AN EXAMINATION OF MECHANISMS CONTRIBUTING TO CELL DEATH INDUCED BY METABOLIC STRESS IN CARDIOMYOBLASTS AND IMMUNE CELLS

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Abstract

Metabolic syndrome is an underestimated, growing global health crisis, plaguing millions of people worldwide. In this thesis, I assessed mechanisms of two important features of metabolic dysregulation; hyperglycemia and iron overload. This was accomplished across three projects; 1) examining mechanisms of adiponectin action in attenuating high glucose-induced cell death in cardiac cells and tissue, 2) observing inflammatory response in macrophages upon combining high glucose and iron treatments and 3) examining shifts in frequency of immune cell subsets in PBMCs treated with high glucose and iron. Briefly, results from study #1 revealed that an adiponectin mimetic, ALY688, was able to successfully attenuate high glucose-induced cell death in cardiomyoblasts and ventricular mouse tissue, in an autophagy-dependent manner. Interestingly, inflammatory agents released from cardiomyoblasts were eliciting inflammatory pathway activation in macrophages, which led to the formation of study #2. This study explored how two relevant metabolic stressors, high glucose and iron, could induce inflammation in macrophages. This study found that combining high glucose and iron led to significant activation of key inflammatory pathways, cytokine release and cell death in bone marrow-derived macrophages. These findings became the foundation for the next study to elucidate whether this robust response would be observed in primary human immune cells; peripheral blood mononuclear cells (PBMCs). Results from this study found no significant difference in frequency of T cells, though frequency of natural killer T cells were elevated upon treatment with high glucose and iron. In addition, combining high glucose and iron in PBMCs resulted in a profound increase in cytokine production, oxidative stress, mitochondrial dysfunction, and cell death. Overall, this thesis elucidates mechanisms of cellular damage induced by highly relevant metabolic stressors in cardiomyoblasts and immune cells. It further evaluates a therapeutic intervention and provides insight into axes that can be investigated to combat adverse metabolic outcomes.

Dedication

To my parents, with gratitude for their endless love, patience, and sacrifices. Thank you.

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BMI	body mass index
CMD	cardiometabolic disease
CVD	cardiovascular disease
DAMPs	damage-associated molecular patterns
EGIR	european group for the study of insulin resistance
ETC	electron transport chain
FOXO	forkhead box
G3P	glyceraldehyde-3-phosphate
GAP	gtpase activating proteins
GAPDH	g3p dehydrogenase
GLUTs	glucose transporters
HDL	high-density lipoprotein
IDF	international diabetes federation
IL	interleukin
IO	iron overload
IR	insulin receptor
IRF	interferon regulatory pathway
IRS	insulin receptor substrates
ISGU	insulin-stimulated glucose uptake
JIS	joint interim statement
LDH	lactate dehydrogenase
LDL	low density-lipoprotein
MetS	metabolic syndrome
MPT	mitochondrial permeability transition
mTOR	mammalian target of rapamycin
NCEP ATP III	national cholesterol education program adult treatment panel iii
NFκB	nuclear factor κ b
NHLBI	national heart, lung, and blood institute
NLRs	nod-like receptors
PBMCs	peripheral blood mononuclear cells

PI3K	type 1a phosphatidylinositol 3’ kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-triphosphate
PRRs	pattern recognition receptors
ROS	reactive oxygen species
T2D	type 2 diabetes
TLRs	toll-like receptors
TNF	tumour necrosis factor
TNFR	tnf receptor
WHO	world health organization

Chapter 1 – Introduction

1.1 Metabolic Syndrome

1.1.1 Background

Metabolic syndrome (MetS), an underestimated and growing worldwide concern, is a group of metabolic abnormalities that are significant risk factors for cardiovascular disease (CVD).[1–3] The term cardiometabolic disease (CMD) is used when evaluating the complex interactions between metabolic dysfunction and cardiovascular processes, essentially being the clinical outcome when examining the relationship between the two.[3,4] Interest in MetS and CVD started in the 1900s to cluster hypertension, hyperglycemia and obesity with their associated risks to develop CVD.[5–7] Over the decades, MetS has been given many names, 'Syndrome X' by Gerald Reaven in the 1988 Banting Lecture, 'The Deadly Quartet' by Norman Kaplan in 1989 and 'Insulin Resistance Syndrome' by Samuel Haffner.[6,8,9] Now however, 'Metabolic Syndrome' remains the most well-established and agreed upon term to describe metabolic abnormalities.[10–16]

The precise definition of MetS has evolved over the years to what has now been widely accepted as a group of conditions that increases risk of CVD and type 2 diabetes mellitus (T2D).[1,14,15] Many international organizations such as the World Health Organization (WHO), the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III), the European Group for the study of Insulin Resistance (EGIR), the National Heart, Lung, and Blood Institute (NHLBI) and the International Diabetes Federation (IDF), among others have made efforts to incorporate comprehensive parameters to define MetS.[17] Each organization emphasized different criteria, unable to agree on a uniform definition. The WHO centred on an insulin resistance-centred definition, the NCEP ATP III focused on abdominal obesity, etc.[15,17,18] In 2009, the most widely used definition of MetS that is used today was formed by the aforementioned major health

organizations as the Joint Interim Statement (JIS).[19,20] According to this definition, to be considered a patient with MetS, one must present at least three of the following conditions: impaired fasting blood glucose levels (>100 mg/dL), hypertension (130/85 mm Hg), abdominal obesity (>35 inches waist circumference for women, >40 inches waist circumference for men), high triglycerides levels (>150 mg/dL) and low high-density lipoprotein (HDL) cholesterol (<50 mg/dL for women and <40 mg/dL for men).[19–21] The need for this classification is to detect individuals who are at high risk for CVD and T2D; dyslipidemia is strongly associated with cardiovascular risk and high fasting blood glucose and insulin resistance increases risk of T2D.[17]

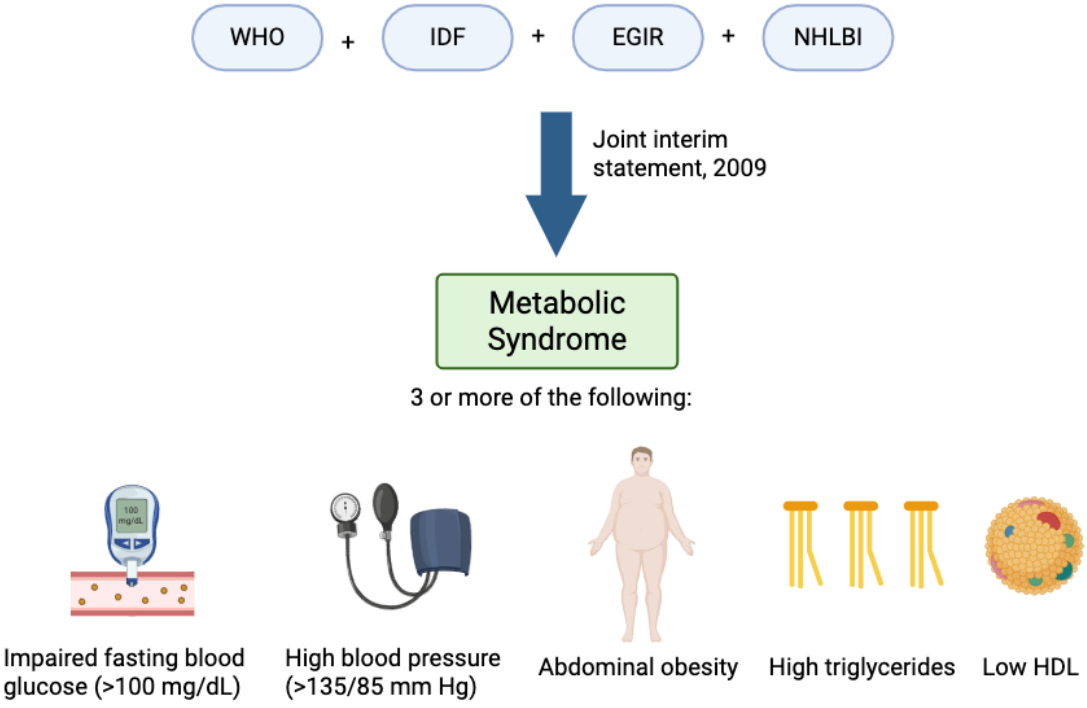

Figure 1.1: Classification of MetS according to the Joint Interim Statement (JIS) released in 2009 by various global health organizations.
According to this statement, patients must present three or more of the listed metabolic abnormalities to be diagnosed with MetS. Image created with BioRender.com.

Prevalence of MetS has been increasing globally, affecting 20-30% of adults worldwide, influenced by a variety of factors.[18] These include age, sex, ethnicity, socioeconomic status, stress, and lifestyle factors.[15,17,18] The trend of higher prevalence of MetS with increased age is a common one seen across varying regions around the world.[15,17] MetS becomes more prevalent with each decade of life, as does obesity and central adiposity, plateauing at the sixth and seventh decades.[15,17] In the younger population, the presence of MetS in youth can be a strong predictor for development of CVD and T2D. However, the problem with identifying MetS in adolescents is there is no established criteria to do so.[15,17] Pediatric growth, ethnic differences, and effects of hormonal changes on insulin sensitivity and lipid profile render it difficult to create criteria to classify youth as MetS patients.[15,17] Regardless, studies have shown that cardiovascular risk factors present during childhood, such as high low density-lipoprotein (LDL), high body mass index (BMI), and insulin resistance increases the risk not only for MetS, but for CVD and T2D in later life.

Risk factors of MetS are not limited to the five listed in the criteria; sedentary behaviour, a poor diet, genetic pre-disposition, age, sleep disturbance and smoking and alcohol intake can have significant impacts on risk of developing MetS. The aforementioned genetic and lifestyle factors contribute to visceral adiposity, which is thought to be the primary trigger for development of MetS. Many studies have highlighted how sedentary behaviour and diets high in sugar and saturated fats can contribute to obesity, dyslipidemia, and insulin resistance, all of which are components of MetS.[22–25] Aging is a natural biological process that is accompanied by reduced insulin sensitivity, increased visceral fat accumulation, and decreased muscle mass; all results of decreased metabolic rate and hormonal changes.[26,27] This shift in body composition influences fat metabolism, energy expenditure and insulin sensitivity, which naturally puts older adults at risk of

MetS.[26,27] Furthermore, tobacco use and alcohol intake have both been associated with worsened lipid profiles that contribute to abdominal adiposity, though there is conflicting literature suggesting there is no additive effect of the two on development of MetS.[28–30] Interestingly, poor sleep quality (too much or too little) has also been found to raise risk of MetS significantly.[31,32] Insomnia has correspondingly been strongly linked to components of MetS, such as hypertension and obesity.[31,32] Taken together, these lifestyle risk factors have repeatedly demonstrated their might in increasing risk of MetS and explain the rise in prevalence of this condition.

The established five criteria of MetS may define the syndrome, but these risk factors largely contribute to each component. To develop therapeutic targets to combat the rising prevalence of MetS and the downstream development of severe conditions such as CVD and T2D, it is imperative to understand the biological mechanisms underlying this condition.

1.1.2 Glucose Metabolism and Homeostasis

Glucose is the main energy source for most tissues of the body and maintaining glucose homeostasis involves a complex network of regulatory pathways and inter-organ crosstalk. Proper glucose homeostasis requires several organ systems working in sync, including skeletal muscle, adipose tissue, liver, pancreas, and the brain.[33,34] There are various processes regulating glucose metabolism including glycolysis, gluconeogenesis, glycogenolysis, and glycogenesis. The following subsections will focus on glucose uptake and downstream processing pathways.

1.1.2.1 Glucose transport

Glucose transporters (GLUTs), belonging to the SLC2A gene family, and sodium-glucose symporters are the two main classes of glucose transporters.[34,35] Currently, there are 14 GLUTs encoded by the human genome, each having a unique role in varying tissues and cell types.[34–36]

Each glucose transporter isoform plays a unique role correlated to glucose handling to regulate metabolism and maintain whole-body glucose homeostasis.

Glucose sensing in cells can occur via direct detection of the molecule, through receptors or transporters described above, or indirectly via changes in metabolic rate or monitoring metabolic derivatives.[34,36] Glucose uptake in cells can be insulin-dependent (ex. GLUT4 in skeletal muscle or heart) or insulin-independent (ex. GLUT1 in most cells and GLUT2 in the liver and pancreas).[34–36] Insulin-dependent mechanisms rely primarily on GLUT4 translocation as the primary regulator, vs insulin-independent mechanisms are led by constitutive GLUT activity or alternate signalling pathways and external stimuli.[34–36]

Insulin-stimulated glucose uptake (ISGU) occurs primarily in skeletal muscle, adipose tissue and the heart via the GLUT4 transporter, which is highly expressed in these tissues.[37,38] Insulin regulates glucose uptake through coordinated cellular responses that lead to translocation of GLUT4-containing intracellular compartments, vesicles, to the plasma membrane.[37,38] This process is governed by numerous cell signalling events including formation of scaffolding complexes, activation of kinases, rearrangement of cytoskeletal components, and formation of machinery that leads to fusion of GLUT4 to the plasma membrane.[37,38]

Insulin, a hormone produced by the pancreatic β cells, regulates metabolism of carbohydrates, lipids, and proteins, inducing glucose uptake into muscle and adipose tissue.[39,40] Under normal physiological conditions, insulin secretion is induced by increased blood glucose levels to enhance glucose uptake in muscle, adipose tissue and the heart. This was first studied in the 1950s when various research groups found that insulin accelerated rate of glucose uptake in these tissues.[41] The mechanism was further elucidated in the 1980s when it was proved that insulin promotes glucose transport from an intracellular region to the plasma membrane.[41] Following this,

GLUT4 was shown to be the major transporter responsible for enhanced glucose uptake in muscle and adipose tissue, upon insulin binding to its receptor in target tissues.[41]

1.1.3 Pathogenesis and Pathophysiology

Pathogenesis of MetS begins with the initiating risk factors mentioned in section 1.1.1, followed by biological disturbances such as insulin resistance, neurohormonal dysregulation, and activation of inflammatory pathways leading to chronic low-grade inflammation. These mechanisms are commonly agreed upon to be central contributors to the progression of MetS, transition to CMD, and eventual development to cardiovascular complications. The following sections will focus on each of the proposed mechanisms as drivers of MetS.

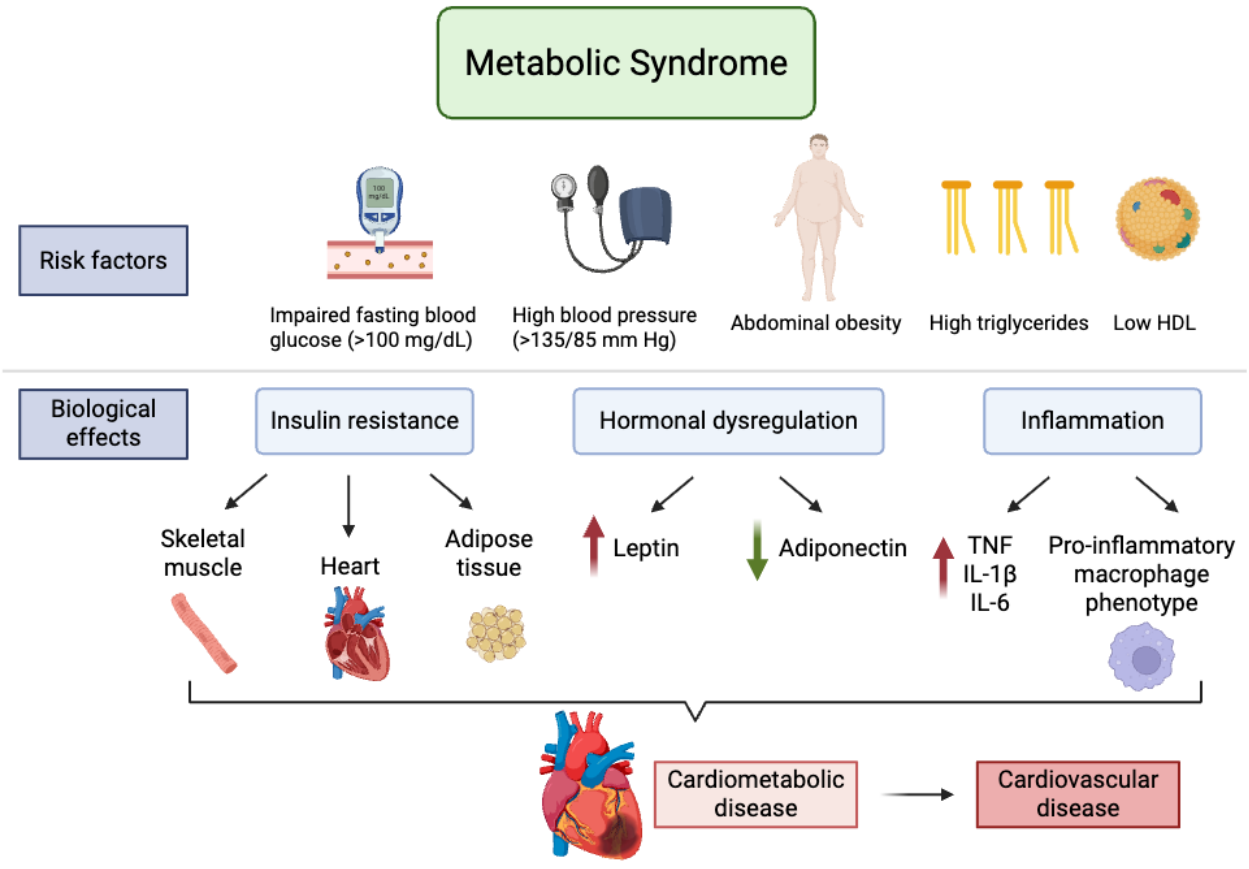

Figure 1.2: Schematic summarizing risk factors of MetS leading to biological effects that can result in CMD, and eventually cardiovascular outcomes.

Development of risk factors of MetS can lead to biological effects in different tissue, including insulin resistance, hormonal dysregulation and inflammation. This can ultimately contribute to the development of CVD. Image created with BioRender.com.

1.1.3.1 Insulin resistance

Insulin, as mentioned in section 1.1.2.1.1, is an important hormone released by the pancreas that stimulates glucose uptake in the liver, muscle, adipose tissue and heart. Insulin resistance is a pathological response where the aforementioned target tissues have built up a tolerance to insulin and therefore stop responding to it, rendering the peptide less effective.[42] This has intense metabolic consequences as it induces pancreatic β-cells to produce more insulin and can ultimately lead to chronically increased blood glucose levels, or.[42] Not only does insulin promote glucose uptake, it also inhibits lipolysis and gluconeogenesis (formation of glucose from non-hexose precursors).[14,43] When insulin resistance occurs, insulin-mediated inhibition of lipolysis is reversed and this leads to increased circulation of free fatty acids (FFAs).[44,45] FFAs then go on to reduce glucose uptake and increase kinase activation to promote gluconeogenesis and lipogenesis. [44,45] This leads to hyperinsulemia to maintain normal glucose levels, but ultimately results in decreased insulin levels which is only worsened by the FFAs exerting lipotoxic effects on β cells that are producing insulin.[45] This points to the conclusion that this is a bidirectional relationship, where oxidative stress can contribute to insulin resistance, but insuin resistance in return can also exacerbate oxidative stress and mitochondrial dysfunction.

1.1.3.1.1 Insulin resistance in adipose tissue

Adipose tissue is thermal regulator and lipid storage unit, but it also has significant endocrine function in that it releases adipokines which serve as signalling molecules to influence bodily functions.[14] Imbalance in this hormonal homeostasis leads to inflammation and development of MetS. Adipokines released by adipose tissue include hormones (adiponectin,

leptin) and inflammatory cytokines, both of which play a role in insulin resistance.[14] Within adipose tissue itself, insulin resistance manifests as impaired glucose uptake and reduced inhibition of lipolysis. In particular, visceral fat deposits have been found to contribute significantly to the development of insulin resistance compared to subcutaneous fat.[46] Visceral fat is inherently diabetogenic, responsible for secretion of adipokines that impair insulin sensitivity in the liver and muscle.[46] Visceral lipolysis increases FFAs in the liver via splanchnic circulation, leading to increased synthesis of triglycerides and cholesterol.[46] Chronically elevated FFAs can decrease insulin sensitivity and induce a pro-inflammatory state that overlaps with insulin resistance. Visceral adipocytes secrete pro-inflammatory cytokines such as interleukin (IL)-6 and tumour necrosis factor (TNF), which can directly impair insulin signalling in various insulin-dependent tissue. IL-6 has been found to be secreted in higher abundance from visceral fat compared to subcutaneous, whereas TNF had no difference in gene expression among the two types of adipose tissue.[47,48] Essentially, secretion of these pro-inflammatory agents leads to recruitment of monocytes which differentiate into macrophages and can polarize to a pro-inflammatory M1-like phenotype. These M1-like macrophages then secrete additional pro-inflammatory cytokines that recruit more monocytes and lead to adipose tissue dysfunction. In insulin signalling, increases in systemic TNF promotes activity of cell signalling pathways (IKK, p38 MAPK, JNK), which target serine residue of IRS and this impairs tyrosine phosphorylation of this protein.[49,50] Similarly, IL-6 has also been found to exert inhibitory effects on IRS-1 protein expression, as well as reduced GLUT4 mRNA expression accompanied by decreased glucose uptake.[51] Inflammation plays a major role in driving insulin resistance in various tissue, this aspect is explored further in sections 1.1.3.3 and 1.2.

1.1.3.1.2 Insulin resistance in muscle

Skeletal muscle is essential to maintain glucose homeostasis and prevent dysfunction of adipose tissue. The main drivers of insulin resistance in muscle are oxidative stress, mitochondrial dysfunction, and lipid accumulation.[52] Insulin resistance manifests itself as significantly decreased glucose uptake and decreased glycogen synthesis in muscle, presenting itself long before persistent hyperglycemia.[53] Insulin signalling defects have been reported with decreased IRS-1 and tyrosine phosphorylation, as well as reduced PI3K and Akt activity, leading to reduced GLUT4 translocation and glucose uptake.[44,54,55] Lipid metabolites, such as ceramides and diacylglycerols, can cause a negative effect on insulin signalling in skeletal muscle by interfering with insulin signalling.[56,57] Insulin is a potent inhibitor of lipolysis, preventing the release of FFAs from adipocytes.[58] However, under insulin resistant conditions, the ability of insulin to reduce plasma FFAs is impaired and these elevated plasma levels of FFAs can cause insulin resistance in skeletal muscle.[59–61]

Oxidative stress and mitochondrial dysfunction contribute to insulin resistance in skeletal muscle. ROS and byproducts of ROS can impair insulin sensitivity by directly acting on proteins involved in insulin signalling, activating pathways such as p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinases (JNK), and nuclear factor κ B (NFκB) to increase serine phosphorylation of IRS, inhibiting tyrosine phosphorylation.[62–64] When it comes to the mitochondria, differentiating between mitochondrial dysfunction causing insulin resistance or vice versa remains a controversial topic. Excessive ROS production can overwhelm mitochondrial antioxidant capacity, leading to its dysfunction.[65,66] Spillage of high energy electrons from the ETC damages mitochondrial antioxidant defence mechanisms.[65,66] On the other hand, insulin

resistances has been implicated in reduced mitochondrial oxidative function and energy production, as well as increased susceptibility to oxidative stress.[67]

1.1.3.1.3 Insulin resistance in the heart

Insulin signaling in the heart occurs through the PI3K and Akt pathway. The MAPK signalling pathway has also been implicated in the heart, phosphorylating IRS-1 leading to eventual activation of extracellular signal-regulated kinase (ERK) to respond to growth and remodelling responses to myocardial hypertrophy, cardiac fibrosis, and death of myocardial cells.[68,69] The point at which all signalling pathways contributing to insulin resistance meet is IRS; phosphorylation of various serine residues on IRS can attenuate tyrosine phosphorylation and lead to diminished activation of associated downstream proteins.[68,69] Instead, this triggers proteasomal degradation of IRS, contributing to impaired insulin signalling and resistance.[68,69]

Cardiac insulin resistance can develop independent of systemic insulin resistance, as a consequence of increased circulating glucose levels, oxidative stress and altered hormonal and cytokine release.[70] Overnutrition and excess caloric intake are central to development of obesity, a key feature of MetS, and increased visceral fat mass as a result of adipocyte hypertrophy.[68,70] As mentioned in section 1.1.3.1.1, these adipocytes release inflammatory agents that lead to recruitment of immune cells which exacerbates the immune response of adipocytes, resulting in increased circulating fatty acids, diacylglycerol, and ceramide. This can cause deposition of lipid species into various organs, including the heart, contributing to insulin resistance by promoting serine phosphorylation of IRS-1.[68,70] Dysregulated adipokine function and cytokine release, such as TNF and IL-6, can activate proteasomal degradation pathways such as MAPK and mTOR.[68,70]

The primary mode of cardiac metabolism has been well-established as fatty acid oxidation, accounting for about 70% of energy production in the adult myocardium, with the remaining 30%

of accounted for by glucose metabolism.[71] During insulin resistance, the heart is abundant in fatty acids and glucose. Increased levels of insulin in response to this can increase uptake of FFAs in the heart.[72] This leads to increased synthesis of FFA transporters, mitochondrial FFA transporting proteins, and enzymes involved in fatty acid oxidation.[72] Under conditions of insulin resistance, the heart becomes increasingly reliant on FFA oxidation, culminating metabolites that can inhibit production of glycolytic enzymes and ultimately inducing glucotoxicity.[73,74] Decreased ability to use fatty acids in the heart leads to myocardial lipid accumulation such as diacylglycerol and ceramides, this can contribute to myocardial apoptosis, hypertrophy, and mitochondrial dysfunction.[73,75]

To conclude, insulin resistance can develop in these tissues and induce tissue-specific effects that eventually lead to cellular dysfunction. Essentially, all pathways converge at IRS and its proteasomal degradation because of serine phosphorylation of this protein through activation of regulatory pathways. Overnutrition, leading to abnormal glucose and lipid levels, and obesity causing adipose tissue expansion and release of pro-inflammatory agents, remain at the root of the development of insulin resistance in various organs. This can lead to hyperglycemia which is a key trigger for metabolic changes in T2D. To summarize, these include further promotion of insulin resistance, oxidative stress, inflammation and apoptosis which contributes to progression of cardiac damage and DCM.

