448, Integrated DNA Technologies) purified from an agarose gel. 50 pmol of probe was denatured, random primed with Klenow fragment using the Random Primers Labeling Kit (#18187013, Life Technologies Inc), and radioactively labeled with [α -³²P]dCTP (#BLU013H250UC, Perkin Elmer Health Sciences). A cDNA probe for 18S RNA (control) was radioactively end-labeled with [γ -³²P]-ATP (#BLU502Z250UC, Perkin Elmer Health Sciences) in a T4 Polynucleotide Kinase (#M0201S, New England Biolabs) reaction. Radioactive probes were added to 10 mL of fresh hybridization solution and incubated with the pre-hybridized membrane overnight at 65°C. The next day, the membrane was transferred to a tray and washed 3 times with 0.2% SDS in 2X SSPE buffer. The membrane was then washed 2 times in 0.2% SDS in 0.2X SSPE buffer in a 50°C bath for 15 minutes each. The membrane was covered in plastic wrap and exposed to a storage Phosphor Screen overnight and image was developed on a STORM 860 phosphorimager (Amersham Biosciences).

Preparation of cDNA for Microarray Analysis

Microarray analysis was performed using Mouse Gene 1.0 ST Arrays (Affymetrix). Total RNA was isolated and purified from mouse heart, skeletal muscle, and liver as described previously, and samples were sent to the Affymetrix Microarray Facility at StemCore Laboratories (Ottawa Hospital Research Institute). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (#G2943CA, Agilent Technologies), which provides an RNA integrity number (RIN) from microfluidics analysis. The Ambion Whole-Transcript Expression kit was used in conjunction with the Affymetrix GeneChip Whole-Transcript Terminal Labeling and Controls Reagent kit to prepare samples for the Mouse Gene 1.0 ST Array.

RNA for microarray analysis was prepared by generating sense-strand cDNA for fragmentation and labeling with the Ambion Whole-Transcript Expression Kit (#4411974, Life Technologies). First-strand cDNA was synthesized by reverse-transcription from total RNA and second-strand cDNA was synthesized using DNA polymerase and RNase H simultaneously to degrade the RNA. Antisense cRNA was synthesized and amplified by *in vitro* transcription of the second-strand cDNA template using T7 RNA polymerase. cRNA was purified using Nucleic Acid Binding Beads, washed twice with Nucleic Acid Wash Solution, and eluted with 55°C preheated Elution Solution. The cRNA was briefly placed on ice

and its yield was assessed by UV absorbance at 260 nm. Next, 10 µg cRNA was mixed with random primers to synthesize 2nd-cycle cDNA by reverse transcription. The cRNA template was degraded with RNase H, and the cDNA was purified to remove enzymes, salts, and unincorporated dNTPs.

Histology and Immunofluorescence Microscopy

Tissues harvested for H&E staining were fixed in 4% paraformaldehyde (PFA) in PBS for 24–48 hours, then transferred to 70% ethanol. Samples were paraffin-embedded, sectioned at 5 µm thickness, then H&E stained by the University of Ottawa Department of Pathology and Laboratory Medicine. For immunofluorescence, samples were fixed in 4% PFA, dehydrated in 30% sucrose overnight and frozen 10 µm sections were processed for immunofluorescence. Antigen retrieval was carried out in citrate buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0) at 95°C for 20 minutes. Sections were blocked in 10% normal serum of the species used for secondary antibody. Antibodies were as follows: mouse monoclonal to the macrophage-specific antigen CD68 (mab101141 R&D Systems Inc.) was revealed with a chicken anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (#A-21441, ThermoFisher Inc.), the PD-L1 (CD274) antibody was a rabbit polyclonal (SAB4301882, Sigma Aldrich Inc.), the lymphocyte-specific antigen CD74 was a mouse monoclonal conjugated to Alexa Fluor 647 (FAB35901R, R&D Systems Inc., Minneapolis, MN), the Kupffer cell specific antigen Clec4f (mab2784, 1:200, R&D Systems Inc., Minneapolis, MN), Barr bodies were revealed by an anti-ubiquitinyl-Histone H2A antibody, as described (42), clone E6C5 (Sigma Aldrich Inc.) revealed with a goat anti mouse IgG, IgM(H+L) secondary antibody conjugated to Alexa Fluor 488 (A-10680, ThermoFisher Inc.). The rabbit anti-Irf2bp2 antibody was described previously (5). Nuclei were revealed with DAPI and sections were treated with Vector® TrueVIEW® Autofluorescence Quenching Kit to minimize autofluorescence (VECTSP8400, Vectorlabs Inc.) and mounted with coverslips in VECTASHIELD® Antifade Mounting Medium (VECTH1000, Vectorlabs Inc.). Images were acquired on a Zeiss M1 microscope.

In Situ Hybridization

Paraffin sections from liver tissue isolated from adult mice were probed with a Y chromosome-specific fluorescent probe (Creative Bioarray) and counterstained with DAPI. The probe was incubated overnight at 37°C for 16 hours, followed by autofluorescence quenching for 5 minutes (Vector Laboratories Vector TrueVIEW Autofluorescence Quenching Kit #SP-8400), then stained with DAPI for 10 minutes and mounted with a coverslip in VECTASHIELD mounting medium.

Ingenuity Pathway Analysis

Differentially expressed genes identified by array analysis were further compared by Ingenuity® pathway analysis (Qiagen) to identify upstream transcription factors whose target genes are upregulated or downregulated by Irf2bp2 ablation.

Statistical Analysis

The Robust Multi-array Average (RMA) algorithm was used to transform and normalize the raw intensity values of the probes using Affymetrix Expression Console™ Software. To identify differential gene expression between wildtype and knockout tissues, a Bayesian t-test analysis was performed using the Cyber-T analysis package for R (43). The Benjamini-Hochberg (BH) False Discovery Rate (FDR) method was used to correct for multiple testing and the significance threshold was set at 0.05, equal to an FDR of 5% (44). Probes that were not linked to a gene through annotation were excluded from analysis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: ArrayExpress accession E-MTAB-11558.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Ottawa Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

RV, TH, AD, NH, KK, and FS obtained and analyzed the data, RV, H-HC, and AS wrote the manuscript, and H-HC and AS obtained research funding. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.868053/full#supplementary-material>

Supplementary Figure 1 | Heat map of differentially expressed genes in liver of Irf2bp2 null mice compared to WT controls.

Supplementary Figure 2 | Heat map of differentially expressed genes in skeletal muscle of Irf2bp2 null mice compared to WT controls.

Supplementary Figure 3 | Heat map of differentially expressed genes in heart of Irf2bp2 null mice compared to WT controls.

Supplementary Figure 4 | Hematology profile from an adult Irf2bp2-null mouse.

Supplementary Figure 5 | Hematology profile from an adult hemizygous Irf2bp2 mouse.

Supplementary Figure 6 | Lymphocyte infiltration into the ventricular myocardium of an Irf2bp2-null mouse with lymphoma.

Supplementary Figure 7 | Absence of Barr bodies in liver of Irf2bp2-null male mouse.

Supplementary Table 1 | Lists of differentially expressed genes in Irf2bp2-null mice.

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