1.1.3.2 Hormonal regulation

The endocrine function of adipose tissue contributes another mechanism to the development of MetS. Adipokines are defined as hormones, cytokines and growth factors secreted by adipocytes that regulate bodily functions to maintain metabolic homeostasis.[76] Currently, over 600 secretory proteins from adipose tissue have been profiles, though not all have been

characterized.[77] Adipokines play a multi-faceted role in regulating appetite, insulin sensitivity, fat distribution, endothelial function, inflammation, and blood pressure.[76] In different organs, there are varying proportions of adipokines depending on the biological processes being carried out.[76] Hence, alterations in adipokine secretion can link obesity to inflammatory, metabolic and cardiovascular complications.

Adipose tissue is a dynamic organ, increasing in size with imbalanced energy as a result of genetic and lifestyle factors.[77] Expansion of adipocytes significantly influences its biology through size and cellular composition, which can lead to impaired adipocyte function.[77] Adipocyte hypertrophy is a key event that is associated with insulin resistance, individuals with larger adipocytes have elevated inflammatory agents such as leptin, IL-6, TNF, and reduced levels of hormones that attenuate insulin resistance, such as adiponectin and IL-10.[78–80] Not only do these factors affect adipose tissue dysfunction, but their release into circulation can lead to development of MetS, CVD and inflammatory diseases.[76] The following sections will briefly discuss adiponectin, a key adipokine of import and the subject of numerous studies on MetS.

1.1.3.2.1 Adiponectin

Adiponectin, a circulating adipose-derived cytokine, has been identified as an insulin sensitizing adipokine.[81,82] As mentioned previously, insulin resistance is considered a major risk factor for diabetes and cardiovascular disease. Patients with these conditions or increased adipose tissue mass have been found to have low levels of circulating adiponectin and higher levels of leptin.[43] Increased levels of adiponectin have shown insulin-sensitizing, anti-inflammatory and anti-atherosclerotic properties, making it a therapeutic target for cardiovascular, diabetic, and metabolic diseases.[83,84] Many studies have shown that adiponectin, in both human and animal

models, is a key component in the relationship between insulin resistance, inflammation, MetS, and CVD, as explored in this section.

Adiponectin can be found in either globular form (gAd) or the full-length form (fAd). gAd has a molecular weight of 17 kDa while fAd can exist in three forms according to its polymerization: low molecular weight (LMW) 90 kDa trimer, middle molecular weight (MMW) 180 kDa hexamer, and high molecular weight (HMW) at more than 400 kDa.[83,85–87] The HMW form of adiponectin is considered the most biologically active form and presents the ability to exert anti-diabetic and anti-inflammatory actions that have the potential to aid in the prevention and development of cardiovascular disease.[88] Adiponectin receptors, AdipoR1 and AdipoR2 are the two major receptors that mediate its metabolic actions. They are receptors for both fAd and gAd and are involved in the uptake of glucose and fatty acid oxidation.[83] These receptors trigger two different signalling pathways; AdipoR1 activates the AMPK signalling pathway, while AdipoR2 enhances the PPAR-α cascade.[87] The activation of the AMPK signalling pathway affects a variety of different cellular processes in vascular endothelial and heart cells which leads to anti-apoptotic effects and reduces myocardial infarction in mice models of heart ischemia-reperfusion.[89] Essentially, adiponectin is able to protect the heart by increasing oxidative phosphorylation in heart tissue and insulin-stimulated glucose uptake in cardiomyocytes and muscle.[89]

Studies in knockout mice on the importance of these receptors have shown that adiponectin-induced activation of AMPK is blocked in mice without AdipoR1 which leads to insulin resistance and glucose intolerance.[88] Adiponectin is able to exert its cardioprotective effects on a variety of components in the vascular and cardiac systems, including directly on the myocardium. In studies where mice lack adiponectin, the mice show an insufficient response to cardiac stress and are more susceptible to insulin resistance.[82,90] With the addition of adiponectin,

these damaging effects were reversed and mice showed improved insulin sensitivity.[82,90] The ability of adiponectin to protect cardiac function is rooted in its mediating role in inflammation and apoptosis.[88] The anti-apoptotic effects of adiponectin depend on activation of the AMPK pathway, an important process in mediating adiponectin action on a cellular level. AMPK, a sensor of low intracellular ATP levels, responds to low levels of cellular energy by activating pathways that produce energy and inhibit energy consuming pathways.[84] In the context of inflammation, higher molecular weight isoforms of adiponectin have actually shown activation of nuclear factor κ B (NFκB) in myocytes, fibroblasts, and endothelial cells.[91–93] The anti-inflammatory effects of adiponectin is rooted in its ability to suppress NFκB in macrophages and TNF induced activation of inflammatory pathways.[94,95] Adiponectin can improve cardiac function and increase insulin sensitization through a variety of mechanisms, providing an important target for therapeutic strategies for cardiovascular disease and MetS.

1.1.2.3 Inflammation

Inflammation is the coordinated response of the immune system, essential for host defence against invading pathogens and infections.[96–98] Stimuli such as infection or injury lead to the recruitment of inflammatory cells that phagocytose the infectious agents and develop the adaptive immune response.[96–98] The inflammatory response is essential for tissue repair and wound healing. Inflammation that occurs in the absence of microorganisms is termed ‘sterile inflammation’, which is the result of trauma or chemically induced injury, and underlies the pathogenesis of a variety of diseases.[96–98] These pathogen-free stimuli induce the release of danger-associated molecular patterns (DAMPs), which activate a sterile inflammatory reaction.[96–98] Briefly, this includes DAMPs interacting with pattern recognition receptors (PRRs) of resident innate immune cells, which can activate downstream transcription factors and signalling pathways.[96,98] This then leads

to inflammasome assembly and activation, and subsequent release of pro-inflammatory cytokines such as IL-1β and IL-18, which attract immune cells such as neutrophils and macrophages to the site of inflammation.[96,98]

The inflammatory component of MetS reflects a chronic, maladaptive state opposed to an acute, transient response to injection or injury. Continuous metabolic stress such as nutrient excess, adipocyte hypertrophy, and oxidative stress can persistently stimulate the immune response to produce inflammatory mediators.[43,99] This leads to a chronic state of inflammation in MetS where cytokine release can rewire metabolic pathways, leading to cardiometabolic dysfunction.[100] On a molecular level, metabolic stressors (nutrient excess, saturated fatty acids, ROS) can activate various inflammatory signalling pathways, including NFκB, TLR4, and JNK in adipose tissue, liver, heart, skeletal muscle, and the heart.[43,99,100] In adipose tissue, chronic inflammatory cues recruit and polarize macrophages, amplifying TNF and IL-1β release which can also enhance lipolysis.[101] In liver and muscle, persistent activation of NFκB and JNK disrupts mitochondrial function, increases oxidative stress, creating additional DAMPs that can sustain immune activation.[102] Cardiac macrophages and fibroblasts can also respond to inflammatory cues similar to liver and skeletal muscle, but also secrete pro-fibrotic mediators, perpetuating tissue remodelling and stiffening.[102–104]

Inflammation is considered one of the main mechanisms underlying complications in MetS. In fact, T2D is characterized by chronic inflammation, leading to damaged blood vessels and organs which can contribute to various diabetes-related complications, including CVD. Hyperglycemia can lead to increased expression of genes involved in insulin resistance and inflammation, leading to severe cardiovascular injury.[105–107] The exact mechanism through which this occurs is not well understood, as the cause of undergoing hyperglycemia is unknown. The

inducible NF-κB is an important transcription factor that controls many pathological processes in the myocardium, including inflammation in cardiomyocytes and ultimately apoptosis. Studies have recently shown that NF-κB signalling is involved in hyperglycemia-induced cardiac cell damage.[106–108] Activation of NF-κB by TNF regulates expression of various pro-inflammatory cytokines, such as IL-6 and IL-1β, and this is thought to be increased under hyperglycemic conditions.[106–108] Acute inflammatory responses have been linked to regenerative processes in cardiac tissue, however chronic low-grade inflammation has contributed to functional impairment and adverse remodelling in the heart.[109]

Activation of inflammatory pathways, elaborated on in section 1.2, has widely been accepted as the common pathway that leads to the clinical manifestation of MetS. Various inflammatory markers have been found to be elevated in MetS patients because of insulin resistance and oxidative stress, which can trigger inflammatory cascades and lead to MetS, as well as other chronic diseases.

1.1.4 Cardiometabolic Disease

1.1.4.1 Background

Cardiometabolic disease (CMD) is a relatively newer umbrella term describing the interrelated spectrum of metabolic and cardiovascular abnormalities that increase risk of T2D, CVD, and heart failure.[17,100,110] There is significant overlap with MetS in that CMD encompasses obesity, dyslipidemia, insulin resistance, hypertension and chronic inflammation.[17,100,110] While MetS is more of a diagnostic label with parameters that need to be met, CMD stresses the clinical consequences of the metabolic abnormalities in MetS.[19,110] These include insulin resistance, impaired glucose homeostasis, myocardial dysfunction and increased risk of cardiovascular events

and mortality.[19,110] Essentially, MetS encompasses a high risk phenotype, whereas CMD captures the downstream vascular consequences that produce a clinical outcome.[19,110]

Pathophysiology of CMD integrates insulin resistance, vascular dysfunction, and inflammation, similar to MetS. Impaired insulin signalling in the muscle, liver, heart, and adipose tissue disrupts normal metabolic function of these organs, leading to increased circulating glucose levels.[17,100] Adipose tissue dysfunction and inflammation contribute as mentioned in previous sections, with adipocyte expansion and the release of inflammatory agents.[17,100] Additionally, oxidative stress and dyslipidemia contribute to CMD, interacting with the other mechanisms in a continuous cycle.[17,100,110] Increased lipid accumulation can cause insulin resistance, which can further exacerbate dyslipidemia, insulin signalling and endothelial function.[17,100,110] Inflammation sustains this cycle, promoting vascular injury and impeding essential cellular processes. In the heart, hyperglycemia and dyslipidemia can lead to mitochondrial dysfunction, recruitment of inflammatory immune cells, production of ROS, and ultimately apoptosis.[17,100,110,111] All these events combined can contribute to cardiomyocyte hypertrophy, impaired contractility, and ultimately heart failure.[17,100,110,111]

CMD represents a convergence of MetS and CVD, bridging symptoms of MetS and its clinical outcomes. Insulin resistance, inflammation, oxidative stress, mitochondrial dysfunction lead to alterations that can lead to T2D, atherosclerosis, and heart failure.[17,100,110,111] It is therefore essential to understand these mechanisms to develop targeted interventions that can address both metabolic and cardiovascular risks.

1.1.4.2 Mechanisms of CMD

CMD is clinically centred on the heart and its vasculature, though it really is a multi-organ disease involving metabolic, cardiovascular and immune system disturbances. Therefore,

mechanisms of CMD occur not only in cardiomyocytes to lead to diabetic cardiomyopathy, but also endothelial cells to drive atherosclerosis, immune cells to modulate the inflammatory response, and adipocytes and hepatocytes to influence insulin resistance and lipotoxicity. The following section will elaborate on different mechanisms of CMD and how they can vary in different cell types.

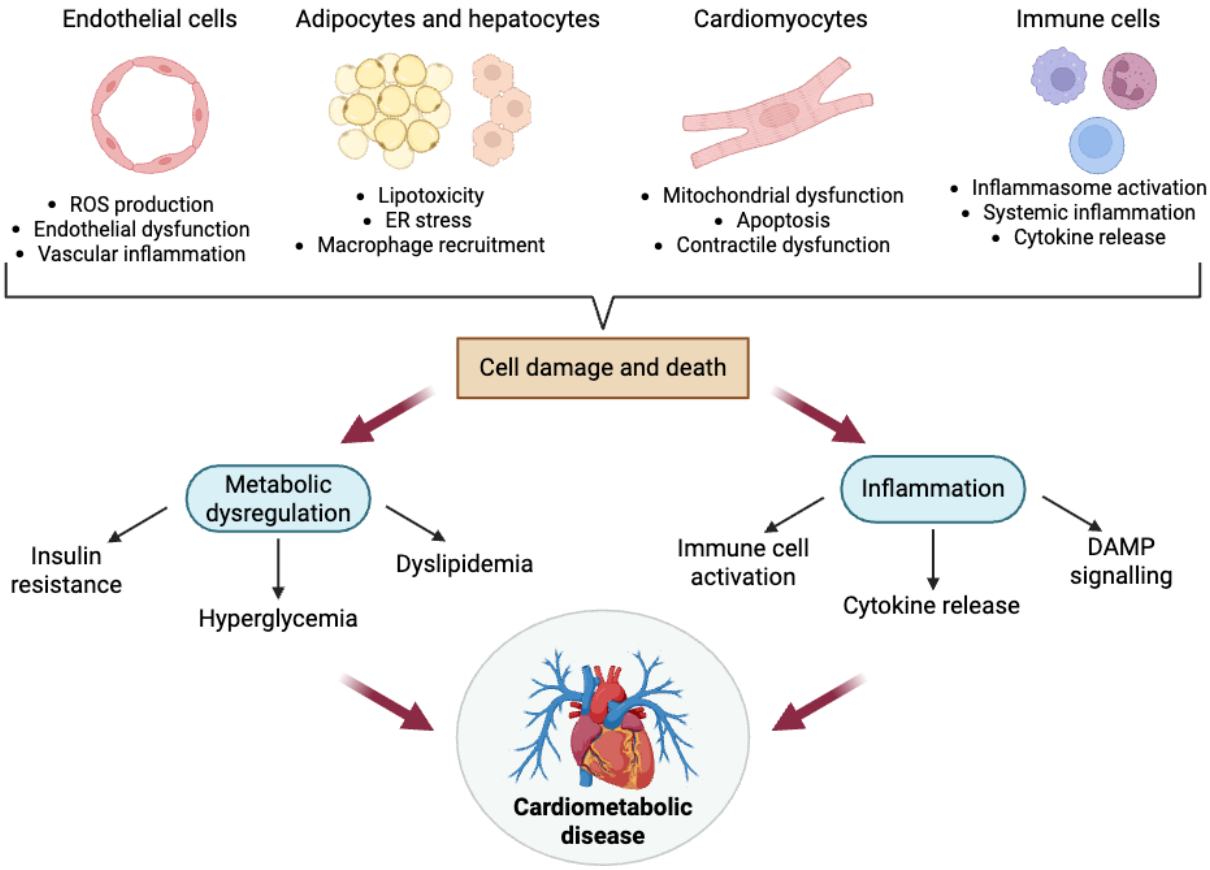

Figure 1.3: Schematic of various cell types contributing to metabolic dysregulation and inflammation leading to cardiometabolic disease (CMD).
Cell types affected by metabolic stress include endothelial cells, adipocytes, hepatocytes, cardiomyocytes and immune cells. Damage of these cells can lead to metabolic dysregulation and inflammation, hallmarks of CMD. Image created with BioRender.com.

1.1.4.2.1 Autophagy

Autophagy is an essential catabolic process that maintains cellular homeostasis and survival upon exposure to extra- or intracellular stressors.[112,113] It delivers cytoplasmic material to the

lysosome for degradation and promotes cell survival by eliminating damaged organelles, proteins, and cellular structures.[114] While autophagy is considered a cell survival mechanism, it can also be an indicator of cell health and whether cells are under conditions of stress. The process of autophagy, induced by stressors such as starvation, begins with the formation of the phagophore where an isolation membrane encloses a portion of the cytoplasm (including any damaged organelles).[112–117] The membrane is then elongated to evolve into the autophagosome, which is still enclosed around the cellular components to be delivered to the lysosome.[112–117] The autophagosome then fuses with the lysosome to form the autolysosome and the enclosed cargo is degraded by hydrolytic enzymes and the resulting macromolecules are released into the cytosol.[112–117]

Three types of autophagy have so far been identified, microautophagy, chaperone-mediated autophagy and macroautophagy, most commonly referred to as autophagy, which is the most well-characterized. Autophagy can be divided into four key steps; initiation and nucleation of the phagophore, elongation and cargo recruitment to form autophagosome, fusion with the lysosome, and degradation and release of autophagic cargo.[113,115] Autophagy is initiated by AMPK, a cellular energy sensor, and inhibited by mTOR, a cell growth regulator.[118] The unc-51-like kinase 1 (Ulk1) complex, is activated by active AMPK by phosphorylation at any of the AMPK serine residues, such as Ser555 and Ser777.[118–120] Under high nutrient conditions, mTOR activity then prevents Ulk1 and AMPK interaction by phosphorylating Ulk1 at Ser757.[118] Activated Ulk1 then phosphorylates and activates class III phosphatidylinositol 3-kinase (PI3K) complex, which then goes on to phosphorylate phosphatidylinositol-3-phosphase (PI3P), which define the region of phagophore initation.[113] Following these initiation steps, autophagy-related gene (Atg) proteins are recruited to promote lipidation at the phagophore membrane to generate the double-membrane

autophagosome which is engulfing the cellular cargo. The Atg proteins then dissociate from the outer membrane of the autophagosome.[121,122]

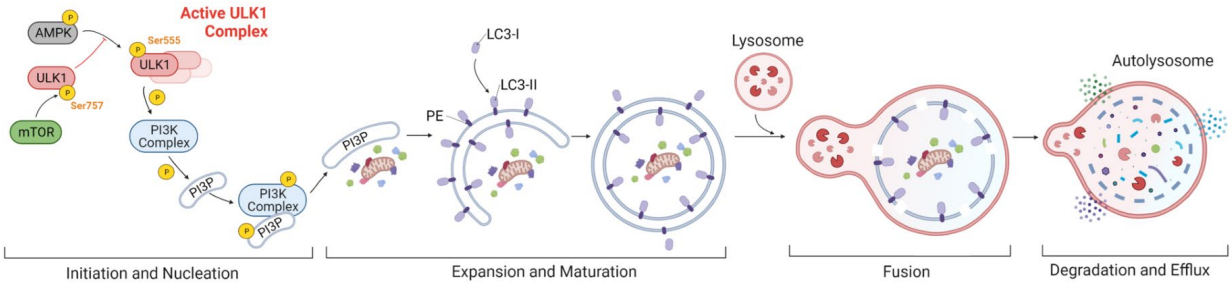

Figure 1.4: Core steps of autophagy beginning with activation of PI3K complex and ending with formation of the autolysosome, resulting in degradation and efflux of cellular components.
Autophagy can be initiated downstream of AMPK activation, starting with phosphorylation of ULK1 and formation of the PI3K complex, leading to phagophore formation. The phagophore is then elongated to form the autophagosome, which fuses with a lysosome becoming an autolysosome that degrades the cargo and releases macromolecules into the cytosol. Image created with BioRender.com.

In the final stages of autophagy, the autophagosome fuses with the lysosome to form the autolysosome, and the sequestered cargo on the inner membrane of the autophagosome is exposed to lysosomal hydrolases for degradation.[113] The small molecule products of these degradation reactions are released into the cytosol through membrane permeases for reuse or to act as substrates for catabolic reactions.[113] Autophagy is viewed as a double edged sword, where too much or too little can both have detrimental effects on cells.[123–125] It is a protective mechanism to eliminate damaged organelles and proteins, but an excessive amount can increase tissue damage and impair the immune response, while too little can lead to a buildup of toxic cellular components.[123,125]

Autophagy has been heavily implicated in cardiac dysfunction, studies have found mice with autophagy deficiencies have reduce cardiac contractibility, with some even suffering from serious cardiomyopathy.[126,127] In addition, cardiac-specific deletion of Atg5 displayed dysfunctional mitochondria and impaired sarcomere structure for six months, followed by

increased mortality.[128] Another study showed cardiac hypertrophy, left ventricular dilation and contractile dysfunction in Atg5 cardiac specific ablation.[129] On the other hand, GFP-LC3 mice transgenic mice used to monitor autophagosome formation, showed increased autophagosome numbers, which is important for cardiac function and mitochondrial turnover.[130] Furthermore, numerous studies have shown that increased autophagy is associated with decreased apoptosis and attenuated cardiac dysfunction in varying disease models.[131–135]

Autophagy plays a crucial role in maintaining metabolic and cardiovascular homeostasis, and its dysregulation has been implicated in CMD and diabetic cardiomyopathy. Basal levels of autophagy preserves cellular homeostasis by degrading damaged organelles and unwanted molecules.[136,137] Under conditions of hyperglycemia, nutrient overload and lipotoxicity can impair autophagic flux across various tissues including the heart, liver, adipose tissue and immune cells.[136,137] This can lead to exacerbated insulin resistance, oxidative stress and inflammation.[136,137] For example, mouse models of obesity and insulin resistance mimicking MetS, impaired autophagy in the heart was observed marked by decreased LC3-II and accumulation of p62.[138] This was associated with increased mitochondrial dysfunction, ROS production, and promotion of diabetic cardiomyopathy.[138] Restoration of autophagy through activation of AMPK and inhibition of mTOR has shown to improve metabolic balance and reduce lipotoxic damage in diabetic models.[139,140] Subtypes of autophagy, such as mitophagy, have proven critical in removing damaged mitochondria and reducing ROS production, thereby decreasing apoptosis. Beyond the heart, altered autophagic flux in vascular or immune cells can promote endothelial dysfunction and inflammation, further highlighting that balanced autophagy is crucial to maintain cellular and tissue integrity, and that its dysregulation can promote progression of MetS and CMD.[141,142]

There are numerous key proteins involved in the entire process of autophagy, with the current molecular understanding of it emerging in the past two decades. Autophagy was first discovered in mammalian cells, the discovery of Atg genes was in yeast followed by homologs being identified in eukaryotes.[143] The core molecular machinery essential for autophagosome formation can be divided into four groups: the Atg1/unc-51-like kinase (ULK) complex, two ubiquitin-like protein (Atg12 and Atg8) conjugation systems, class III PI3K/Vps34 complex, and Atg9 and VMP1 transmembrane complex.[143–145] In mammals, initiation of autophagy is controlled by the ULK1 complex; comprised of ULK1 and 2, Atg13, FIP200.[143–145] When nutrients are readily available, mTOR suppresses ULK1 activity through inhibitory phosphorylation, upon starvation this is relieved and ULK1 and 2 phosphorylate Atg13 and FIP200, as well as downstream components to induce phagophore nucleation.[143–145] Next, the class III PI3K complex is recruited to the initiation site, comprised of Vps34 (catalytic PI3K subunit), p150 (regulatory PI3K subunit), Beclin-1, and Atg14L.[143–145] Activation of ULK1 leads to phosphorylation of Beclin-1 and Atg14L to enhance Vps34 lipid kinase activity.[143–145] Vps34 then produces PI3P on the nascent phagophore, recruiting effectors that scaffold downstream conjugation systems to extend the phagophore.[143–145] These conjugation systems involve two ubiquitin-like proteins, Atg12 and Atg8/LC3, together these systems coordinate autophagosome expansion and maturation. Atg12 is conjugated to Atg5, a reaction that requires E-like enzyme Atg7 and E2-like enzyme Atg10, this conjugate then noncovalently binds to Atg16L forming an oligomeric complex.[143–145] Atg8/LC3 is cleaved by Atg4 to expose a C-terminal glycine and then the Atg16L complex acts as an E3-like ligase to facilitate Atg7 and Atg3 in conjugating phosphatidylethanolamine (PE) to LC3, forming LC3-II.[143–145] This drives phagophore expansion, cargo recruitment and membrane closure.

Transmembrane proteins Atg9 and VMP1 deliver membrane vesicles to the growing phagophore and ensure cargo is fully incorporated into the autophagosome.[143–145]

Atg5, 7, 9 and Beclin-1 have all been extensively studied in loss and gain of function studies on autophagy. Gain of function studies on have shown that mice overexpressing Atg5 increased autophagic flux and extended lifespan of transgenic mice by 17% compared to wild-type.[146] In addition, Atg5 transgenic mice were found to have increased insulin sensitivity and improved motor skills.[146] Enhancing Atg7 has also been found to confer protection in disease models, with cardiomyocyte-specific overexpression of Atg7 in mice protecting against a proteotoxic model of heart failure.[147] Atg7 overexpression had strong therapeutic effects in reducing protein aggregates, decreasing fibrosis and hypertrophy, and improve ventricular function and life span.[147] Loss-of-function studies have shown that Atg7 KO in mice abolishes LC3 lipidation and autophagosome formation, leading to accumulation of damaged organelles.[148] In adipose tissue, Atg7 KO mice showed increased mitochondrial content, resistance to diet-induced obesity, and improved insulin sensitivity.[149] Atg7 KO in cardiomyocytes led to a dramatic decrease in LC3-II and accumulation of p62, indicating impaired autophagic flux.[150] Functionally, the mice developed contractile dysfunction and worsened ischemia-reperfusion injury.[150] Atg7 also appears to play a role in protecting against DCM; cardiac specific deletion of Atg7 has led to accumulation of lipids, systolic and diastolic dysfunction, and hypertrophy.[151] A review published by Collier *et al.* established that Atg7 is required for heart function to be rescued under metabolic stress, and that loss of Atg7, and by extension impairment of autophagy, can aggravate features of DCM such as lipotoxicity, mitochondrial dysfunction, fibrosis and contractile function.[152]

To summarize, autophagy serves as an essential process to maintain cellular homeostasis, and during metabolic stress, autophagy removes damaged organelles to mitigate the formation of

ROS. However, persistent metabolic stress can impair autophagic flux, leading to mitochondrial dysfunction and oxidative stress. This can lead to activation of inflammatory pathways and apoptotic cascades.

1.1.4.2.2 ROS production and mitochondrial dysfunction

Reactive oxygen species (ROS) are highly reactive molecules derived from oxygen that are signalling intermediates and sources of oxidative stress.[153] Under normal physiological conditions, ROS are constantly generated from various intracellular sources including mitochondrial oxidative phosphorylation, NADPH oxidases (NOX) and uncoupled nitric oxide synthase (NOS).[153,154] The mitochondria are a dynamic organelle with the primary purpose of performing oxidative phosphorylation to generate ATP through the ETC.[155,156] It also regulates apoptosis, calcium homeostasis, and redox signalling.[155,156] ROS can be mitochondrial or cytosolic derived, with mitochondrial ROS acting as signalling molecules in insulin signalling and adaptive stress responses through redox-dependent pathways such as AMPK, PI3K/Akt, and MAPK.[157,158] Cytosolic ROS are produced by NOX enzymes and contribute to redox signalling in endothelial cells, adipocytes and immune cells.[153,157] In order to maintain homeostasis, cellular antioxidant defence systems exist to tightly regulate ROS levels and prevent oxidative damage.[159] This balance is essential to support cellular differentiation, glucose metabolism, and immune response.[159] Antioxidants such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), tightly regulate ROS at low levels.[159] However, when ROS generation exceeds capacity of these defence systems, as in the case of metabolic stress, oxidation of lipids, proteins and mitochondrial DNA.[159] This marks a shift from physiological to pathological redox signalling contributes to the pathogenesis of CMD, leading to mitochondrial dysfunction and inflammation.[153,157,159,160]

The mitochondria are central regulators of cellular metabolic state, performing oxidative phosphorylation and generating ROS along the way. A study by Ni *et al.* found that mice treated with mitochondria-targeted antioxidants reduced mitochondrial ROS and reduced cardiac apoptosis, hypertrophy and overall improved cardiac function.[161] Song *et al.* showed that inhibiting mitochondrial ROS endothelial ion-channel responses, providing mechanistic evidence that mitochondrial ROS can influence coronary dysfunction in diabetes.[162] Additionally, Wu *et al.* found that ETC activity was reduced and mitochondrial membrane potential was lost in diabetic mouse hearts.[163] Upon deletion of a gene that is a component of a protein that can increase ROS output, mitochondrial membrane potential was rescued and ROS production decreased.[163] In macrophages, a pro-inflammatory M1-like phenotype is observed in diabetic mice, alongside impaired mitochondrial function and autophagy.[164,165] Yuan *et al.* found that treatment with a mitochondrial ROS inhibitor however, this M1-like macrophage polarization was reversed.[165] This highlights the role of mitochondrial function and ROS production under metabolic stress, and how it closely relates to autophagy and the inflammatory response.[165]

Production of ROS and mitochondrial dynamics play an intimate role with fellow mechanism of both MetS and CMD; autophagy, apoptosis and inflammation. Low levels of ROS act as signalling molecules that can induce autophagy through activation of AMPK and inhibition of mTOR, this promotes clearance of damaged mitochondria.[158,166] However, under metabolic stress sustained ROS production can overwhelm antioxidant systems, impairing autophagy and leading to accumulation of damaged cell organelles.[167,168] This can lead to apoptosis through cytochrome c release in the mitochondria.[167] In addition, these damaged cellular organelles and ROS molecules can act as signals to induce an inflammatory response.[167]

In cardiomyocytes, impaired autophagy has been directly implicated in cardiomyopathy and heart failure. As an example, diabetic mice that were deficient in the leptin receptor showed reduced LC3-II and increased p62 in the heart.[163,169,170] This was indicative of inhibition of autophagy accompanies by mitochondrial swelling and oxidative damage. Restoring autophagy was observed to improve mitochondrial quality and promote cardiac remodelling.[171] In the context of immune cell activation and inflammation, ROS have been found to be messengers of NLRP3 inflammasome activation, which promotes maturation and release of pro-inflammatory cytokine IL-1β.[172,173] Under metabolic stress, failure to remove damaged mitochondria can lead to excessive ROS production and release of mitochondrial DNA, both of which are potent activators of the inflammasome.[172,173] In adipose tissue, defective autophagy and excess ROS production can promote macrophage infiltration and a pro-inflammatory M1-like phenotype, contributing to insulin resistance as well.[174,175]

1.1.4.2.3 Apoptosis

Apoptosis, or programmed cell death, is a tightly regulated process essential for tissue repair and homeostasis, removing damaged or dysfunctional cells.[176] In CMD, the above mentioned mechanisms and metabolic stress can lead to activation of apoptotic pathways, contributing to the loss of cardiomyocytes, endothelial cells, and pancreatic β-cells.[176] Two main apoptotic pathways have been identified; the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway.[176] There is evidence that the two may be linked and can influence one another, however this section will focus on the intrinsic pathway.

First, there are certain biochemical features of apoptosis that are common across the different types. Apoptotic cells exhibit protein cleavage, DNA breakdown and protein cross-linking.[177] A proteolytic cascade mediated by the protein family of caspases amplifies apoptosis

and results in cell death.[176] Caspases possess proteolytic activity that can cleave proteins at the aspartic residue, or recognize other specific amino acids.[176] Activation of caspases marks irreversible commitment to cell death.[176] Caspase proteins have been classified into initiator caspases (-2, -8, -9, -10), effector or executioner caspases (-3, -6, -7) and inflammatory caspases (-1, -4, -5).[178,179] Another biochemical feature of apoptosis is expression of cell surface markers that lead to phagocytotic recognition of the apoptotic cell by adjacent cells.[176] This allows quick phagocytosis with minimal effect on surrounding tissue. Externalization of phosphatidylserine, a key lipid component of the plasma membrane, is a well-known ligand for phagocytosis, alongside Annexin V.[180]

Intrinsic apoptosis involves production of intracellular signals that converge at the mitochondria. These intracellular signals can be positive or negative factors. Negative stimuli could be the absence of hormones, growth factors, and cytokines, whereas positive signals could be hypoxia, toxins, infections or free radicals.[176] These stimuli can induce changes in the inner mitochondrial membrane, resulting in the opening of the mitochondrial permeability transition (MPT) pore, loss of mitochondrial membrane potential, and release of two groups of pro-apoptotic proteins that are normally sequestered in the intermembrane space to the cytosol.[181] The first group of these proteins consists of cytochrome c, Smac/DIABLO, and a serine protease, HtrA2/Omi. Cytochrome c binds and activates apoptotic protease-activating factor 1 (Apaf1) as well as procaspase-9, forming the "apoptosome".[176,182,183] Clustering of procaspase9 in this way leads to activation of caspase-9, while Smac/DIABLO and HtrA2/Omi promote apoptosis by inhibiting activity of a group of proteins referred to as inhibitors of apoptosis proteins (IAP).[183] The second group comprises of AIF, endonuclease G, and CAD, which are released during the later stages of apoptosis.[176] All three of these proteins translocate to the nucleus, AIF facilitates DNA

fragmentation and chromatin condensation.[184,185] Endonuclease G cleaves chromatin to produce oligonucleosomal DNA fragments.[186] AIF and endonuclease G function in a caspase-independent manner but CAD is cleaved by caspase-3 following translocation to the nucleus, where it then leads to more pronounced DNA fragmentation and advances chromatin condensation.[184,185,187] All of these apoptotic events are under the control of members of the Bcl-2 family of proteins, which governs mitochondrial membrane permeability and cytochrome c release.[188]

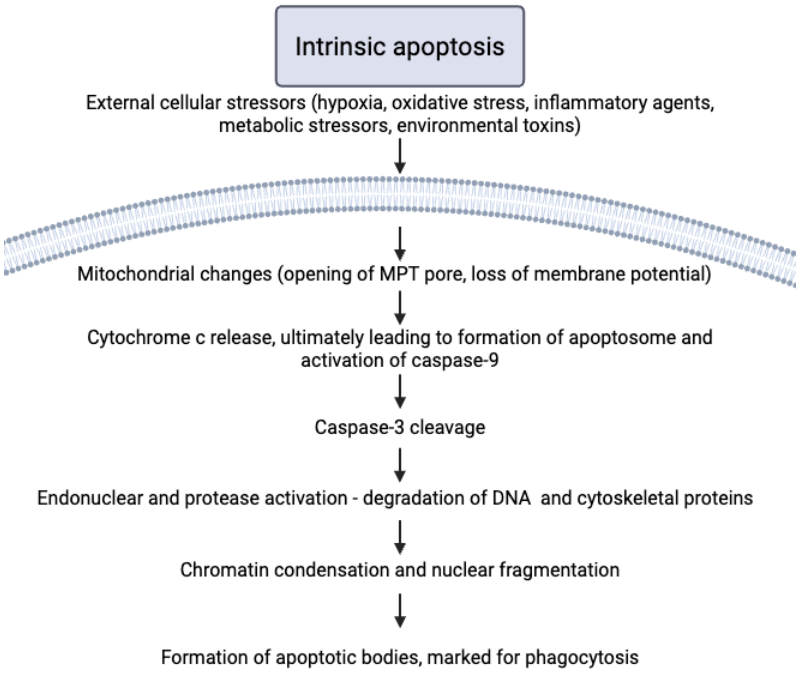

Figure 1.5: Summary of intrinsic apoptosis induced by external cellular stressors.
Intrinsic apoptosis begins with cellular stressors that lead to mitochondrial changes and cytochrome c release. This is followed by formation of the apoptosome which can cleave caspase-3, triggering protease and nuclear activity and resulting in formation of apoptotic bodies. Figure adapted from Elmore, 2017. Image created with BioRender.com.

Essentially, intrinsic apoptosis starts with changes in mitochondrial membrane permeability, triggered by cellular stressors.[176] This leads to cytochrome c release and formation of the apoptosome, followed by caspase-9 activation.[176] Caspase-9 goes on to cleave caspase-3,

initiating the execution portion of this pathway.[176] This results in chromatin condensation, DNA fragmentation facilitated by proteins mentioned above, and degradation of cytoskeletal proteins.[176] It ends with formation of apoptotic bodies that are marked for phagocytosis.[176]

Intrinsic apoptosis can be triggered by cellular stressors as seen in CMD; ROS production, calcium or iron overload, DNA damage, impaired autophagy and mitochondrial dysfunction. Mitochondrial dysfunction and elevated ROS are particularly influential in activating intrinsic apoptosis. In diabetic hearts, hyperglycemia can overwhelm the mitochondria leading to cytochrome c release, elevated pro-apoptotic Bax/Bcl-2 ratio and cleavage of caspase-3.[189–192] Studies on diabetic or obese rodents have consistently shown myocardial apoptosis, and interventions that induced autophagy or enhanced mitochondrial function reduced apoptosis and improve cardiac function.[189–192] Beyond the heart, nutrient overload can trigger apoptosis in adipose tissue which can promote macrophage infiltration and chronic inflammation, contributing to insulin resistance.[193] In pancreatic β cells, oxidative and ER stress induce intrinsic apoptosis, contributing to loss of β cells and insulin resistance, leading to elevated blood glucose levels which can damage other tissues as well.[194] As such, apoptosis is a critical cellular endpoint influenced by metabolic stress and tissue dysfunction in CMD.

Taken together, it is evident that these cellular mechanisms play an intertwined role in development of CMD in various tissue. Normally, autophagy suppresses apoptosis through removal of damaged organelles, however when it is impaired as seen in CMD, accumulation of these organelles leads to ROS production and release of inflammatory factors, resulting in pro-apoptotic signalling. Furthermore, apoptotic cell remnants and ROS can further serve as activators of inflammasome activation, leading to a more robust immune response which can impair insulin

signalling and cause tissue damage. Targeting these closely related mechanisms can represent a promising therapeutic approach to reduce cardiovascular-related consequences of MetS.

1.2 Inflammatory Pathways in MetS

1.2.1 Introduction

Chronic low-grade inflammation is a hallmark of MetS, a key feature of obesity, T2D, and CVD. This inflammatory state is characterized by innate immune activation, leading to the release of pro-inflammatory agents and recruitment of immune cells to affected tissues. There is a clear and tight link between metabolic disturbances and inflammation as key players in pathogenesis and progression of MetS.

Following metabolic stress or tissue injury, the immune system responds through coordination of both innate and adaptive arms of immunity. Unlike inflammation triggered by microbial infections, metabolic inflammation occurs in a sterile environment such as nutrient overload, hyperglycemia, lipid accumulation and oxidative stress.[96,97] The innate immune system is the first response in this setting, mediated by resident macrophages, dendritic cells, and endothelial cells that are able to sense metabolic dangers through pattern recognition receptors (PPRs).[96,97] These include toll-like receptors (TLRs) and nod-like receptors (NLRs) which can detected damage-associated molecular patterns (DAMPs) released from stressed cells such as cardiomyocytes, adipocytes and hepatocytes.[195] Once the metabolic stress persists, the adaptive immune response becomes engaged, activating antigen-presenting cells (macrophages, dendritic cells) and promoting increase in regulatory and helper T cells.[96] Cytokine production by B cells can also play a role in exacerbating tissue inflammation.[96] This persistent interplay between the innate and adaptive immune responses maintains a chronically inflammatory environment which is distinctive to MetS and CMD.

Upon metabolic stress or injury, DAMPs (such as ROS) are released from the stressed cells or the dying cells themselves act as DAMPS and are recognized by PRRs on resident macrophages or cardiomyocytes.[195] This can trigger activation of NFκB or interferon regulatory factor pathway (IRF), leading to release of pro-inflammatory cytokines or chemokines that can further recruit circulating immune cells [195] These include monocytes, neutrophils, and lymphocytes. Macrophages are components of the innate immune system, involved in both initiation and resolution of inflammation in cardiac tissue.[196] Both macrophages and PBMCs can influence the inflammatory state in patients with metabolic disease, releasing pro-inflammatory agents and contributing to sustained inflammation.[196,197]

It is imperative to understand the mechanisms underscoring inflammation in MetS, studies have continuously demonstrated the importance of modulating inflammatory pathways to attenuate adverse effects of chronic inflammation in MetS. The following sections will dive into key inflammatory pathways and cell types involved in metabolic inflammation.

1.2.2 NFκB signalling

NFκB is one of the most extensively studied inflammatory pathways in the context of MetS. The NFκB family is essentially a master transcriptional regulator of inflammatory genes. This family is comprised of five related members; p50, p52, p65 (RelA), RelB, and c-Rel, all of which mediate transcription of genes by binding to specific DNA elements.[198] Normally, these proteins are sequestered in the cytoplasm by a family of inhibitory proteins called IκB, the most important member of which is called IκBα.[198] There are two main signalling pathways for activation of NFκB, canonical and non-canonical.

The canonical pathway, activated by diverse stimuli, includes ligands of various cytokine receptors, PRRs, and TNF receptor (TNFR) superfamily members.[102,198,199] The primary

mechanism for canonical NFκB activation starts with the IκB kinase (IKK) complex, which is composed of two catalytic subunits; IKKα and IKKβ and one regulatory subunit called NFκB essential modulator (NEMO).[102,198,199] IKK can be activated by various growth factors, cytokines, mitogens, and stress agents.[102,198,199] Upon activation, IKK phosphorylates IκBα at two N-terminal serine residues, triggering ubiquitin-dependent IκBα degradation.[102,198,199] This results in rapid and transient translocation of NFκB members, predominantly the p50/p65 and p50/c-Rel dimers, to the nucleus for transcription of inflammatory genes.[102,198,199]

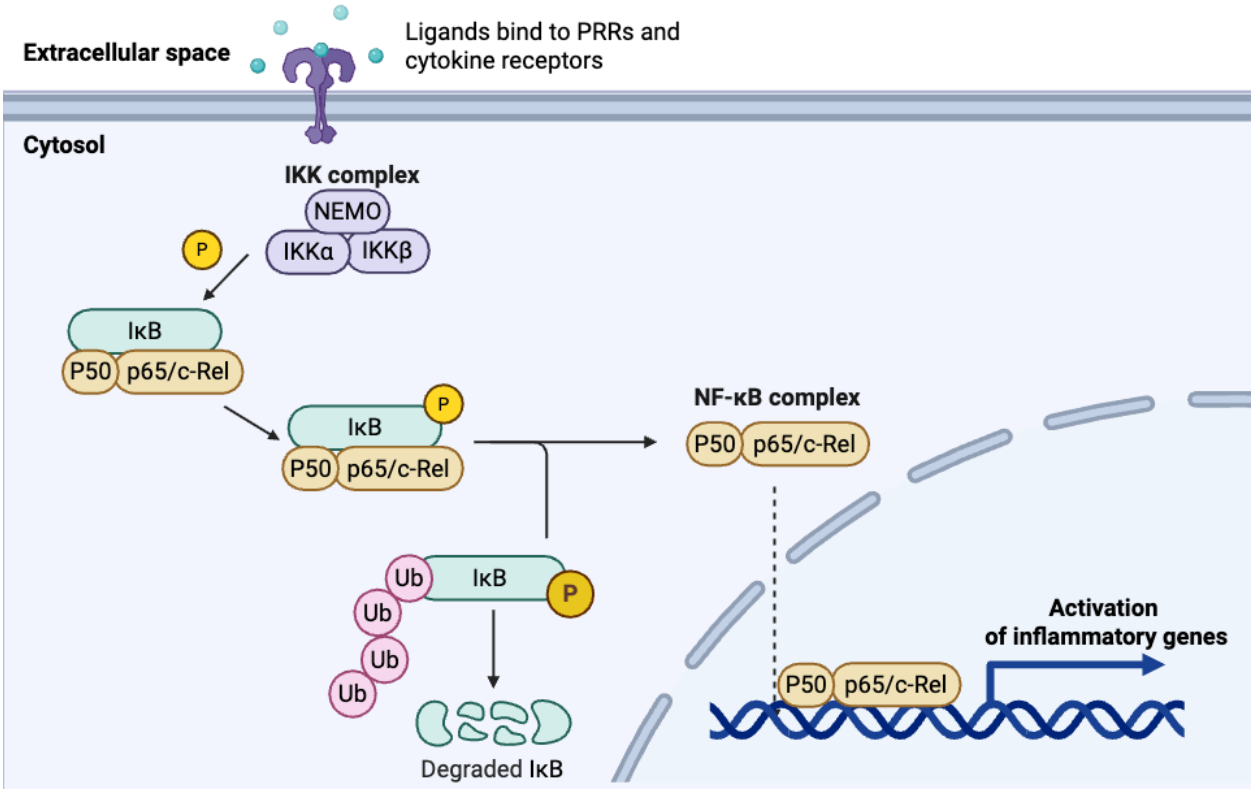

Figure 1.6: Canonical NFκB signalling pathway involving degradation of IκB, translocation of p65 subunit to the nucleus, leading to transcription of various genes involved in inflammation, immune regulation, adhesion and cell survival.
Ligands bind to their PRRs, leading to recruitment of adaptor proteins to activate the IKK complex, which phosphorylates IκB. IκB holds active subunits of NFκB and once phosphorylated IκB is marked for degradation and NFκB is released. NFκB translocates to the nucleus and can transcribe inflammatory genes. Image created with BioRender.com.

Chronic nutrient excess and oxidative stress in metabolic tissues can induced NFκB activation. In cardiomyocytes, high glucose exposure can stimulate NFκB-dependent expression of IL-6, TNF, and MCP-1, thereby promoting local and systemic inflammation.[200,201] This inflammatory response can contribute to myocardial hypertrophy, fibrosis, and apoptosis.[200,201] Interestingly, adiponectin has shown cardioprotective and anti-inflammatory properties by attenuating NFκB activation through AMPK signalling, suppressing production of inflammatory agents and maintaining cell viability.[202] In macrophages, NFκB is an important regulatory of polarization and cytokine production. Activation of the canonical pathway can promote an M1-like pro-inflammatory phenotype, and inhibition can lead to an anti-inflammatory phenotype.[196,199] Metabolic overload can sustain NFκB activation in macrophages, leading to inflammation and recruitment of other immune cells in adipose tissue and the heart.[102] Essentially, production of ROS, accumulation of damage organelles as a result of impaired autophagy and dead cells themselves can act as DAMPs that can lead to activation of NFκB, leading to increased production of pro-inflammatory genes such as IL-6 and TNF, which can in turn recruit other immune cells to produce a more robust inflammatory response.

1.2.3 IRF pathway

The IRF pathway is a family of transcription factors, primarily associated with antiviral and immune signalling. That said, this pathway can also modulate inflammation and macrophage polarization under metabolic stress. IRF3, IRF5, and IRF7 are pro-inflammatory regulators, activation typically occurs under PRRs leading to transcription of interferon-related genes and cytokines.[203] In metabolic tissues, IRF signalling can occur as a result of inflammatory and metabolic sensors, shaping macrophage phenotypes.[204,205]

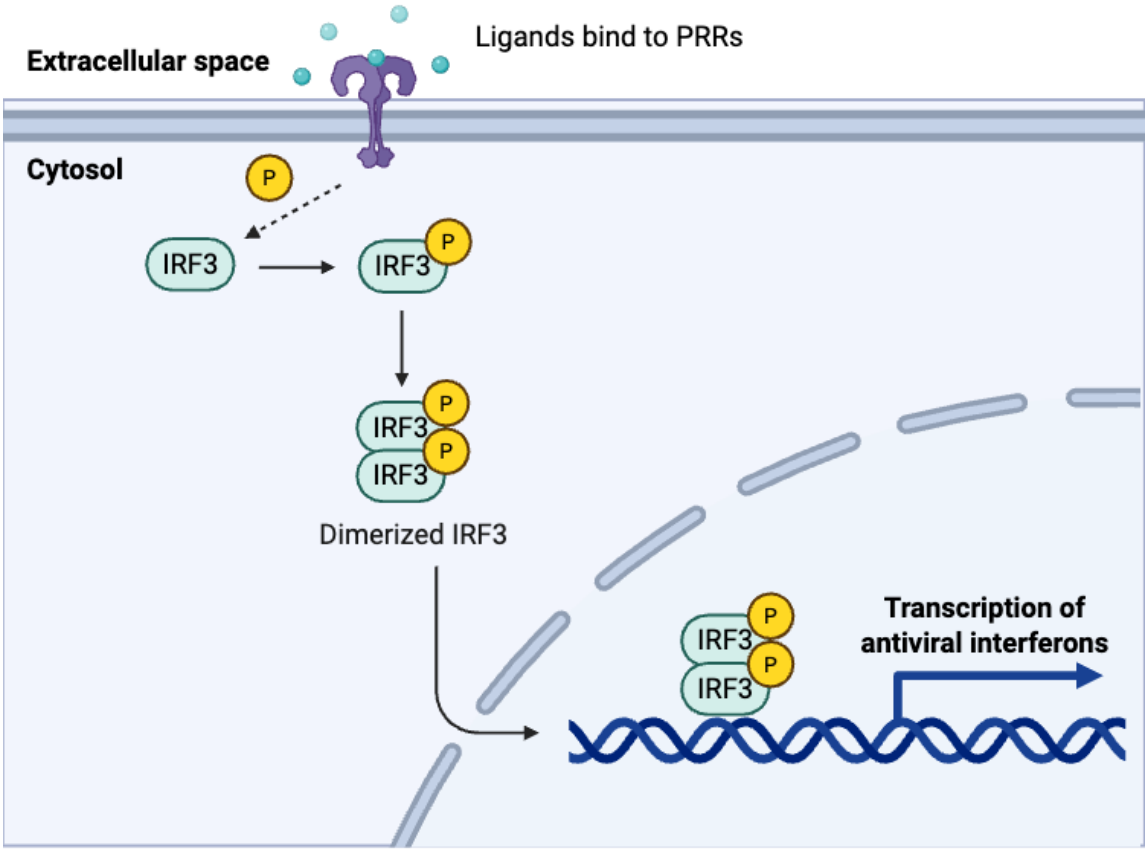

Figure 1.7: Activation and translocation of IRF3 leading to transcription of inflammatory and IFN-related genes.
Ligands bind to their PRRs, leading to phosphorylation of IRF3 and subsequent dimerization of IRF3, allowing this dimer to translocate to the nucleus and transcribe inflammatory genes. Image created with BioRender.com.

There are three types of interferon receptors, type I, II, and III, which bind these same classes of IFN ligands.[203] The most well-characterized type I IFNs are IFNα and IFNβ, which bind to a heterodimeric receptor composed of two binding chains; IFNAR1 and IFNAR2, which signal through recruitment of janus-activated kinases (JAKs), TYK2 and JAK1. JAK activation causes tyrosine phosphorylation of signal transducers and activators of transcription (STAT)1 and STAT2, which leads to the formation of the complex, interferon stimulated gene (ISG) factor 3 (ISGF3) with IRF9.[203] ISGF3 then translocates to the nucleus and binds IFN-stimulated response elements

(ISREs) in promoter regions of IFN-inducible genes.[203] Binding of type II and type III interferons can similarly lead to transcription of ISGs, albeit downstream of different receptor complexes.[203]

Emerging evidence suggests that metabolic stress can trigger IRF3, even without microbial stimuli. In metabolic tissues, IRF3 can be activated by mitochondrial damage, fatty acids, and cytosolic DNA released from damaged organelles.[206–208] Mitochondrial damage and DNA released during metabolic stress can activate cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING)-IRF3 axis, and this can also promote NFκB-dependent inflammatory gene expression in metabolic tissue, producing a pro-inflammatory outcome that leads to tissue damage.[209,210] Once activated by these stimuli via cGAS-STING-TBK1 or TLR4, IRF3 is phosphorylated, dimerizes and then translocates to the nucleus, transcribing inflammatory genes, such as TNF, IL-6, and IFNβ (Figure 1.8).[206] Interestingly, there is a duality when it comes to IRF3 in promoting or restraining features of metabolic disease. In the liver for instance, overexpression of IRF3 suppressed NFκB signalling through direct interaction with a subunit of IKK, reducing inflammation, insulin resistance, and lipid accumulation.[211] On the other hand, IRF3 expression is elevated in adipocytes of obese mice and humans and its signalling promotes insulin resistance. Knocking down of IRF3 reduced adipose and systemic inflammation in an obese mouse model.[212] In cardiomyocytes cultured to mimic lipotoxicity and DCM, mitochondrial damage and DNA release led to activation of cGAS-STING and subsequent activation of IRF3 and NFκB, increasing cytokine production.[213] In this same model, downregulation of STING attenuated inflammation and cell death in cardiomyocytes, suggesting the cGAS-STING-IRF3 axis plays a role in myocardial injury under metabolic stress.[213] In a pressure-overload mouse model, suppression of STING, associated with decreased phosphorylation of IRF3, led to ameliorated oxidative stress, cardiac remodelling, inflammation and mitochondrial fragmentation in the heart.[214] While these

studies can suggest that IRF3 contributes to inflammation, insulin resistance, lipid accumulation and tissue remodelling, the fact remains that this pathway is not extensively studied in the context of metabolic disease. Considerable research is still needed to understand the specific molecular mechanisms, if in existence, by which IRF3 participates in consequences of MetS.

1.2.4 Inflammation in key cell types

Inflammatory processes manifest differently in different cell types that are all vital in MetS, the following section will focus on cardiomyocytes, macrophages, and PBMCs and their responses to metabolic stress.

1.2.4.1 Cardiomyocytes

Under hyperglycemic conditions, cardiomyocytes exhibit increased activation of NFκB and production of pro-inflammatory cytokines, elevated ROS, and increased cell death.[200,215–218] This finding has been reported in numerous studies *in vitro* and *in vivo*. Upon an initial metabolic stress (glucose, iron, lipids), these metabolites accumulate in the cells leading to mitochondrial overload, electron leakage and ROS generation. In response to this, antioxidant gene transcription is increased to counteract the sudden increase in ROS.[219] If the antioxidant system continues to be overburdened, ROS accumulation continues and associated cellular stress kinases (MAPK) phosphorylate downstream substrates.[219,220] NFκB becomes activated and translocates to the nucleus, inducing transcription of pro-inflammatory cytokines.[221] At the same time, these pathways intertwine with MAPK promoting NFκB and ROS enhancing MAPK.[220,221] Cytokine release promotes recruitment of additional immune cells and this combined with mitochondrial damage, loss of membrane potential, can lead to apoptosis. Ultimately, this can induce structural changes in the heart including hypertrophy, contractile dysfunction and fibrosis.

1.2.4.2 Macrophages

Resident and recruited macrophages exposed to metabolic stressors shift to a significantly inflammatory environment compared to without. This shifts macrophages to a pro-inflammatory phenotype and transcription of NFκB, IRF, and inflammasome components can be upregulated.[222] In dilated cardiomyopathy patients, CCR2+ monocytes higher expression of inflammatory mediators and NLRP3 inflammasome components, when glucose uptake is reduced, the inflammatory output is diminished.[223] In mice given a high fat diet, elevated inflammatory macrophage accumulation contributed to diastolic dysfunction, reducing that pro-inflammatory phenotype and blocking IL-1β attenuated ROS generation in cardiomyocytes and improve diastolic function.[224] This highlights that macrophages can detect metabolic disturbances and diminishing the signalling pathways that exacerbate release of inflammatory agents that cause tissue damage remains a central axis to target.

When macrophages are exposed to high glucose, they increase glucose uptake and glycolytic flux via GLUT transporters, providing substrate not only for energy but for production of inflammatory agents.[225] This also triggers metabolic reprogramming, with increased glycolysis and ROS production through mitochondria or NOX.[226] High glucose levels can also trigger activation of the hexosamine biosynthetic pathway, modifying intracellular proteins to influence regulation of inflammatory gene transcription.[227] Inhibiting this pathway has been found to reduce IL-1β and IL-12 expression, providing an additional mode to target to reduce inflammatory cytokine release.[227] Production of ROS and other metabolic intermediates can promote activation of pro-inflammatory pathways; NFκB, IRF, and MAPK.[225] This leads to transcription of pro-inflammatory cytokines such as TNF, IL-6, IL-1β, and chemokine MCP-1.[225] Elevated pro-inflammatory cytokines can then act in an autocrine and paracrine manner, exacerbating

inflammation and reducing insulin signalling in neighbouring cells.[226] In addition, phagocytic capabilities become compromised during periods of metabolic stress, high glucose conditions have been shown to reduce macrophage ability to phagocytose pathogens or apoptotic cells.[226] Cell viability can become impaired under metabolic stressors such as high glucose and iron, oxidative damage can trigger cell death.[226] Beyond macrophages themselves, cytokine production can also impair glucose metabolism, for example IL-6 has been shown to suppress AMPK activity in the heart and liver, and can also systemically impair insulin signalling.[104] Accumulation of ROS, as mentioned previously, can damage mitochondria, leading to mitochondrial ROS production which can activate NFκB or inflammasome formation. This is essentially a positive feedback loop which sustains inflammatory activation, presenting multiple metabolic enzymes and pathways as a target to reduce inflammatory cytokine production.

1.2.4.3 PBMCs

Peripheral blood mononuclear cells (PBMCs) are a heterogenous population of cells made up of monocytes, lymphocytes, and a small number of dendritic cells, all of which can contribute to systemic inflammation as seen in MetS.[228] In MetS, PBMCs exhibit altered bioenergetics, oxidative stress and increased cytokine production, acting as sensors and amplifiers of metabolic dysregulation.[228] Monocytes are central players in this, being the most responsive to metabolic stress, chronic exposure to which activates PRRs leading to activation of NFκB and sometimes IRF signalling events, promoting release of pro-inflammatory cytokines which can propagate inflammation.[229] Functionally, monocytes typically exhibit "trained immunity" as a results of metabolic and epigenetic reprogramming characterized by increased glucose uptake, ROS production and rapid responses to additional stimuli.[230] This can contribute to vascular and tissue dysfunction and insulin resistance. There are three classes of monocytes; classical, non-classical

and intermediate, shifting among these populations depending on the context. Under metabolic stress, classical CD14+CD16+ monocytes shift toward a non-classical or intermediate phenotype, associated with oxidative stress and release of inflammatory agents.[231] Lymphocytes encompass T cells, B cells and NK cells. CD4+ and CD8+ T cells can also be regulated by metabolic status, with increased expression of activation markers (CD69, HLA-DR), shifting towards a pro-inflammatory phenotype to release cytokines, thereby exacerbating tissue inflammation and insulin resistance.[232] Another subset of T cells called regulatory T cells or Tregs, restrain inflammation with release of anti-inflammatory agents such as IL-10 and TGFβ.[233] In MetS, depletion of Tregs can lead to activation of effector T cells and macrophages, promoting tissue inflammation. NK cells exhibit increased cytotoxicity and cytokine release in the context of metabolic stress, enhancing T cell activation and promoting systemic inflammation.[234] Chronic activation of NK cells can reduce the protective NK cell subset (CD56 bright), expanding cytotoxic NK cells (CD56 dim), which is also correlated with vascular dysfunction and insulin resistance.[235]

PBMCs are systemically exposed to metabolic stressors and can therefore reflect the systemic inflammatory status, acting as lookouts for whole-body inflammation and reflecting functional changes upon metabolic stress.[228]Acute and chronic high glucose can increase glucose uptake and shift PBMCs to elevated glycolytic flux, providing energy for inflammatory responses.[229] Several studies have documented mitochondrial dysfunction in patients with poor glycemic control, as well as elevated ROS and endoplasmic reticulum (ER) stress.[228,236,237] As ROS and ER stress surge, PBMCs, similar to other cell types, activate redox-sensitive pathways (NFκB, IRF, and MAPK), leading to increased production of pro-inflammatory agents.[238] Additionally, autophagy and mitophagy are upregulated in PBMCs under metabolic stress as a compensatory

mechanism in response to oxidative stress; this can reduce high glucose-induced inflammatory gene expression and apoptosis.[237,239]

Additionally, metabolic stress can trigger signalling cascades, upregulate cytokine production, and increase activation of subtypes such as monocytes and lymphocytes.[237,240] Activated PBMCs release inflammatory agents into circulation, promoting systemic inflammation which can affect other tissues as well.[237,240] Oxidative stress in PBMCs can alter surface activation and homing markers in the different subsets of cells in PBMCs, promoting vascular inflammation and increased leukocyte infiltration into tissues.[228,237] Upon prolonged exposure to metabolic stress, PBMCs can show markers for apoptosis and cell death. Examining PBMCs can reliably reflect systemic metabolic control, as impaired mitochondrial dysfunction, elevated ROS and cytokines, and autophagy markers, can indicated conditions of the metabolic state of patients, such as poor HbA1c being associated with PBMC bioenergetics.[228] This makes PBMCs useful in examining effects of metabolic stress to reflect whole-body inflammation.

1.3 Role of Iron in Metabolic Dysregulation

1.3.1 Iron Homeostasis and Regulation

Iron is an essential element involved in many different cellular and metabolic processes. Total amount of iron is tightly regulated and exists bound to transferrin, but when it exceeds transferrin capacity iron homeostasis becomes imbalanced and this can contribute to insulin resistance and diabetes.[242,243] Iron overload (IO) cardiomyopathy is a form of cardiomyopathy resulting from the accumulation of iron in the myocardium due to iron metabolism dysfunction.[244] While IO affects several organs, the heart is the main one affected by accumulation of iron as it eventually leads to cardiac dysfunction and failure.[244,245]

Regulation of iron metabolism is essential to maintain the balance of bodily iron levels. There are specific uptake and efflux mechanisms involved, as well as intracellular iron chaperones. Dietary iron is present in its oxidized form, Fe^{3+} and loaded onto transferrin, the main iron plasma carrier to various tissues and organs. Transferrin prevents iron from being freely available to have toxic effects. Iron transport occurs at barriers such as heart barrier, blood-brain barrier or the liver barrier, from serum into these tissue systems.[246,247] Uptake of transferrin-Fe^{3+} complex is through transferrin receptor 1 (TRF1) through receptor mediated endocytosis.[246,247] For iron to leave, it must be reduced in the acidic environment of the endosome by the ferriducatse, six-transmembrane epithelial antigen of the prostate 3, STEAP3 and then it leaves the endosome through the divalent metal transporter 1, DMT1.[246,247] Iron is then stored in ferritin and exported into the blood through ferroportin, a transmembrane protein that transport iron out of the cell, and the only known mammalian iron exporter.[246,247] Hepcidin is an important hormone involved in regulating the availability of iron available for bodily functions, such as acting as a cofactor for oxygen transport in red blood cells and limiting the amount of iron available for cells to use by binding to ferroportin, decreasing iron efflux.[248] Together, these proteins are vital in iron metabolism and cellular homeostasis.

Transferrin can carry up to two molecules of iron, IO occurs when iron exceeds the buffering capacity of transferrin and non-transferrin bound iron are taken up in organs such as the heart, liver, and skeletal muscle.[246,247,249–252] These highly reactive forms of non-transferrin bound iron can cause oxidative damage, inflammation, insulin resistance, and cell death, leading to organ dysfunction.[246,247,252] These consequences, which incidentally overlap with those of hyperglycemia, can lead to diseases such as cirrhosis, cardiomyopathy and T2D.

1.3.2 Iron Overload in MetS

Iron overload is the result of underlying mechanisms that lead to accumulation of iron in the body.[253] There are a multitude of ways for iron overload to arise in an individual, these can be classified as genetic iron overload or acquired iron overload. Genetic iron overload includes hemochromatosis, where there are mutations in the *HFE* gene leading to excessive iron absorption in the intestine and accumulation in the liver, pancreas, and heart.[254] Acquired iron overload encompasses individuals with thalassemia, liver disease, excess iron intake in diet, and metabolic conditions.[255,256] Thalassemia is a genetic disorder of hemoglobin, characterized by reduced hemoglobin production and subsequent anemia.[255] Iron overload is a significant consequence of this disorder given that thalassemia patients require frequent blood transfusions.[255]

Beyond the primary and secondary genetic disorders mentioned above that result in iron overload, iron overload has also been observed in approximately 30% of patients with MetS.[257–259] ere are a variety of factors that can distinguish those who develop iron overload, although the exact cause and underlying mechanism is unclear in MetS patients. These factors include development of hepcidin resistance, differences in liver phenotype, degree of insulin resistance, inflammatory patterns, genetic background, and diet.[259,260] Accumulation of iron and dysregulated iron handling are integral components of the pathophysiology of MetS.[253] Many studies have linked even modest increases of iron stores with insulin resistance, T2D, NAFLD and CVD.[253] On a systemic level, disrupting the hepcidin-ferroportin axis is a crucial mechanism linking inflammation and iron retention in MetS.[261] Release of pro-inflammatory cytokines such as IL-6 can stimulate hepcidin expression, thereby reducing ferroportin-mediated iron export from macrophages and increasing iron sequestration in tissues.[261] On the other hand, obesity and insulin resistance can change hepcidin expression through metabolic and genetic pathways.[262] According

to a consensus statement on defining metabolic hyperferritinaemia, insulin resistance, NAFLD, aging, iron accumulation, and alcohol consumption are all contributing factors to iron accumulation in patients with metabolic dysfunction.[262]

Iron overload can affect a variety of tissues and cells, including the liver, adipose tissue, heart, and immune cells. In the liver, iron accumulation can exacerbate lipotoxicity and oxidative stress, resulting in NAFLD. Rat models combining a high fat diet with iron overload saw consequences of inflammation and oxidative damage, contributing to progression of liver disease.[263] Adipose tissue present another organ that is influenced by iron in metabolic signalling, where iron overload in adipocytes and resident macrophages can impair adipokine secretion, promote insulin resistance, and skew macrophage phenotype to an M1-like state.[253,264,265] Interestingly, increased adipose iron has also been found to be associated with reduced adiponectin levels in humans, suggesting a mechanistic role in reducing adipokine secretion in the context of MetS.[264–266] Cardiac tissue is particularly prone to iron-induced injury, myocardial iron accumulation increases ROS production, induces mitochondrial damage and has been heavily implicated in cardiomyopathy.[267] Iron can also regulate immune cell function in MetS, with macrophage polarization heavily influences by iron handling in various tissue.[268] Activated M1-like macrophages retain iron, promoting inflammatory signalling, while M2-like macrophages promote iron export.[268]

The pro-oxidant properties of labile iron (through the Fenton reaction) is the central event that links iron to tissue injury, iron-induced ROS damages lipid, proteins and DNA, which can go on to prime inflammatory pathways such as NFκB and inflammasome activation.[261] Excess iron can also increase susceptibility to lipid peroxidation and ferroptotic cell death.[269] Using iron as a biomarker of metabolic conditions remains controversial, as iron has not been well-established as

a key metabolic risk feature.[253] Therapeutically however, interventions that reduce iron have shown metabolic benefits in certain clinical and pre-clinical models.[253,269] Currently, targeting oxidative damage and inflammatory mediators remains the focus in cardiovascular and metabolic contexts.

1.3.3 Mechanisms of Iron-Induced Dysfunction

Mechanisms of iron-induced function occur in various tissues through oxidative stress, mitochondrial dysfunction, impaired insulin signalling, and activation of inflammatory pathways, ultimately leading to adverse outcomes seen in CMD. A summary of mechanisms of iron-induced dysfunction in tissue is outlined in Figure 1.9.

1.3.3.1 Oxidative stress and mitochondrial function

Excess iron accumulation can lead to production of ROS through the Fenton reaction, where iron catalyzes the conversion of hydrogen peroxide to reactive hydroxyl radicals.[270,271] These ROS damage cellular components, leading to dysfunction and apoptosis. Increased ROS in this context can promote inflammation, fibrosis and insulin resistance.[270] This oxidative damage is particularly harmful in the liver, pancreas, skeletal muscle and the heart.[270] Iron accumulation in the mitochondria also leads to impaired oxidative phosphorylation and ATP synthesis [270] As previously discussed, mitochondrial dysfunction can directly lead to apoptosis in muscle, liver and the heart. Essentially labile iron undergoes various biochemical reactions to produce ROS, which can oxidize lipids, proteins, and DNA.[272] Lipid peroxidation, deactivation of key enzymes, and DNA damage can all trigger the intrinsic apoptosis pathway.[272] Thus, the central mechanism for iron-induced cellular damage is elevated ROS and mitochondrial dysfunction.

1.3.3.3 Insulin signalling

Accumulation of iron can directly interfere with insulin signalling, thus contributing to insulin resistance. Elevated iron can impair tyrosine phosphorylation of IRS, reducing its sensitivity.[272] This can affect glucose metabolism in key tissues such as adipose tissue and skeletal muscle.[272] IO has also been associated with decreased β-cell function, leading to impaired insulin secretion.[272]

1.3.3.4 Inflammatory pathway activation

Iron overload can activate NFκB and inflammasome formation.[261] Production of pro-inflammatory cytokines plays a key role in insulin resistance, as mentioned in previous sections. Iron-induced inflammation can impair adipocyte and macrophage function, leading to altered adipokine secretion which also contributes to insulin resistance and metabolic disturbances.[261]

1.4 Conclusion

MetS is a multifaceted group of abnormalities that remain the centre of cardiovascular-related outcomes. While cardiovascular disease is the main outcome of this group of conditions, there are numerous organs that contribute to this outcome, including the skeletal muscle, adipose tissue and liver. There are several key pathways that contribute to the tissue damage and sustained inflammatory state seen in MetS. Beyond that, they are closely intertwined and influence each other, revealing MetS as a complex, multidimensional issue that affects millions of people worldwide.

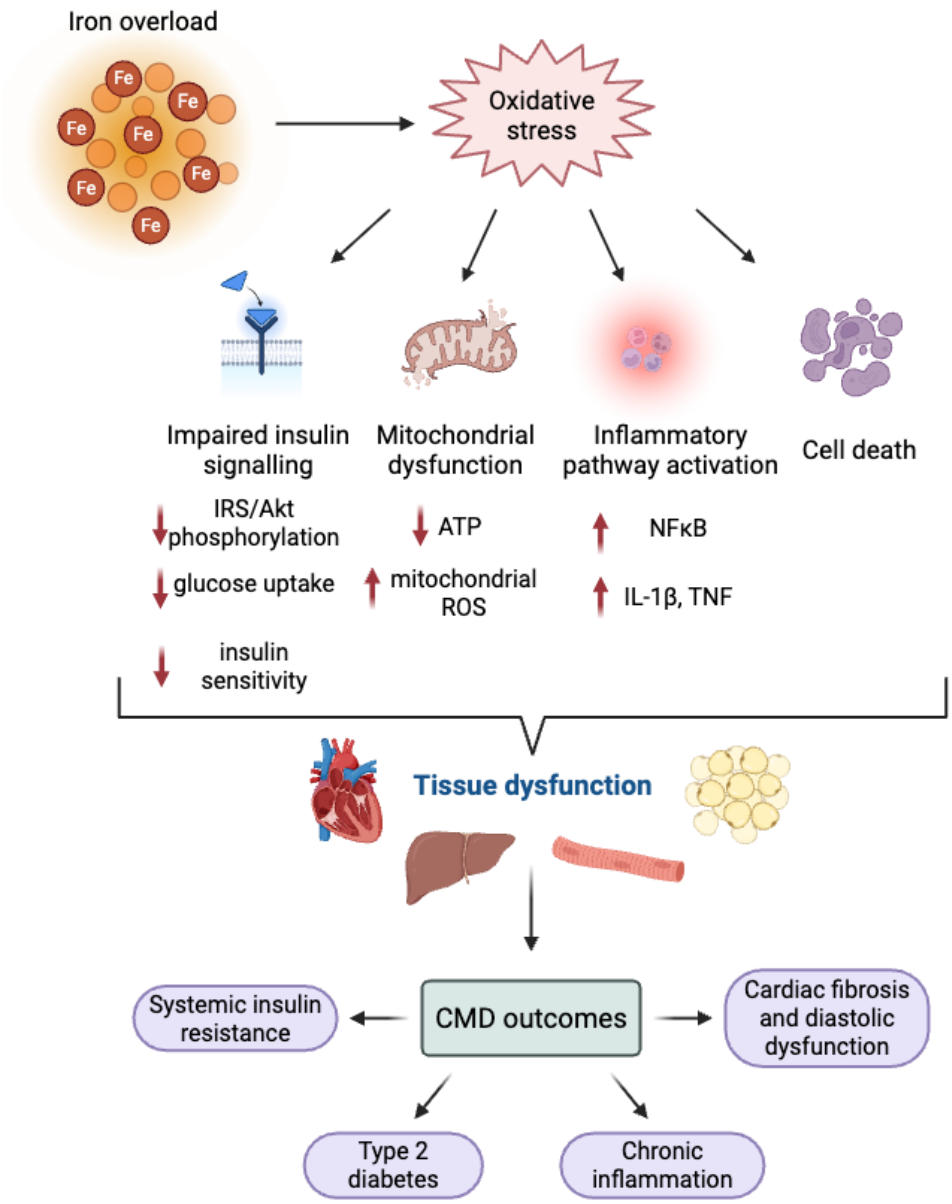

Figure 1.8: Mechanisms of iron-induced dysfunction leading to adverse CMD outcomes. Excess intracellular iron promotes oxidative stress, disrupting multiple cellular pathways. These include impaired insulin signalling, mitochondrial dysfunction, inflammatory pathway activation and cell death. This can ultimately contribute to tissue dysfunction in the heart, liver, skeletal muscle and adipose tissue, leading to CMD outcomes. Image created with BioRender.com.

1.5 Research Aims and Hypotheses

The ability of metabolic stressors, such as high glucose and iron, to induce cell death, inflammation and ROS production, is an imperative area of study given the relevance of these disturbances in MetS. These metabolic perturbances lead to adverse outcomes in multiple tissues and organs, rendering them highly relevant research subjects to tackle. The overall goal of this dissertation is to elucidate the different cellular events caused by high glucose, and whether these effects can be mitigated with a potential therapeutic target, or exacerbated with the addition of iron in different cell types. This will be accomplished using three aims, with each chapter of the dissertation addressing its corresponding aim.

Aim 1: Examine the ability of adiponectin-mimetic peptide ALY688 to attenuate high glucose-induced cell death via regulation of autophagy and inflammation in cardiomyoblasts.

Hypothesis: ALY688 will exert anti-apoptotic, anti-inflammatory and autophagy-boosting effects in H9c2 rat cardiomyoblasts upon high glucose treatment. Additionally, caspase-3 activity and pro-inflammatory gene expression will be blunted in ventricles of hyperglycemic mice.

Aim 2: Determine whether combining high glucose and iron overload in macrophages will elicit a robust inflammatory response and oxidative stress eventually leading to cell death.

Hypothesis: Combining high glucose and iron in macrophages will provide the necessary signals to induce NFκB and prime and activate NLRP3 inflammasome, leading to IL-1β release. Additionally, I hypothesize that combining these two stressors will lead to oxidative stress increased transcription of inflammatory genes, more than each stimulus alone in an additive or synergistic manner.

Aim 3: Observe changes in immune cell dynamics, ROS production, and cell death in peripheral blood mononuclear cells (PBMCs) upon treatment with high glucose and iron.

Hypothesis: Combining high glucose and iron treatments will induce changes in the composition of PBMCs, including cytotoxic T cells, natural killer cells, and monocytes. Furthermore, I hypothesize combining these two stressors will lead to increased pro-inflammatory cytokine production and oxidative stress in cellular subsets, in comparison to either treatment independently.

Overall, I hypothesize that the metabolic stressors of high glucose and iron will cause cell death as a result of oxidative stress and pro-inflammatory cytokine production in immune cells, and that ALY688 will attenuate high glucose-induced cell death by promoting autophagy and reducing inflammation in cardiomyoblasts and ventricular heart tissue.

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Chapter 2 – The adiponectin mimetic peptide ALY688 mitigates high glucose-induced cell death via an autophagy-dependent mechanism

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Author contributions:

I performed all experiments, including; cell culturing, cell treatment, preparation of cells and ventricular tissue for downstream applications, data analyses, and writing of the manuscript. The following contributions were made by co-authors:

J Tang: Blind confocal imaging of VC3AI cell line (Figure 2.1J).

K Nguyen: Blind confocal imaging of HiBiT cell line (Figure 2.2D)

HK Sung: Experimental guidance and troubleshooting aid

D Burger: Providing hearts isolated from C57BL/6 injected with/without STZ and ALY688

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Abstract

High glucose (HG) levels in diabetes are associated with cardiovascular-related complications. Here we examined the protective effects of ALY688, an adiponectin-mimetic peptide on HG-induced cell death in H9c2 cardiomyoblasts. Using a caspase-3 activatable fluorescent probe and time-lapse microscopy, we found that HG induced caspase-3 activation after approximately 16 hours. This was verified by western blot and real-time monitoring of activated fluorescent caspase-3 at 24 hours. Subsequently, cell death was confirmed using live/dead flow cytometry, ReadyProbe cell viability probe microscopy and determination of lactate dehydrogenase release. Both HG-induced caspase-3 activation and cell death were attenuated by overnight ALY688 pretreatment for 16 hours. ALY688 was no longer able to mediate these effects in cells with CRISPR-mediated Atg7 knockout to render them autophagy-deficient. We verified that ALY688 stimulated autophagy using western blot for LC3 and p62 and the HiBiT LC3 reporter system assay. Next, we examined whether H9c2 cell death released factors that resulted in activation of macrophage inflammatory pathways. We observed that RAW 264.7 macrophages treated with conditioned media from HG-treated, but not ALY688/HG-treated cells, elicited elevated inflammatory signaling and cytokine gene expression. These include upregulated gene expression of IFNβ, TNF, and IL-1β, downstream of significantly increased activation NFκB. Translating these findings to examining the myocardium of mice with prolonged hyperglycemia due to streptozotocin injection, we observed elevated caspase-3 cleavage which was attenuated by intervention with 8 weeks of daily subcutaneous ALY688 injection. HG-induced increases in myocardial pro-inflammatory cytokines, including IL-1β, TNF, IL-6 and IL-12 were reduced by ALY688. In conclusion, adiponectin signaling can reduce glucose-induced cell death via promoting autophagy, reducing the subsequent impact on induction of macrophage inflammation.

2.1 Introduction

Cardiac complications remain a leading cause of death in diabetic patients [1]. For example, diabetic cardiomyopathy (DCM) is characterized by abnormal heart structure or function that occurs in individuals with diabetes in the absence of other underlying abnormalities in the heart [2]. Hyperglycemia, which occurs in the setting of poorly controlled diabetes, is a major contributor to the pathogenesis of DCM [2]. Hyperglycemia is a key trigger for metabolic changes in diabetes, and can promote insulin resistance, oxidative stress, apoptosis, and inflammation to contribute to the progression of cardiac damage and DCM [2]. Activation of several pro-inflammatory signalling pathways have been implicated in DCM, notably nuclear factor-κ B (NF-κB) [3].

Cell death is a critical event that contributes to cellular development and tissue homeostasis [4]. Conventional regulated cell death pathways include apoptosis, occurring through a stringent biochemical pathway, and necrosis, the rupture of acutely injured cells [4]. Apoptosis is the most extensively studied form of programmed cell death and is characterized by morphological changes in the cell mediated by proteolytic enzymes named caspases [5,6]. In the heart, cardiomyocyte apoptosis has been implicated in progression of cardiovascular disease, contributing to impaired cardiac function and adverse remodelling events, ultimately leading to heart failure [7–9]. On a molecular level, inflammation, impaired autophagy and oxidative stress significantly drive myocardial apoptosis [10–12]. When autophagy is impaired, damaged cellular components accumulate and this can lead to the release of pro-apoptotic factors particularly in the context of DCM [13,14]. This is particularly relevant in the heart, as cardiac apoptosis is a component of the development of DCM [2,3]. Diabetic patients have substantially increased risk of suffering from cardiovascular-complications that account for at least 50% of deaths in the diabetic population

[15,16]. In addition, risk of suffering from heart failure in men and women are increases by 2.4-fold and 5-fold, respectively [2].

Inflammation has been implicated in a variety of metabolic abnormalities that can lead to cardiometabolic disease, given its ability to disrupt insulin signalling, promote oxidative stress, ad drive malapative tissue remodelling [17]. In particular, hyperglycemia can lead to increased expression of inflammatory genes across multiple tissues, exacerbating cardiomyocyte apoptosis and cardiovascular injury [18–20]. The inducible nuclear factor-κ B (NF-κB), is an important transcription factor that controls many pathological processes in the myocardium, including inflammation in cardiomyocytes and ultimately apoptosis [19,21]. Studies have recently shown that NF-κB signalling is involved in hyperglycemia-induced cardiac cell damage [19–21]. Activation of NF-κB by TNF regulates expression of various pro-inflammatory cytokines, such as IL-6, IL-17, and IL-1β and this is thought to be increased under hyperglycemic conditions [19–21].

Autophagy is an essential catabolic process that maintains cellular homeostasis and survival upon exposure to extra- or intracellular stressors [22,23]. While autophagy is primarily considered a cell survival mechanism, it can also be an indicator of cell health and whether cells are under conditions of stress. Autophagy is viewed as a double edged sword, where too much or too little can both have detrimental effects on cells [24–26]. Autophagy is a protective mechanism to eliminate damaged organelles and proteins, but an excessive amount can increase tissue damage and impair the immune response, while too little can lead to a buildup of toxic cellular components [24,26].

Adiponectin, is a circulating adipose-derived cytokine involved in regulating glucose levels [27,28]. Individuals with diabetes and cardiovascular disease have been found to have low levels

of circulating adiponectin [29]. Increased levels of adiponectin have shown anti-apoptotic, anti-inflammatory, and anti-atherosclerotic properties, making it a therapeutic target for cardiovascular and metabolic diseases [30,31]. Many studies have shown that adiponectin, in both human and animal models, is a key component in the relationship between autophagy, inflammation, and cardiac cell death [32].Adiponectin is known to stimulate autophagic flux in skeletal muscle, cardiomyocytes, liver, and adipose tissue, with increased levels of autophagy promoting anti-apoptotic and anti-inflammatory outcomes, ultimately leading to improved tissue function [33–37]. In streptozotocin (STZ)-induced diabetic mouse models, animals have suppressed autophagy and when an intervention to promote autophagy was used a cardioprotective effect was observed [38–40]. In addition, induction of autophagy is a key cellular process that can prevent high glucose-induced cell death in cardiomyocytes [41]. ALY688 is an adiponectin mimetic peptide that has been shown to act via both adiponectin receptors, AdipoR1 and AdipoR2 [42,43]. This peptide was generated by identifying the biologically active domain of adiponectin and consists of 10 modified non-natural amino acids.

In this study, we used ALY688 as an adiponectin receptor agonist to examine its protective role in high glucose-induced cell death, the mechanistic role of autophagy and the impact to subsequently attenuate macrophage inflammation.

2.2 Methods

2.2.1 Cell culture

H9c2 rat cardiomyoblasts were cultured with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in Dulbecco's Modified Eagle Medium (DMEM) and grown to 80-90% confluency, depending on the target assay. Subsequently, groups undergoing treatment with ALY688 (300 nM) were pre-treated overnight for 16-18 hours in normal glucose media (5.5mM), control groups were pre-treated with PBS. Following pre-treatment, cells were treated for 24 hours in 0.5% FBS media with either normal glucose (5.5 mM) or high glucose (25 mM) media, supplemented with and without 300 nM of ALY688. To investigate the role of autophagy, Atg7 knockout (Atg7 KO) H9c2 cells were used, generated via the CRISPR-Cas9 system [44].

2.2.2 Western blotting

H9c2 cells were seeded into 6-well plates and treated with normal glucose and high glucose with and without ALY688 (300 nM). Following treatment, cells were washed with cold PBS and prepared using lysis buffer, lysates were syringed to ensure adequate lysis of cells. The resuspension was then centrifuged at 12,000 rpm for 15 minutes at 4°C, supernatant was collected and boiled at 95°C for 5 min. Lysates were then run on the appropriate percentage of polyacrylamide gels at 90 V for 30 min for the proteins to leave the stacking gel, then run at 120 V for 1 h. The proteins on the gel were then transferred to polyvinylidene difluoride (PVDF) membranes using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories Ltd). Membranes were then submerged in 3% bovine serum albumin (BSA) blocking buffer for 1 h, followed by overnight incubation in 1:1000 dilution of primary antibodies: caspase-3 (Cell Signalling, 9662), p62 (Cell Signalling, 5114), and LC3B (Cell Signalling, 2775) at 4°C. Membranes were then washed with TBS-T three times for 10 min before incubation with a 1:5000 dilution of an anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody at room

temperature for 1h. Following secondary antibody incubation, membranes were washed with TBS-T three times for 10 min. Membranes were then quickly rinsed with Clarity Western ECL solution (Bio-Rad Laboratories Ltd) and visualized using the Chemi-doc MP Imaging System (Bio-Rad Laboratories Ltd). Western blot band intensity was quantified using ImageLab software and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signalling, 2118).

2.2.3 Flow cytometry to study cell death

Wild-type (WT) and Atg7 KO cells were seeded into a 6-well plate, grown to 90% confluency and treated accordingly with normal glucose and high glucose media, with and without ALY688 pretreatment for 24 hours. Following treatment, cells were harvested and stained with the appropriate stain or antibody. Cells were harvested and stained with a live-dead stain (Thermofisher, cat#L23105) for 30 minutes (RT, protected from light), fixed in IC fixation buffer (Thermofisher, 88-8824-00) for 30 minutes (RT, protected from light), permeabilized in cold methanol (Sigma, 322415) for 20 minutes (4°C, protected from light), and then stained with a cleaved caspase-3 antibody (Cell Signalling, 8788) conjugated to pacific blue for 30 minutes (RT, protected from light). Following staining, cells were washed in FACS buffer (2% FCS in PBS) and run on the Aurora 5L flow cytometer (Cytek) and subsequent analyses performed using the FlowJo software.

2.2.4 Confocal and fluorescence microscopy to study autophagy and cell death

Cells were seeded into a 96-well plate, grown to 90% confluency and treated accordingly with normal glucose and high glucose media, with and without ALY688 pretreatment for 24 hours. Following treatment, cells were washed with warm DPBS (with Ca^{2+}/Mg^{2+}) and stained using a cell viability kit (ReadyProbes, Thermofisher, R37609) for 30 minutes. After staining, cells were

imaged using the EVOS FL Auto 2 Imaging System. Quantitative analysis was performed as a percentage of dead cells (stained green) over total cells (stained blue).

Genetically engineered H9c2 cardiomyocytes with a genetically encoded switch-on fluorescence-based indicators that contain a caspase-3 cleavage site as a switch were used to visualize caspase-3-like activity [45]. Upon cleavage by caspase-3-like proteases the non-fluorescent indicator becomes rapidly fluorescent, emitting a GFP signal, and allowing for detection of caspases. This cell line is termed "VC3AI cell line" and was grown in the same way as wild-type H9c2 cells. These cells were seeded and treated with normal glucose and high glucose media, both with and without ALY688. Following treatment, cells were stained with a nuclear mask stain (ThermoFisher, H10325) for 30 min, fixed with formaldehyde for 30 min at room temperature and mounted on microscope slides.

An LC3 HiBiT Reporter Assay system (Promega, GA1040) was also used to measure autophagic flux. H9c2 cells were transfected with the LC3 HiBiT reporter, consisting of a HiBiT tag fused to the human LC3 protein with a Halo-Tag spacer. Incubation with a HaloTag ligand allows for visualization of protein trafficking and turnover in the autophagosome. Cells were pre-treated overnight with and without ALY688 (300 nM), followed by 24 hours of normal or high glucose treatment. Cells were then incubated with a nuclear mask stain (Thermofisher, H10325) and the Janelia Fluor 646 HaloTag ligand (Promega, HT1060) for 30 min. Cells were fixed with formaldehyde for 20 min at room temperature and mounted on microscope slides. All slides were imaged using an A1 Nikon Confocal Microscope and subsequent analysis on fluorescent intensity was done using ImageJ software.

2.2.5 Quantitative polymerase chain reaction (qPCR)

WT H9c2 cells were cultured in a 6-well plate as described above and following treatment cells were harvested for RNA isolation. RNA was isolated from the cells using the PureLink RNA Mini Kit (Invitrogen, 12183025) following the manufacturer's instructions using TRIzol Reagent (Thermofisher, 15596018). Complementary cDNA was made from 1 ug of RNA using the RevertAid RT kit (Thermofisher, K1691), per the manufacturer's protocol. Following conversion to cDNA, 100 ng was used to assemble 10 uL qPCR reactions using the Sso Advanced Universal SYBR Green Supermix (Bio-Rad Laboratories Ltd, 1725270). The following appropriate primer sets synthesized by ThermoFisher were used to examine the following genes: IL-6, IL-1β, IFNβ, iNOS, TNF, TGFβ, and IL-10. Relative gene expression analysis was conducted on CFX Maestro and normalized to RPLP0. Table 1 summarizes the primers used in this study.

Table 2.1: List of rat primers

Name	Type	Sequence (5' – 3')
IL-6	Forward Reverse	TCCAGCCAGTTGCCTTCT GTATCCTCTGTGAAGTCTCCTCTC
IL-1β	Forward Reverse	TCATTGTGGCTGTGGAGAAG GGTGCTTGGGTCCTCATC
IL-10	Forward Reverse	CTGGCTGGAGTGAAGACC CTGGCTGACTGGGAAGTG
IFNβ	Forward Reverse	CGTTCCTGCTGTGCTTCT ATGTCCGAATGCTAGTGCTT
iNOS	Forward Reverse	ACAGCCTCAGAGTCCTTC ACCAGCCAAATCCAGTCT
TNF	Forward Reverse	TCAGCCTCTTCTCATTCC ACTTCTCCTCCTTGTTGG
RPLP0	Forward Reverse	ACAATGGCAGCATCTACAG CGGACACCCTCTAGGAAG

2.2.6 Measuring NFκB activity

RAW-Dual™ 264.7 cells, a murine macrophage cell line, express two reporter genes encoding secreted embryonic alkaline phosphatase (SEAP) and lucia luciferase (Invivogen, rawd-ismip). The luciferase gene is downstream an ISG54 promoter in conjunction with 5 interferon-stimulated response elements (ISRE). Hence, when luciferase activity is detected, it is indicative of activation of interferon regulatory factors (IRFs). On the other hand, SEAP expression is dependent on activation of endogenous CXCL2, a chemokine produced in an NFκB-dependent manner. Therefore, enhanced SEAP expression reports activation of NFκB.

These Raw-Dual reporter macrophages were seeded in a 96-well plate and grown to full confluency. The macrophages were then treated with the conditioned media from the H9c2 cells (outlined in section 2.1) for the following times: 3, 6, and 12 hours. Following treatment, the supernatant was incubated with the Quanti-blue SEAP detection reagent for 2 hours and absorbance was measured at 620 nm.

2.2.7 Treatment of macrophages with conditioned media for western blotting and qPCR to examine inflammatory pathways

Conditioned media from experiments described in Section 2.1 in the H9c2 cells were used to treat raw 264.7 murine macrophages for 3, 6, and 12 hours. Following treatment cells were washed in PBS and resuspended in 0.1% NP40 lysis buffer. Following lysis, cells were centrifuged at 12,000 rpm for 15 minutes at 4°C, supernatant was collected and boiled at 95°C for 5 min. Lysates were then run on the appropriate percentage of polyacrylamide gels at 90 V for 30 min for the proteins to leave the stacking gel, then run at 120 V for 1 h. The proteins on the gel were then transferred to polyvinylidene difluoride (PVDF) membranes using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories Ltd). Membranes were then submerged in 3% bovine serum albumin (BSA) blocking buffer for 1 h, followed by overnight incubation in 1:1000 dilution of

primary antibody: phospho-NFκB p65 (Cell Signalling, 3033) at 4°C. Membranes were then washed with TBS-T three times for 10 min before incubation with a 1:5000 dilution of an anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody at room temperature for 1h. Following secondary antibody incubation, membranes were washed with TBS-T three times for 10 min. Membranes were then quickly rinsed with Clarity Western ECL solution (Bio-Rad Laboratories Ltd) and visualized using the Chemi-doc MP Imaging System (Bio-Rad Laboratories Ltd). Western blot band intensity was quantified using ImageLab software, normalized to β-actin (Thermofisher, MA5-15739).

Conditioned media from experiments described in Section 2.1 in the H9c2 cells were used to treat raw 264.7 murine mouse macrophages for 3, 6, and 12 hours. Following treatment, RNA was extracted and converted to cDNA as per the protocol mentioned in section 2.6. Gene expression of the following cytokines was examined: TNF, IL-1β, iNOS. Relative gene expression analysis was conducted on CFX Maestro and normalized to RPLP0.

2.2.8 Examining the role of ALY688 in reducing cell death, autophagy, and inflammation in STZ mice

8-week old C57/BL6 mice (n=24) were divided into three groups including vehicle control, streptozotocin (STZ), STZ and ALY688. All mice were housed at the University of Ottawa Animal Care Facility with free access to food and water. Protocols were approved by the University of Ottawa Animal Care Committee and conducted in accordance with the guidelines of the Canadian Council on Animal Care. STZ mice were injected intraperitoneally with 150 mg/kg of streptozotocin (STZ), at 8 weeks of age one group was injected subcutaneously with ALY688 (15 mg/kg body weight), followed by termination at 16 weeks. The heart was isolated and both ventricles were lysed in tissue lysis buffer for Western blotting and Trizol for RNA extraction and subsequent gene expression analysis of inflammatory genes TNF, IL-1β, IL-6, and IL-12, as well

as autophagy-related genes via qPCR. Steps for Western blotting and qPCR are outlined in sections 2.2 and 2.6, primer list used for in vivo experiments are outlined in Table 2.

Table 2.2: List of mouse primers

Name	Type	Sequence (5' – 3')
IL-1β	Forward Reverse	GCAGCACATCAACAAGAG CAGCAGGTTATCATCATCATC
iNOS	Forward Reverse	ATAACTGCACCCACTTCCCA GGGCATCACTTCTACCAGGT
TNF	Forward Reverse	AGAATGAGGCTGGATAAGAT GAGGCAACAAGGTAGAGA
Atg3	Forward Reverse	AGGAATCAAAATTTAAGGAAA TTTGTCTGTCGGAAGATATG
Atg10	Forward Reverse	CAAAACACAGTTTCGAATAA TCATGTTTAATCACTTCTGC
Atg12	Forward Reverse	GGAGACACTCCTATAATGAAA ATAAATAAACAACTGTTCCGA
Atg16	Forward Reverse	AGATGAATGAAGCAAAGATT AGTAAAAGTAATCTGCAGGG
p62	Forward Reverse	TGGGCAAGGAGGAGGCGACC CCTCATCGCGGTAGTGCGCC
LC3	Forward Reverse	CGTCCTGGACAAGACCAAGT ACCATGTACAGGAAGCCGTC
RPLP0	Forward Reverse	TTGGAGTGACATCGTCTT ATCTTGAGGAAGTAGTTGGA

2.2.9 Statistical Analyses

To compare experimental treatment conditions in the cell culture study, a two-way ANOVA was used to examine the influence of PBS or ALY688 on normal and high glucose conditions, followed by Tukeys posthoc test to adjust multiple comparisons. For in vivo analysis, a two-way ANOVA with Tukeys multiple comparison analysis was used. All such analyses were carried out using GraphPad Prism version 10.5.0 for Mac. P values of < 0.05 were considered statistically significant.

2.3 Results

2.3.1 ALY688 attenuates high glucose-induced cell death in H9c2 cells

To examine the protective effects of ALY688 on high glucose-induced cell death *in vitro*, H9c2 rat cardiomyoblasts were pre-treated with ALY688 or PBS, followed by 24 hours of normal (5 mM) or high glucose (25 mM) treatment and subsequent cell death assays were performed. Figure 1A shows histograms of cells stained with a viability marker and analyzed by flow cytometry. Quantification of percentage dead cells is shown in Figure 1B where there is a statistically significant increase upon high glucose treatment, which is attenuated with ALY688. This is further shown in Figure 1C - D, where nuclei of dead cells are visualized via fluorescence microscopy upon treatment with high glucose with/without ALY688. High glucose was also shown to significantly increase cell rupture events measured by LDH release, and ALY688 significantly attenuated this effect (Figure 1E). Consistently, high glucose-induced cell death was significant following 24 hours of treatment in H9c2 cells, which decreased to near basal-levels upon pretreatment with ALY688. Next, we moved on to examined cleaved caspase-3, an executioner caspase and critical marker of apoptosis.

Various assays were used to examine caspase-3 and caspase-3 like activation in H9c2 cells, including measuring fluorescent caspase-3/7 activity over the course of 24 hours (Figure 1F - G) and Western blotting of cleaved caspase-3 (Figure 1H - I). A genetically engineered cell line (VC3AI) with a switch-on fluorescence-based indicator containing a caspase-3 cleavage site was used to visualize cleaved caspase-3-like activity (Figure 1J). The non-fluorescent indicator becomes fluorescent when cleaved by caspase-3-like proteases, rendering it detector for caspase-3 activation We found that there was a significant increase in caspase-3-like activity upon high glucose treatment (Figure 1K), which was attenuated by ALY688.

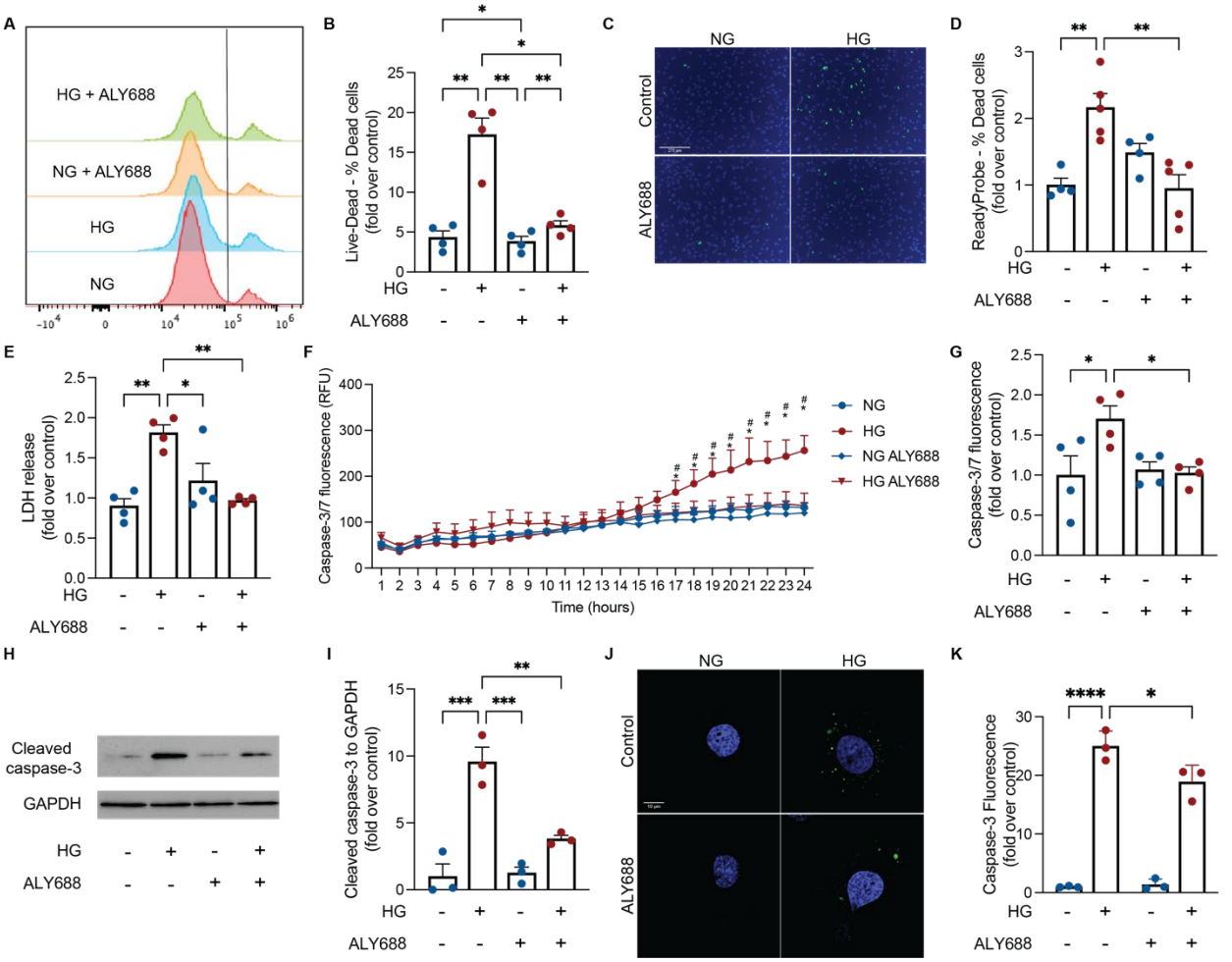

Figure 2.9: ALY688 (300 nM) rescues cardiomyoblasts from high glucose (25 mM)-induced cell death.

A) Overlayed histograms showing mean fluorescence intensity (MFI) of Live-Dead stain. B) Quantification of % dead cells using Live-Dead via flow cytometry C-D) Representative fluorescence microscopy images of nuclei of dead and total cells using the ReadyProbe cell viability kit and associated quantification of percentage of dead cells expressed as fold change. E) LDH release measured as absorbance, fold over control. F-G) Representative kinetic plot of caspase-3/7 activity over a period of 24 hours and end-point quantification of caspase-3/7 activity at 24 hours, #P<0.05 vs NG, *P<0.05 vs HG ALY. H-I) Representative Western blot of cleaved caspase-3 and respective quantification of cleaved caspase-3 over GAPDH, fold over control. J-K) Representative confocal microscopy images visualizing caspase-3-like activity and caspase-3 fluorescence (RFU) expressed as fold change. Values are mean ± s.e.m (n=3-4), *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. NG, normal glucose; HG, high glucose.

2.3.2 Stimulation of autophagy as a mechanism of ALY688 action in H9c2 cells

To elucidate the role of autophagy in protecting against high glucose-induced cell death, H9c2 cells were treated as above and Western blotting of key markers of autophagy, p62 and LC3, were examined. We first verified that ALY688 can induce autophagy via Western blotting of p62

and LC3 (Figure 2A), with rapamycin used as a positive control. We saw a significant increase in LC3-II and decrease in p62 upon treatment with ALY688 for 60 minutes, indicating enhanced autophagic flux characterized by cargo degradation and autophagosome formation. Using an LC3-reporter assay we found a significant accumulation of LC3-II after high glucose (Figure 2B - C), which was relieved with ALY688. In addition to that, there was a significant accumulation of p62 and LC3-II under high glucose conditions (Figure 2D - F), indicating blockage of autophagic flux. Upon treatment with ALY688, there was a reduction in both proteins, alleviating the high glucose-induced inhibition of autophagy. The increase in LC3-II, and thus autophagosome formation, demonstrated that autophagy is being initiated, while the increase in p62 implied that clearance of these autophagosomes was being impaired by the high glucose.

To further examine the role of autophagy in mediating the protective effects of ALY688, autophagy deficient H9c2 cells was used, based upon Atg7 KO. Both Atg7 KO and WT H9c2 cells were treated as described in section 2.1, and subsequent assays to examine cell death and apoptosis were conducted. Figures 3A - B show nuclei of dead cells in WT and Atg7 KO cells. Again, we observed a significant increase in dead cells in the Atg7 KO cells upon high glucose treatment compared to the WT cells. Additionally, ALY688 did not alleviate cell death induced by high glucose in the Atg7 KO cells. Figure 3C shows metrics of cell viability measured via LDH release. In the WT cells, we saw the expected trend as outlined in section 3.1. In Atg7 KO cells, there was a significant increase in cell death upon high glucose treatment compared to the high glucose treatment in the WT cells, which showed that cell death is exacerbated in the absence of autophagy. In addition, we saw that there was no significant difference between the high glucose and high glucose + ALY688 groups in Atg7 KO cells, suggesting that the protective effects of ALY688 were dependent on autophagy.

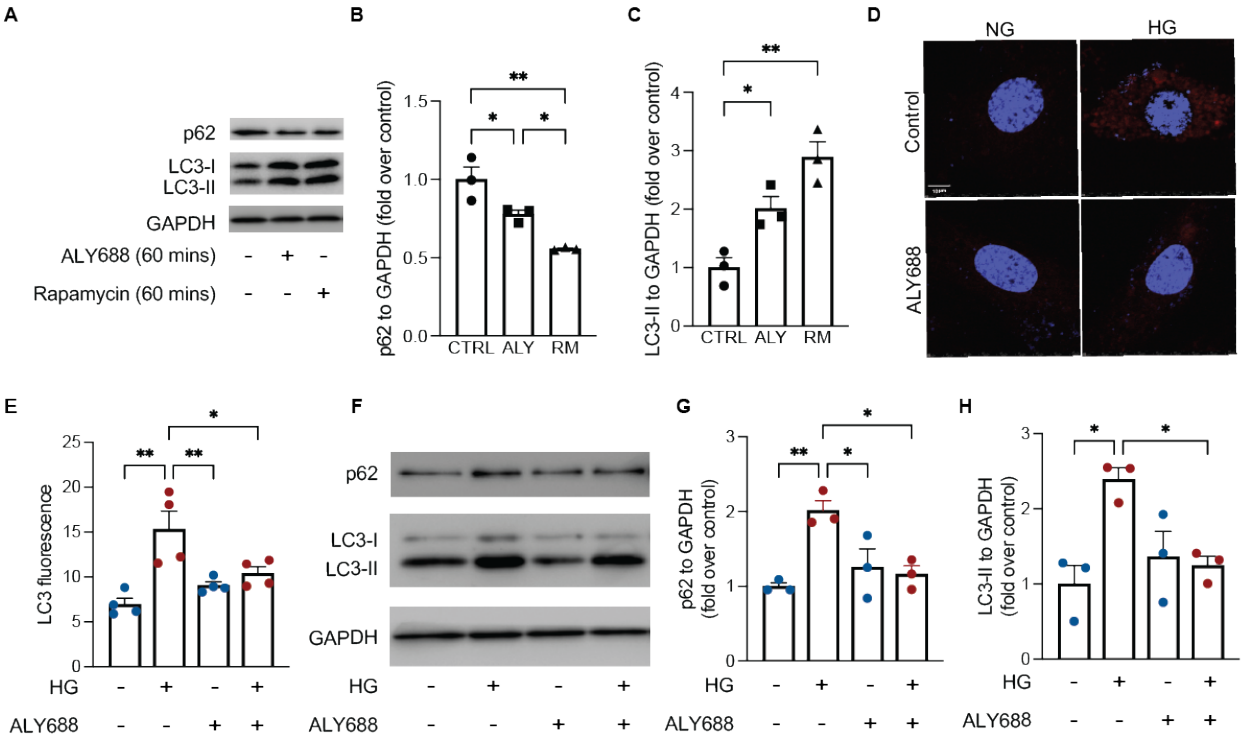

Figure 2.10: ALY688 can lower high glucose-induced autophagosome accumulation.
A) Western blotting of p62 and LC3 showing that ALY688 induces autophagy, rapamycin used as a positive control. B) Representative confocal microscopy images monitoring autophagic flux using an LC3 reporter. C) Quantification of LC3 fluorescence (RFU) in H9c2 cardiomyoblasts transfected with HiBiT LC3 reporter. D) Representative Western blotting images of p62 and LC3. E) Quantification of p62 over GAPDH, fold over control. E) Quantification of LC3-II over GAPDH, fold over control. Values are mean ± s.e.m (n=3-4), *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. CTRL, control; ALY, ALY688; RM, rapamycin; NG, normal glucose; HG, high glucose.

Cleaved caspase-3 levels were also examined in WT and Atg7 KO cells via flow cytometry (Figure 3D - E). There was a significant increase in cleaved caspase-3 in the Atg7 KO cells under high glucose conditions compared to WT, which was not reduced upon treatment with ALY688. In WT cells, we observed consistent results in that high glucose significantly increased cell death when treated with high glucose for 24 hours, which is mitigated with ALY688. Across a variety of assays shown in Figure 3 in the Atg7 KO cells, there was exacerbation of high glucose-induced cell death and caspase-3 activity which was not attenuated with ALY688.

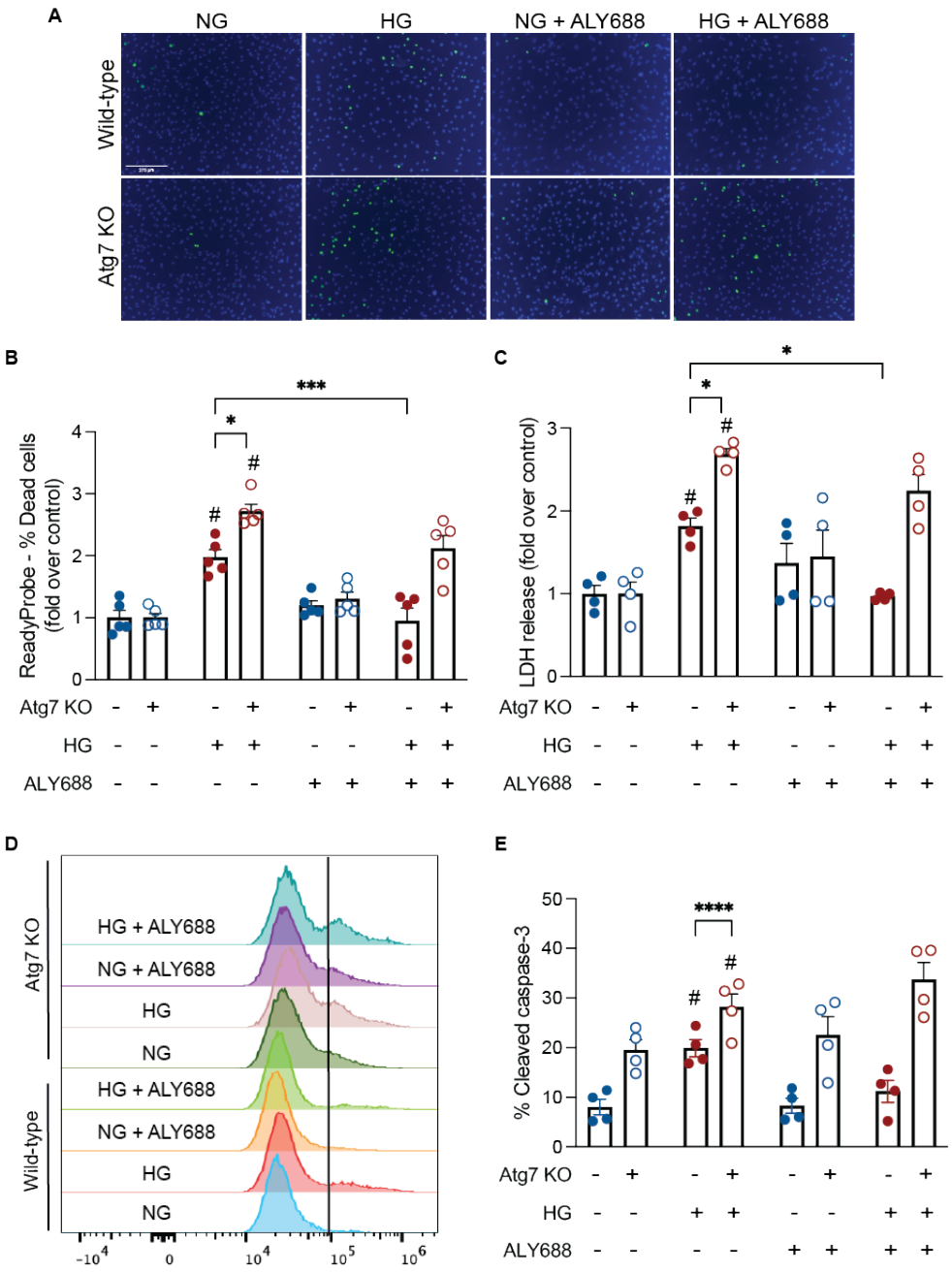

Figure 2.11: Protective effects of ALY688 are dependent on autophagy.
A-B) Representative fluorescence microscopy images of nuclei of dead and total cells using the ReadyProbe cell viability kit in WT and Atg7 KO H9c2 cells, with respective quantification. C) LDH released measured as absorbance, fold over control in WT and Atg7 KO H9c2 cardiomyoblasts. D-E) Overlayed histograms showing MFI of cleaved caspase-3 in WT and Atg7 KO H9c2 cells, with respective quantification. Values are mean ± s.e.m (n=4-5), #P<0.05 vs NG, *P<0.05, ***P<0.001, ****P<0.0001. NG, normal glucose; HG, high glucose.

2.3.3 ALY688 can reduce high glucose-induced inflammation and cytokine release in H9c2 cells and macrophages

A previous study showed that ALY688 attenuated pro-inflammatory cytokine release in LPS-induced cellular and animal models of inflammation [46]. Given the anti-inflammatory potential of ALY688 in mimicking effects of adiponectin, the release of pro-inflammatory agents upon high glucose treatment was also examined in H9c2 cells. Figure 4A shows a heatmap of gene expression of various inflammatory cytokines in H9c2 cells. We saw upregulation of pro-inflammatory genes such as IL-1β, IL-6, TNF, and IFNβ in the high glucose treatment compared to the normal glucose control. These genes were downregulated with ALY688, and we also observed upregulation of anti-inflammatory cytokine IL-10 in the ALY688 treated group, confirming anti-inflammatory properties.

To further delve into the role of inflammation in high glucose-induced cellular damage and the ability of ALY688 to rescue cells from these consequences, RAW murine macrophages were treated with conditioned media from H9c2 cells. Figure 4B shows Western blotting of phospho-NFκB p65, a key transcription factor that controls inflammatory gene expression, in macrophages treated with conditioned media for different times. We saw an increase in phosphorylation of the p65 subunit of NFκB at 3 hours with conditioned high glucose treatment, and this becomes statistically significant following 6 and 12 hours of treatment. There was a significant reduction in this phosphorylation induced by high glucose in macrophages which were treated with conditioned media containing ALY688. To further substantiate that NFκB was being activated, Raw-Dual macrophages were treated with conditioned media from H9c2 cells and SEAP activity, indicative of CXCL2, a chemokine produced in an NFκB-dependent manner, was measured (Figure 4C). We first saw an upward trend of activation of NFκB in macrophages treated with high glucose

conditioned media after 3 hours, with significant activation observed at 6 hours. Alongside ALY688, we saw a reduction in activation of this pathway at 3 hours again, which became significant following 6 hours of treatment.

In addition, inflammatory cytokine gene expression was also examined at 3, 6 and 12 hours in raw murine macrophages treated with conditioned media via qPCR. Figures 4D - F show ΔΔCt values of TNF, IL-1β, and iNOS. We saw significantly increased gene expression of TNF and IL-1β as early as 3 hours upon high glucose treatment and this is reduced with ALY688. We also saw a significant reduction in IL-1β in the high glucose + ALY688 group, compared to high glucose alone. There was a significant increase in iNOS under high glucose conditions following 6 hours of treatment, which was decreased when treated with ALY688. Taken together, it became evident that high glucose treatment in both H9c2 cells and macrophages led to activation of NFκB, a major inflammatory pathway, followed by release of pro-inflammatory cytokines which can further exacerbate cellular damage.

2.3.4 Examining cell death, autophagy, and inflammation in ventricular heart tissue from STZ-diabetic mice

Having demonstrated that ALY688 significantly reduces cellular damage and death *in vitro*, we performed translational studies to investigate its potential to reduce cell death and inflammatory cytokine release using a mouse model. STZ induced hyperglycemia-associated cardiac damage was used and the following mouse groups were injected with/without STZ and ALY688: vehicle control, STZ only, and STZ + ALY688. Mice were sacrificed eight weeks post-injection and hearts were perfused and isolated. Further downstream applications were conducted only on ventricles of the heart.

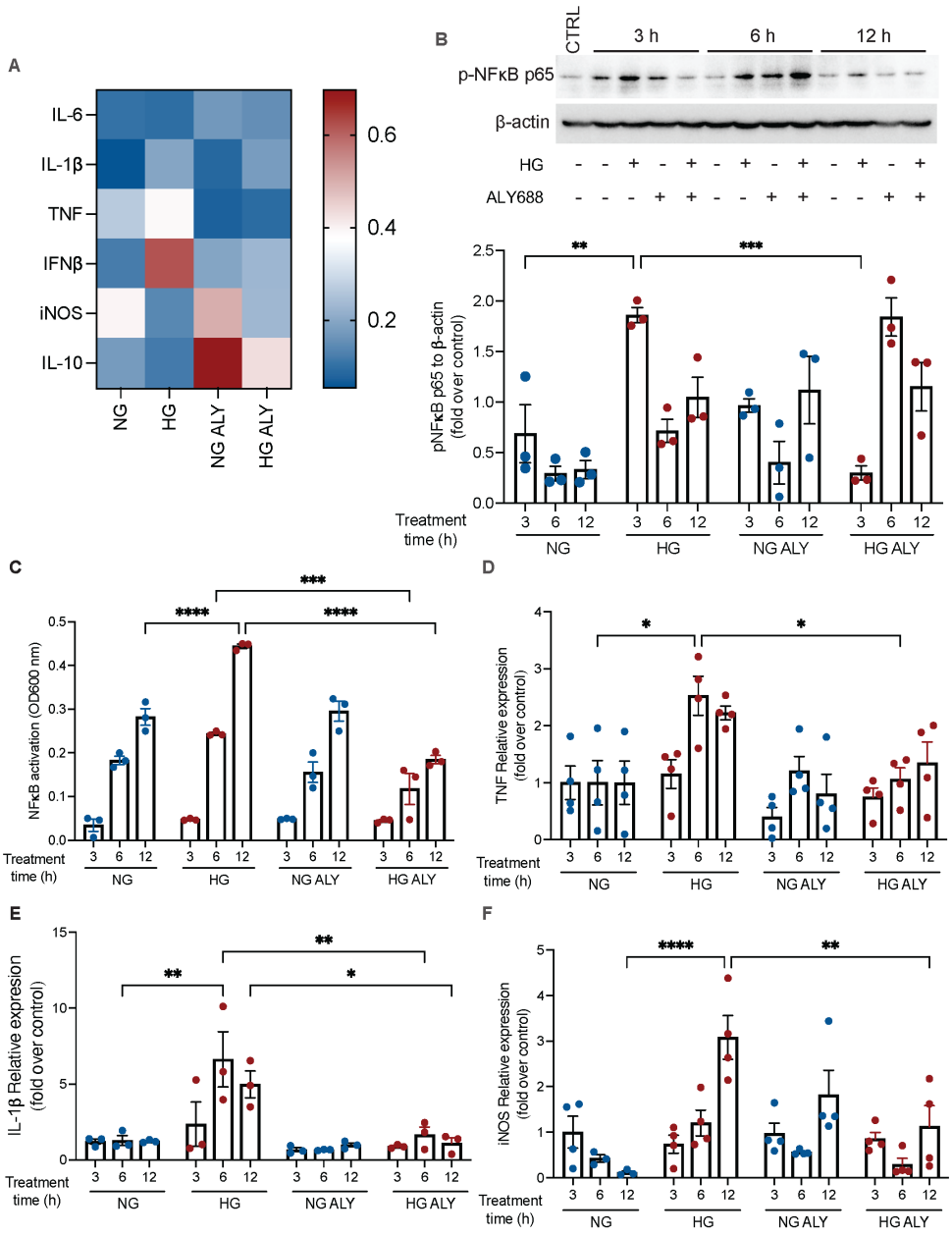

Figure 2.12: ALY688 reduces production of pro-inflammatory cytokines in H9c2 cells and macrophages.

A) Relative pro-inflammatory cytokine gene expressions in H9c2 cardiomyoblasts, normalized to RPLP0. B) Representative Western blotting of phospho-NFκB p65 in murine mouse macrophages treated with conditioned media from H9c2 cardiomyoblasts at different time points, with quantification over β-actin. C) NFκB activation in Raw-Dual macrophages treated with conditioned media from H9c2 cardiomyoblasts at different time points, expressed as OD620 nm. D-F) Relative gene expressions of TNF, IL-1β, and iNOS in murine macrophages treated with conditioned media from H9c2 cardiomyoblasts at different time points, normalized to RPLP0. Values are mean ± s.e.m (n=3-4), *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. NG, normal glucose; HG, high glucose.

To determine the mode of cell death in the ventricles, caspase-3 and caspase-7 were detected in protein lysates (Figure 5A). We saw a significant increase in caspase-3/7 levels in the STZ treated mice compared to the vehicle controls, and this was significantly mitigated with ALY688. In addition, there was a significant increase in the ratio of Bax/Bcl-2 in the STZ group compared to the control, which was reduced with ALY688 (Figure 5B,D). Examination of these key proteins involved in apoptosis all together showed a significant increase in apoptosis in ventricular heart tissue from STZ mice, which was substantially improved in the STZ + ALY688 group.

Western blotting (Figure 5C,E,F) and gene expression analysis of p62 and LC3 (Figure 5G) of various autophagy-related proteins was also done. We found a significant accumulation of p62 in the STZ + ALY688 group compared to the control group and STZ alone, as well as an increase in the LC3-II to LC3-I ratio in both STZ groups compared to the control. We also observed upregulation of various autophagy-related genes in the STZ group alone, compared to the control and STZ + ALY688, namely those involved in formation of autophagosomes such as Atg3, Atg10, Atg12, Atg16 and p62.

Gene expression of pro-inflammatory cytokines in ventricular heart tissue was also examined (Figure 5H - K). There was a significant increase in ΔΔCt values of every cytokine (TNF, IL-1β, IL-6, IL-12) in the STZ group, which was reduced in the STZ + ALY688 mice. Overall, the anti-apoptotic and anti-inflammatory effects of ALY688 were evident both *in vitro* and *in vivo*.

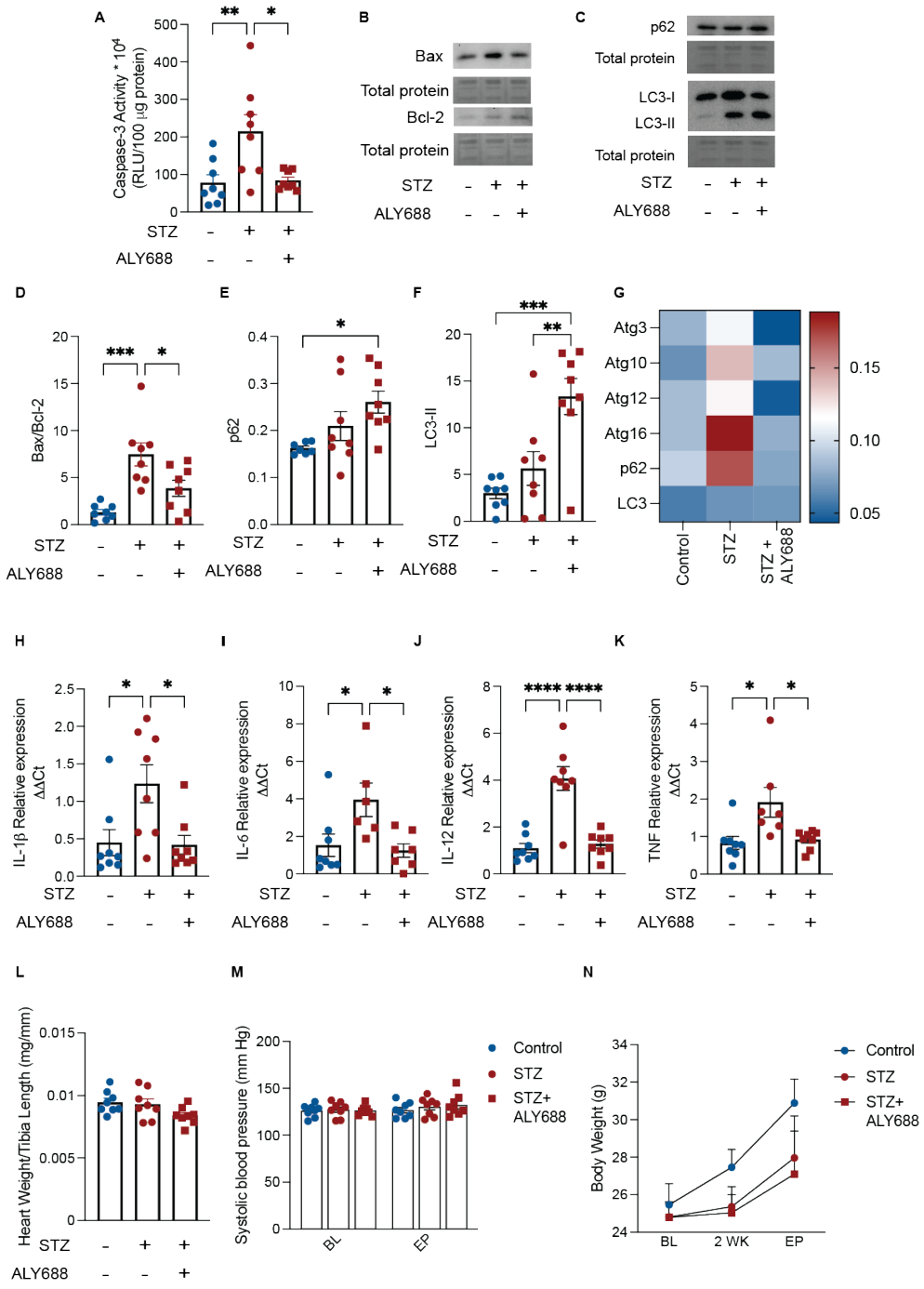

Figure 2.5: Protective role of ALY688 in heart tissue of STZ-treated mice.
A) Representative Western blotting images of Bax and Bcl-2 and total protein stain in heart tissue. C) Representative Western blotting images of p62 and LC3, and total protein stain. D-F) Quantification of Western blotting of Bax to Bcl-2, p62 and LC3-II. G-J) Relative gene expressions of TNF, IL-1β, and IL-6, and IL-12, normalized to RPLP0 in heart tissue. K) Heat map showing relative gene expression of autophagy-related genes in heart tissue, normalized to RPLP0. L) Heart weight normalized to tibia length. M) Systolic blood pressure. N) Body weight. Values are mean ± s.e.m (n=7-8), *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

2.5 Discussion

Numerous studies have reported that hyperglycemia induces apoptosis in the heart, in both cellular and mouse models [2,3,30]. Both autophagy and inflammation have been identified as key mechanisms that regulate cardiac cellular damage and apoptosis. While high glucose has been shown to induce inflammation or cell death in a variety of tissues, the role of adiponectin in preventing against high glucose-induced cell death in the heart via restoration of autophagy and reduction of inflammation has not been extensively studied [31–33]. In the context of DCM, autophagosome formation or autophagosome-lysosome fusion is impaired. In fact, studies have found that defects in autophagy can contribute to loss of protection during cardiac stress and that boosting autophagy via activation of the AMPK pathway reduces ROS production and inflammatory cytokine release [24,33,34]. Our data is entirely in keeping with this dogma as we have shown that adiponectin signaling acts, likely via AMPK, to enhance autophagy and mitigate high glucose-induced damage.

Cardiac inflammation is a key driver of the pathogenesis of DCM, high glucose levels act on cardiomyocytes, fibroblasts, and macrophages to activate NF-κB and subsequent release of pro-inflammatory cytokines such as TNF and IL-6 [35]. Essentially, excess availability of glucose and lipids, as seen in patients with metabolic syndrome, can favour ROS production which can enhance activation of NF-κB [35]. Thomas *et al.* found that cardiomyocyte-specific overexpression of IκBα, an inhibitor of NF-κB, preserved systolic and diastolic cardiac function in diabetic mice [32]. Moreover, the mutated diabetic mice showed decreased oxidative stress, in contrast to the diabetic mice, when comparing to the non-diabetic control mice [32]. Inflammatory cytokines can also regulate a range of processes that induce cardiomyocyte apoptosis, leading to intrinsic and extrinsic activation of cell death pathways [35]. This underscores the damaging effects

inflammatory pathways can induce on the heart, further highlighting the importance of targeting this axis when considering therapeutic interventions to reduce consequences of DCM.

Here, we found that ALY688, an adiponectin mimetic peptide, significantly attenuated high glucose-induced cell death and apoptosis in cardiomyoblasts. Translating this to an animal model of chronic high blood glucose, we found that ALY688 significantly reduced caspase-3/7 activity in STZ mice, showing the promise of this adiponectin receptor agonist in reducing cell death in the heart *in vitro* and *in vivo*. Adiponectin has been shown to protect against apoptosis induced by hypoxia-reoxygenation, lipopolysaccharide (LPS), and palmitate in similar cellular models. ALY688 can bind to both AdipoR1 and AdipoR2, the same way full length adiponectin (fAd) is able to, and is an encouraging drug candidate given its small size and potency [25,26].

Adiponectin is a known stimulator of autophagy and ALY688 has already been shown to induce autophagy in muscle tissue, subsequently improving insulin sensitivity [31,36]. Autophagy, often considered a double-edged sword, provides protective effects for cardiomyocyte cell damage and death via activation of AMPK, and inhibition of mammalian target of rapamycin (mTOR) pathways [37]. Autophagy is stimulated by nutrient starvation to provide cells with additional nutrients supplied by the turnover of damaged cellular organelles [38]. Impairment of autophagy via either formation of autophagosomes or fusion of the autophagosome to the lysosome has been implicated in the development of diabetes and cardiac abnormalities [34,39]. Here, we found an accumulation of LC3-II and p62 in cardiomyoblasts upon high glucose treatment, suggesting build-up of autophagosome formation but not completion of autophagy in its entirety. This accumulation of autophagosomes indicated an impairment of autophagic flux which can lead to increased oxidative stress, shifting cells towards apoptosis. Upon treatment with ALY688, we found that LC3-II and p62 returned to normal levels in the high glucose-treated cells, indicating

that autophagic flux was restored. We believe that this relief and restoration of autophagic flux contributes to the beneficial effects of ALY688 in rescuing cells from cell death. To examine the functional significance of autophagy, an autophagy-deficient cell line of cardiomyoblasts was used. Here, we found that high glucose-induced cell death and caspase-3 activation was exacerbated under high glucose conditions in Atg7 deficient cells, compared to the WT cells. In addition to that, the protection from cell death previously provided by ALY688 was mitigated, indicating that the protective effects of ALY688 are dependent on autophagy. A similar finding was reported by Sung *et al.*, where protective effects of adiponectin against ischemia-induced cardiac function was diminished in autophagy-deficient mice and cardiomyoblasts [21]. Hypoxia-induced cell death was reduced in cells treated with adiponectin, but this effect was lost in autophagy-deficient cells, as also consistently observed in the present study upon high glucose treatment. Studies in adiponectin-knockout mice have also shown decreased cardiac function, cell viability, and insulin sensitivity upon various stressors through decreased myocardial autophagy [21,31]. Thus, autophagy plays a key role in the protective effects of adiponectin in reducing high glucose-induced cell death; the accumulation of damaged cellular organelles can lead to oxidative stress and induce an inflammatory response, ultimately leading to cell death in the heart.

To further elucidate mechanisms through which ALY688 exerts its protective effects, gene expression analysis of various pro-inflammatory cytokines was carried out and significant upregulation of these genes upon high glucose treatment was observed. This was reduced upon treatment with ALY688, showing the anti-inflammatory properties of this drug candidate. The inverse relationship between adiponectin and inflammation has been well-studied, decreased serum levels of adiponectin have been closely associated to chronic inflammation in cardiometabolic disorders [40]. In this study, we found that treating raw macrophages with high

glucose conditioned media from cardiomyoblasts caused significant increase in activation of NF-κB and subsequent release of pro-inflammatory cytokines from these macrophages. This finding suggests that damage associated molecular patterns (DAMPs) may be released by H9c2 cells upon high glucose treatment, which in turn, induce an inflammatory response in macrophages, highly reactive immune cells [41]. The role of DAMPs in aggravating myocardial inflammation has been explored, with Kaur *et al.* concluding that release of high-mobility group box 1 (HMGB1) under diabetic conditions led to macrophages polarizing to a pro-inflammatory phenotype [42]. Other studies have also demonstrated that stressed or injured cardiomyocytes release DAMPs that engage pattern recognition receptors (PRRs) on macrophages, activating inflammatory pathways, namely NF-κB [43,44]. Interestingly, ALY688 significantly decreased the activation of NF-κB and production of pro-inflammatory cytokines, implying that ALY688 reduced the release of DAMPs from cardiomyoblasts. The role of adiponectin in protecting against high glucose-induced inflammation in the heart has not been extensively studied, however it has been shown to reduce inflammation in adipocytes and endothelial cells [45,46].

In rodent models, STZ is commonly used to examine effects of high glucose on cellular damage in various tissues. Myocardial cell death has been found in STZ rodent models, where apoptotic gene expression or protein levels of cleaved caspase-3, Bax, and Bcl-2 are significantly elevated compared to control animals [23,30]. Differentiating the type of cardiomyocyte cell death occurring has also been a point of interest. Fiordaliso *et al*., reported a significant increase in cell death in rat cardiomyocytes and determined there was absence of necrosis, concluding that the mode of cell death occurring was through apoptosis [47]. In mice, Guo *et al*., and Marino *et al*., both found a significant increase in TUNEL-positive nuclei in STZ mice compared to control groups, regardless of a type 1 or type 2 diabetes model [23,30]. Evidently, several studies have

consistently reported that STZ models of hyperglycemia induce myocardial apoptotic cell death, rendering this model apt in examining cell death and potential mechanisms leading to it [23,30,47,48]. Myocardial cell death in humans has been examined using similar assays as done in the aforementioned rodent models, showcasing that STZ-induced hyperglycemia can accurately mimic apoptosis in the heart as seen in humans [49].

Here, translating the cellular model of high glucose in cardiomyoblasts to an STZ hyperglycemia model in mice showed significant activation of caspase-3 and increase in pro-apoptotic protein Bax, which was attenuated with ALY688. Gene expression analysis shows upregulation of various autophagy-related genes in the STZ group, implying that cells were experiencing stress to induce autophagy, which was not seen in the STZ+ALY688 group. The increase in p62 suggests that ALY688 could be promoting autophagy as a protective mechanism against STZ-induced cellular damage, however a strong conclusion cannot be made based on this dataset. On the other hand, there was clear production of pro-inflammatory cytokines in the heart tissue in STZ treated mice, showing that cardiac damage induced by 8 weeks of STZ in mice led to an inflammatory response, and ALY688 was able to bring that down to near-basal levels. Many studies have shown that STZ induces inflammation in the heart in mice and rats, leading to cardiac cell death and dysfunction [23,30,50]. Activation of NF-κB is thought to play a pivotal role in STZ-induced cardiac inflammation, leading to cardiac cell apoptosis and hypertrophy [30]. Here we find that ALY688 can reduce cell death and inflammation in heart tissue, proving to be a promising drug candidate to prevent high glucose-induced cardiac cellular damage.

In conclusion, we found that ALY688, an adiponectin receptor agonist, shows promising potential as a drug candidate to reduce high glucose-induced cell death via restoration of autophagy and reduction of inflammation. This study strengthens the rationale for further exploring

adiponectin agonists as therapeutics to reduce damage induced by high glucose on the heart, and eventually aid cardiometabolic patients in improving cardiac health and function.

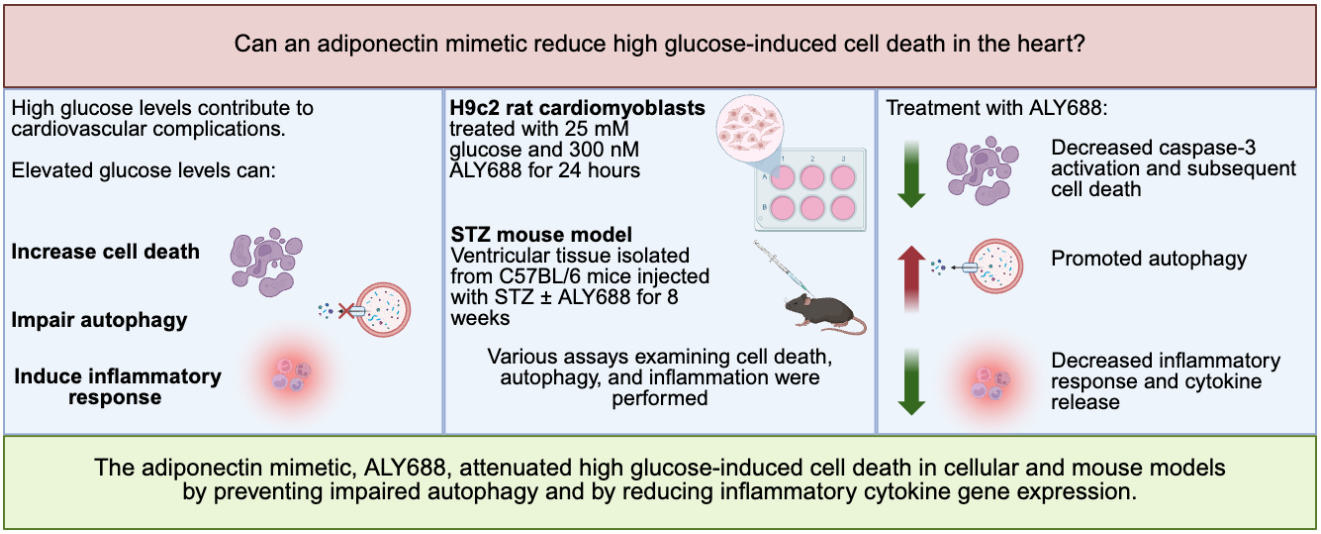

Figure 2.6: Summary graphic on study determining mechanisms of protective effects of ALY688 upon high glucose treatment.
Image created with BioRender.com.

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Disclosure statement

GS, DB and AAS acted as consultants for Allysta Pharmaceuticals who provided ALY688. All other authors confirm that no conflict of interest exists with the contents of this manuscript.

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Chapter 3 – Investigating combined effects of high glucose and iron on inflammatory signalling and cytokine release in macrophages

Author contribution note: I performed all experiments, data analyses, and writing, with guidance from Dr. Ali Abdul-Sater and Dr. Gary Sweeney.

Abstract

Metabolic syndrome (MetS) is characterized by chronic, low-grade inflammation driven by metabolic stressors such as hyperglycemia and dysregulated iron. High glucose and iron have independently been regarded as potent inducers of inflammation, oxidative stress, and cell death in various cell types. However, the combined effect of these metabolic stressors on inflammatory pathway activation in macrophages, and what leads to this, is poorly understood. Macrophages are central players in mediating inflammation. The plasticity of these cells leaves them sensitive to fluctuations in nutrients availability. In this study, mouse RAW 264.7 macrophages and bone marrow-derived macrophages (BMDMs) were used to examine key inflammatory pathway activation (NFκB and IRF3). Subsequent inflammatory gene expression and cytokine release after 6, 12, and 24 hours of treatment with normal glucose (NG), high glucose (HG), iron (Fe) and HG+Fe was assessed. Mechanistically, oxidative stress and cell death were examined as key cellular events that precede and follow inflammatory pathway activation. Results showed that combining high glucose and iron treatments led to significantly increased gene expression of inflammatory cytokines, such as IL-6, TNF, and IL-1β, followed by subsequent release from the cells measured via ELISA. In addition, flow cytometry was used to assess oxidative stress and cell death in cells, where it was found that HG+Fe significantly increased oxidative states and mitochondrial membrane depolarization. This, combined with NFκB and IRF3 pathway activation, led to significantly increased cell death at the end point of treatment. This study emphasizes the importance of studying combined metabolic stressors, to fully gage the target pathways to combat the global health crisis that is metabolic disorders.

3.1 Introduction

Metabolic syndrome (MetS) is a primary cause of cardiovascular morbidity and mortality. A survey conducted by the government of Canada found that 26.1% of adults aged 18-79 years had MetS, and prevalence increased with age; 11.1% for ages 18-39 years, and 44.3% for ages 60-79 years.[1] Among these individuals, high fasting blood glucose was the second most prominent feature (70.6%), preceded only by high waist circumference (90%).[1] High blood glucose is known to have deleterious effects on multiple organs, including endothelial cells, adipose tissue, skeletal muscle, heart, liver, pancreas and the brain.[2,3] Glucose-induced stress or disruption of insulin signalling affects a multitude of cell types; endothelial cells, pancreatic β-cells, hepatocytes, adipocytes, skeletal myocytes, and immune cells.[4–6] Hyperglycemia can disrupt normal cellular function, increasing oxidative stress, inflammation, and cell death, as well as influence insulin resistance.[4–6]

Dysmetabolic iron overload syndrome (DIOS) is associated with increases in liver and bodily iron stores, corresponding to factors of MetS in lieu of other conditions responsible for excess iron (hemochromatosis, thalassemia).[7] Altered iron metabolism and its deleterious effects are a multifaceted sequence of events influenced by environmental, lifestyle, and genetic elements.[7] In fact, increases in serum ferritin levels have become an increasingly acceptable predictor of metabolic syndrome and eventually, type 2 diabetes (T2D).[8–10] A meta-analyses conducted by Zhang *et al.* found a positive correlation between serum ferritin (an indicator of body iron stores) and individual components of MetS.[11]

While iron is an essential element required for a multitude of bodily functions, it has been implicated in insulin resistance in skeletal muscle, cardiomyocytes, and adipocytes.[12–14] There is a growing body of evidence supporting the role of excess iron in chronic diseases such as T2D, non-

alcoholic fatty liver disease, and atherosclerosis.[15] Iron interacts with various cellular agents to modulate its function in cells, including transferring, ferritin, insulin, adipokines and pro-inflammatory molecules.[16–18] Similar to high glucose, iron overload (IO) triggers ROS generation leading to oxidative stress, lipid peroxidation, and DNA damage; all of which can activate inflammatory pathways.[16,17] Release of cytokines as a result of pathway activation can downregulate insulin signalling and decrease adiponectin levels. These processes all contribute to how iron overload can promote insulin resistance, vascular damage and metabolic dysfunction.[16,17]

Macrophages are highly reactive immune cells, sitting at the intersection of metabolic stress, iron overload, and inflammatory signalling.[18] They can be tissue-resident or recruited to the site of injury as monocytes, releasing cytokines that create a chronically inflamed environment in MetS, influencing tissue remodelling and insulin sensitivity.[18] Macrophages are also highly sensitive to extracellular iron levels given their possession of iron-handling machinery such as transferrin receptors, ferritin and ferroportin.[19] Studying macrophages in metabolic dysfunction provides a vital mechanistic platform to determine how metabolic stressors can shape immune signalling, cytokine output and cell damage and death. This is important to study given that persistent tissue and systemic inflammation are key focal points of complications in MetS.

While hyperglycemia and IO have been shown to independently induce oxidative stress and inflammatory signalling in cellular and mouse models, mechanistic studies that gage their combined impact on innate immunity have been scarce. Nuclear factor κ B (NFκB) and interferon regulatory factor 3 (IRF3) are key transcription factors that translocate to the nucleus upon inflammatory and metabolic stimuli for transcription of pro-inflammatory agents. While the IRF3 pathway is typically activated by microbial stimuli, it has emerged as a regulator of metabolic morbidities.[20] This pathway can be engaged via sterile signals such as damage-associated

molecular patterns (DAMPs) and endogenous stimuli, including reactive oxygen species (ROS), nuclear or mitochondrial DNA, and pro-inflammatory cytokines.[20] Both high glucose and IO can generate these stimuli that engage IRF3 activation in immune cells, rendering them as metabolic triggers for immune dysfunction. In addition, both of these stressors have been shown to induce NFκB translocation to the nucleus, leading to transcription of inflammatory genes and components of the NLRP3 inflammasome.[21–23] Neither hyperglycemia, nor iron have been found to fully activate NLRP3, which requires both a priming signal and activation signal.[24] As a result of NFκB translocation upon an inflammatory or metabolic stimuli, components of the NLRP3 inflammasome (NLRP3, ASC, pro-caspase-1) become available for assembly, but are not activated.[24] A second stimulus is required for NLRP3 to oligomerize and recruit the necessary components, ultimately leading to release of IL-1β and inflammatory pyroptotic cell death.[24]

Given that MetS is characterized by hyperglycemia and iron dysregulation, and that these factors converge on oxidative and inflammatory pathways, combining them will present a physiologically relevant model to examine metabolic stress-induced immune dysfunction. This study will aim to elucidate whether treatment with high glucose and iron in macrophages leads to activation of NFκB and/or IRF3 to identify whether combining these stressors induces NLRP3 inflammasome activation, cytokine production, and oxidative stress.

3.2 Materials and Method

3.2.1 Cell culturing

264.7 murine macrophages (wild-type and reporter cells) were thawed in high glucose (25 mM) 10% Dulbecco's Modified Eagle Medium (DMEM), 1% glutathione, sodium pyruvate, penicillin, streptomycin (GPPS) solution. Following the first thaw, when cells reached full confluency and were passaged once, cells were cultured in low glucose (5.5 mM) DMEM with 10% FBS. Subsequently, for the first set of experiments (section 3.3.1) cells were seeded and underwent pre-treatment with either normal glucose (5.5 mM), or iron (50 μM) media, followed by treatment under the following conditions for a given duration of time (6, 12, 24, or 48 hours): normal glucose (NG), high glucose (HG), iron (Fe), or high glucose+iron (HG+Fe). For experiments conducted in section 3.3.2-4, cells did not undergo any pre-treatment and were only treated with normal glucose (NG), high glucose (HG), iron (Fe), or high glucose+iron (HG+Fe) for a given time. All treatments were conducted in DMEM with 0.5% FBS.

3.2.2 Measuring NFκB and IRF activity

RAW-Dual 264.7 cells (InvivoGen, cat#rawd-ismip), a murine macrophage cell line, which expresses two reporter genes encoding secreted embryonic alkaline phosphatase (SEAP) and lucia luciferase. The luciferase gene is downstream an ISG54 promoter in conjunction with 5 interferon-stimulated response elements (ISRE). Hence, when luciferase activity is detected, it is indicative of activation of interferon regulatory factors (IRFs). On the other hand, SEAP expression is dependent on activation of endogenous CXCL2 reporter, a chemokine produced in an NFκB-dependent manner. Therefore, enhanced SEAP expression reports activation of NFκB.

These RAW-Dual reporter macrophages were seeded in a 96-well plate and grown to full confluency. The macrophages were then treated in the conditions outlined above for the following

times: 6, 12, 24, and 48 hours. Following treatment, to detect NFκB activation, the supernatant was incubated with the Quanti-blue SEAP detection reagent (InvivoGen, rep-qbs) for 2 hours and absorbance was measured at 620 nm. To detect activation of IRFs, supernatant was collected and Quanti-Luc luciferase detection reagent (InvivoGen, rep-qlc4lg1) was added, luminescence was measured immediately following addition of the luciferase detection reagent.

3.2.3 Western blotting

RAW 264.7 murine macrophages were treated as outlined in section 2.1. Following the appropriate pre-treatment, cells were treated for 12 or 24 hours under the following conditions: NG, HG, Fe, and HG+Fe. Following treatment, cells were washed in PBS and resuspended in 0.1% NP40 lysis buffer. Following lysis, cells were centrifuged at 12,000 rpm for 15 minutes at 4°C, supernatant was collected and boiled at 95°C for 5 min. Lysates were then run on the appropriate percentage of polyacrylamide gels at 90 V for 30 min for the proteins to leave the stacking gel, then run at 120 V for 1 h. The proteins on the gel were then transferred to polyvinylidene difluoride (PVDF) membranes using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories Ltd). Membranes were then submerged in 3% bovine serum albumin (BSA) blocking buffer for 1 h, followed by overnight incubation in 1:1000 dilution of primary antibody: phospho-NFκB p65 (Cell Signalling, 3033) and phosphor-IRF3 (Cell Signalling, 4947) at 4°C. Membranes were then washed with TBS-T three times for 10 min before incubation with a 1:5000 dilution of an anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody at room temperature for 1h. Following secondary antibody incubation, membranes were washed with TBS-T three times for 10 min. Membranes were then quickly rinsed with Clarity Western ECL solution (Bio-Rad Laboratories Ltd) and visualized using the Chemi-doc MP Imaging System (Bio-Rad

Laboratories Ltd). Western blot band intensity was quantified using ImageLab software, normalized to β-actin.

3.2.4 Quantitative polymerase chain reaction (qPCR)

RAW 264.7 murine macrophages were treated as outlined in section 2.1. Following the appropriate pre-treatment, cells were treated for 12 or 24 hours under the following conditions: NG, HG, Fe, and HG+Fe. Following treatment following treatment cells were harvested for RNA isolation. RNA was isolated from the cells using the PureLink RNA Mini Kit (Invitrogen, cat #12183025) following the manufacturer's instructions. Complementary cDNA was made from 1 ug of RNA using the RevertAid RT kit (Thermofisher, cat #K1691), per the manufacturer's protocol. Following conversion to cDNA, 100 ng was used to assemble 10 uL qPCR reactions using the Sybr Green master mix. The following appropriate primer sets were used to examine the following genes: IL-6, IL-1β, IL-10, IFNβ, iNOS, and TNF. Relative gene expression analysis was conducted on CFX Maestro and normalized to RPLP0. Primer sequences shown in Table 1.

Table 3.3: List of mouse primers

Name	Type	Sequence (5' – 3')
IL-1β	Forward Reverse	GCAGCACATCAACAAGAG CAGCAGGTTATCATCATCATC
IL-6	Forward Reverse	ACAGAAGGAGTGGCTAAG AGAGAACAACATAAGTCAGATAC
IL-10	Forward Reverse	ATAACTGCACCCACTTCCCA GGGCATCACTTCTACCAGGT
iNOS	Forward Reverse	ATAACTGCACCCACTTCCCA GGGCATCACTTCTACCAGGT
TNF	Forward Reverse	AGAATGAGGCTGGATAAGAT GAGGCAACAAGGTAGAGA
IFNβ	Forward Reverse	TCCAAGAAAGGACGAACATTCG TGAGGACATCTCCCACGTCAA
RPLP0	Forward Revers	TTGGAGTGACATCGTCTT ATCTTGAGGAAGTAGTTGGA

3.2.5 Enzyme-linked Immunosorbent Assay (ELISA)

Supernatant from treated cells described in section 3.2.1 was collected, as well as from cells treated with LPS for 12 and 24 hours as a positive control. A mouse IL-1β ELISA kit (Invitrogen, cat#88-7013) and TNF ELISA kit (Invitrogen, cat#88-7324) were used to respectively quantify IL-1β and TNF release into the supernatant from treated cells as per the manufacturers protocol. A standard curve was run with all samples and the equation of the line of best fit was used to quantify protein concentration.

3.2.7 Measuring Cytotoxicity

Supernatant from the aforementioned treatments was collected at each time point to examine lactate dehydrogenase (LDH) release from cells using the CyQuant LDH Cytotoxicity Assay kit (Invitrogen, C20301). Supernatant was incubated with the reaction mixture for 30 minutes in a 96-well flat bottom plate at 37°C as per the manufacturers protocol. Following incubation, absorbance was measured at 490 nm and 620 nm. Background signal at 620 nm was subtracted from the 490 nm absorbance for analysis.

3.2.7 Flow cytometry

RAW 264.7 macrophages were seeded into a 6-well plate, grown to 90% confluency and treated accordingly as described in section 3.2.1. Following treatment, cells were scraped, centrifuged at 12,000 rpm for 5 minutes. Supernatant was discarded and cells were washed with PBS twice. To examine cell death, cells were stained with a live-dead stain (Thermofisher, cat#L23105) for 30 minutes (RT, protected from light), fixed in IC fixation buffer for 30 minutes (RT, protected from light), permeabilized in cold methanol for 20 minutes (4°C, protected from light), and then stained with a cleaved caspase-3 antibody conjugated to pacific blue for 30

minutes (RT, protected from light). Following staining, cells were washed in FACS buffer and run on the Aurora 5L flow cytometer (Cytek).

To examine ROS production and mitochondrial membrane potential, cells were harvested as described above and stained with monobromobimane (mBBr) (Thermofisher, cat#M1378) at a final concentration of 1 μM and tetramethylrhodamine methyl ester (TMRM) (Selleck, cat#E2945) at a final concentration of 2.5 nM for 10 mins at 37°C, protected from light. Following staining, cells were washed in FACS buffer three times and run on the Aurora 5L flow cytometer (Cytek).

All experiments were unmixed to separate fluorescent signals from each dye using the SpectroFlo software (Cytek), and subsequent analyses was performed using the FlowJo software.

3.2.6 Statistical analyses

To compare all experimental treatment conditions, a two-way ANOVA was used to examine the influence of the pre-treating cells with NG, HG, or iron on inflammatory pathway activation in section X. All other analysis comparing the differences between the four treatment groups (NG, HG, Fe, HG+Fe) was done using a one-way ANOVA followed by Tukey's post hoc test with multiple comparisons. All statistical analyses was conducted on GraphPad. v10.5.0.

3.3 Results

3.3.1 NFκB and IRF pathway activation in RAW-Dual 264.7 macrophages under high glucose and iron conditions

NFκB activation was examined in RAW-Dual reporter macrophages pre-treated (PT) with normal glucose (NG), high glucose (HG), or iron (Fe), followed by 6, 12, 24, and 48 hours of treatment with NG, HG, and HG+Fe. It was hypothesized that combining high glucose and iron treatments would cause the release of factors that could activate NFκB, including pro-inflammatory cytokines, chemokines, DAMPs, and mediators of reactive oxygen species. Results showed that there was no induction of NFκB in these treatments, which deviated from what was expected (Figure 3.1A-D). NFκB activation was expected to be the first step in inducing NLRP3 inflammasome activation, leading to the release of IL-1β and various other inflammatory factors resulting in pyroptosis. Evidently, treating the macrophages in this way was not sufficient to activate NFκB. To corroborate this, Western blotting of phospho-p65 showed no statistically significant increase in phospho-p65 in any group (Figure 3.1E). Phospho-p65 is a subunit of NFκB, which becomes active in transcribing inflammatory genes upon its phosphorylation. While not statistically significant, the increase with the combination treatments (HG+Fe) after 24 hours in the PT NG and PT HG groups was evident. It is possible that longer treatment time points may be required to induce NFκB activation, given the long-term chronic exposure to high glucose and iron. Alternatively, NFκB activation could have been activated at a quicker, more acute time point (ex. 1-3 hours), however given the chronic nature of effects of high glucose and iron, this is unlikely.

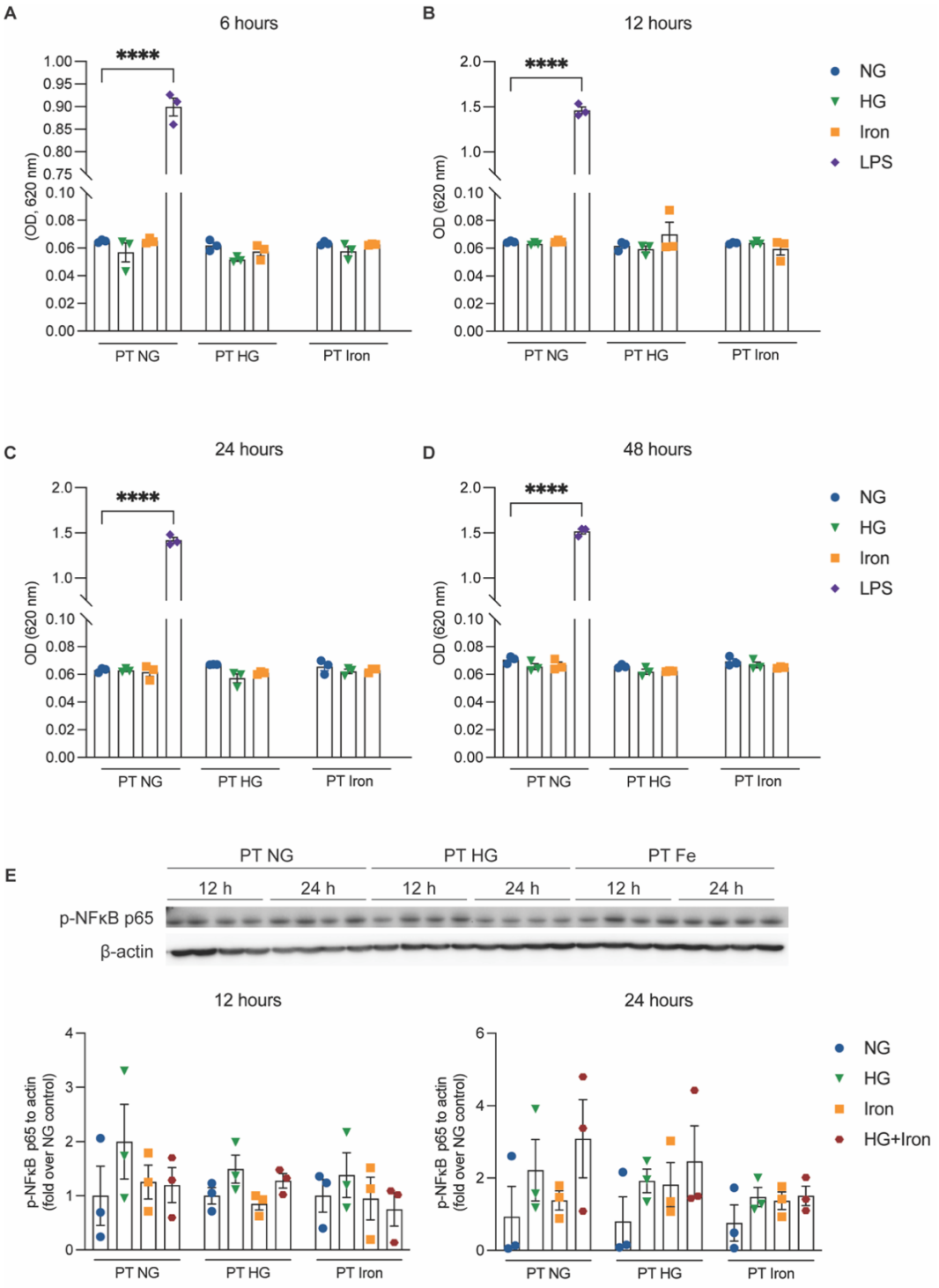

Figure 3.13: NFκB activation in murine macrophages treated with high glucose and iron. A-D) NFκB activation in macrophages pre-treated (PT) overnight with normal glucose (NG), high glucose (HG) or iron (Fe) followed by 6, 12, 24 and 48 hours of treatment with NG, HG, and Fe. LPS used as a positive control. E) Western blotting of NFκB in macrophages pre-treated (PT) overnight with NG, HG or Fe followed by 12 and 24 hours of treatment with NG, HG, Fe, and HG+Fe. Normalized to β-actin and expressed as fold change. Values are mean ± s.e.m (n=3).****P<0.0001. PT, pre-treatment; NG, normal glucose; HG, high glucose.

Additionally, the IRF3 pathway was examined to determine if this is the route through which high glucose and iron treatments could induce an inflammatory response (Figure 3.2). Here, there was evident IRF activation beginning at 6 hours of treatment after pre-treatment with high glucose compared to pre-treatment with normal glucose (PT NG) (Figure 3.2A). While there was no statistical significance among the treatments in the PT HG group, it was evident that prolonged treatment with high glucose was sufficient to induce IRF3 in all groups. Similarly, following 12 hours of treatment with high glucose (Figure 3.2B), IRF3 activation was significantly increased in the PT HG group compared to PT NG. After 24 hours of treatment with high glucose (Figure 3.2C), there was an increase in IRF3 activation in the PT HG group in comparison to PT NG. Evidently, exposing cells for a longer period of time to high glucose induced an IRF-dependent inflammatory response. Interestingly, only after 48 hours of treatment with iron was there a significant increase in IRF3 in the PT HG group, compared to iron in the PT NG group (Figure 3.2D). The same observation was noted for high glucose in the PT HG group, as seen at the other time points. This implied that pre-treating cells with high glucose, followed by either high glucose or iron elicited a similar magnitude of IRF3 activation. Ultimately, these data suggests that prolonged metabolic stress by high glucose activates IRF3, though the impact of iron in altering this is unresolved. Overall, it appears pre-treating cells with iron followed by treatment with high glucose or iron does not cause significant IRF activation. Pre-treatment with normal glucose served as a control to determine whether pre-treatment with high glucose or iron induced IRF activation, which was suspected to be the potent stimuli to activate this pathway. These findings revealed that prolonged treatment with high glucose was primarily responsible for IRF induction.

To further complement these data, Western blotting of phospho-IRF3 was performed (Figure 3.2E), with an additional treatment group of combining high glucose and iron

simultaneously. Here, there was a modest increase in phospho-IRF3 when combining high glucose and iron (HG+Fe) at 12 and 24 hours in the PT HG group. High glucose alone increased phosphorylation of IRF3 compared to normal glucose in all treatment groups, but with the addition of iron there appears to be an additive effect in further exacerbating phospho-IRF3, though this is not supported statistically. These results indicate that combining high glucose and iron treatments has the potential to increase activation of IRF compared to the treatments alone. While these results are not statistically significant, there was an increase of phospho-IRF3 when combining high glucose and iron after pre-treatment with high glucose. This partially supports my hypothesis in the sense that the combination treatment is inducing a more robust inflammatory response, albeit not via NFκB thus far, but potentially through IRF.

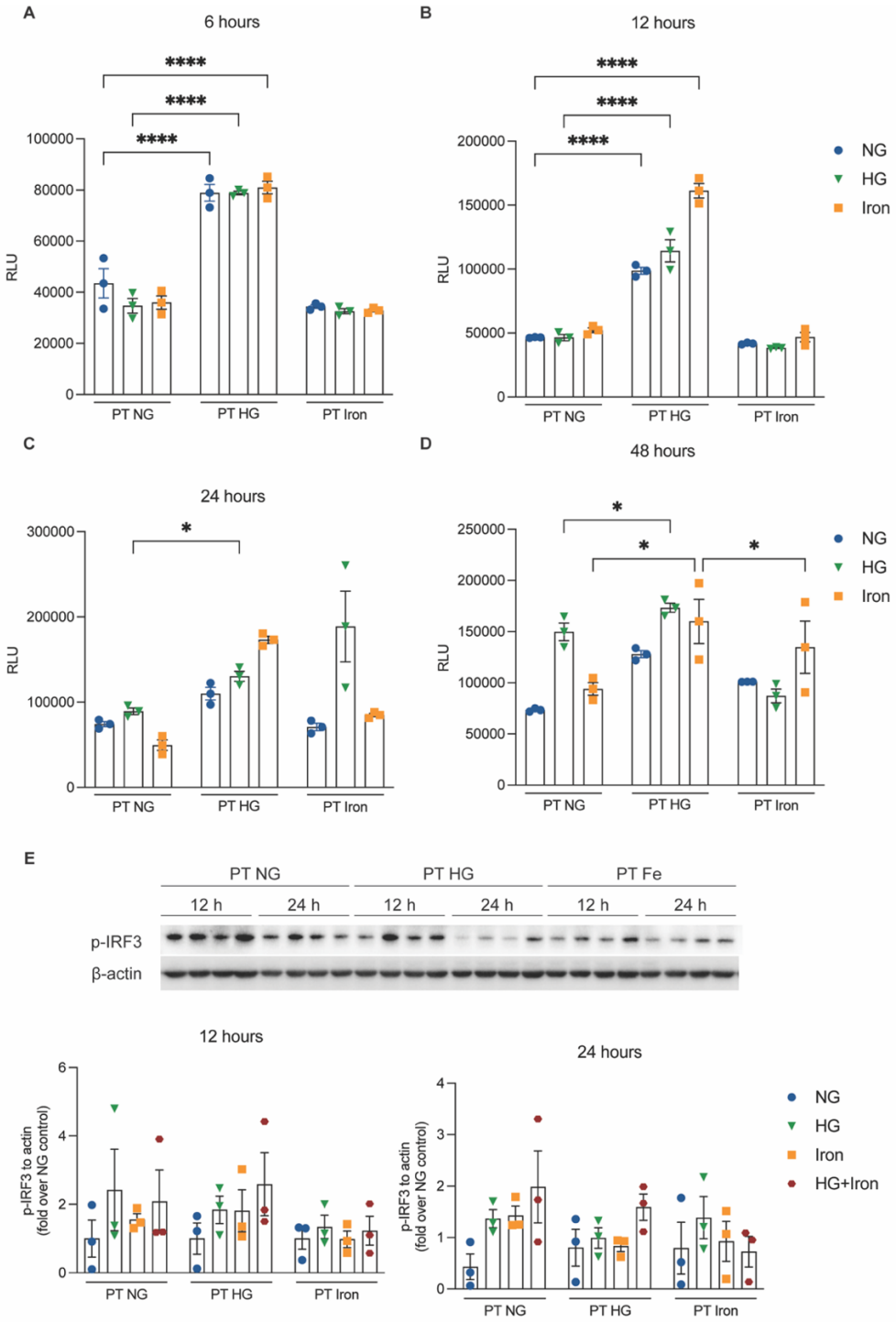

Figure 3.14: IRF3 activation in murine macrophages treated with high glucose and iron. A-D) IRF3 activation in macrophages pre-treated (PT) overnight with normal glucose (NG), high glucose (HG) or iron (Fe) followed by 6, 12, 24 and 48 hours of treatment with NG, HG, and Fe. E) Western blotting of NFκB in macrophages pre-treated (PT) overnight with NG, HG or Fe followed by 12 and 24 hours of treatment with NG, HG, Fe, and HG+Fe. Normalized to β-actin and expressed as fold change. Values are mean ± s.e.m (n=3). *P<0.05, ****P<0.0001. PT, pre-treatment; NG, normal glucose; HG, high glucose.

To assess inflammatory cytokine expression, qPCR on the following genes was done: IL-1β, IL-6, iNOS, TNF, IFNβ and TGFβ. Results for the 12- and 24-hour treatments are shown in Figures 3.3. To start, 12 hours of high glucose alone across all pre-treatment groups slightly increased pro-inflammatory cytokine gene expression of IL-1β, IL-6, iNOS, and TNF, though not within the realm of statistical significance (Figure 3A). In the HG+Fe group, there was significant increase in gene expression of IL-6, TNF, and TGFβ without any pre-treatment. Following 24 hours of treatment (Figure 3B), there was no significant trend among any of the groups, save IFNβ, where combining HG+Fe had significantly increased gene expression in the PT HG group.

Results in the 264.7 macrophages remain inconsistent. The reporter assay showed no induction of NFκB, nor did the Western blot. While there was some evidence to indicate inflammatory gene expression when combining high glucose and iron, this too was largely inconclusive with massive variability among replicates. While the purpose of these experiments was to determine if there was a priming signal by one treatment, followed by an activation signal for inflammasome formation, no such outcome was observed. As such, the treatment times may have been too long, and while there was an attempt at clinically relevant scenarios with different pre-treatments, no substantial change was noted between any of the pre-treatment groups. Given these results, I next sought to use bone marrow-derived macrophages from mice, in the hopes that this primary, physiologically relevant model will yield concrete results.

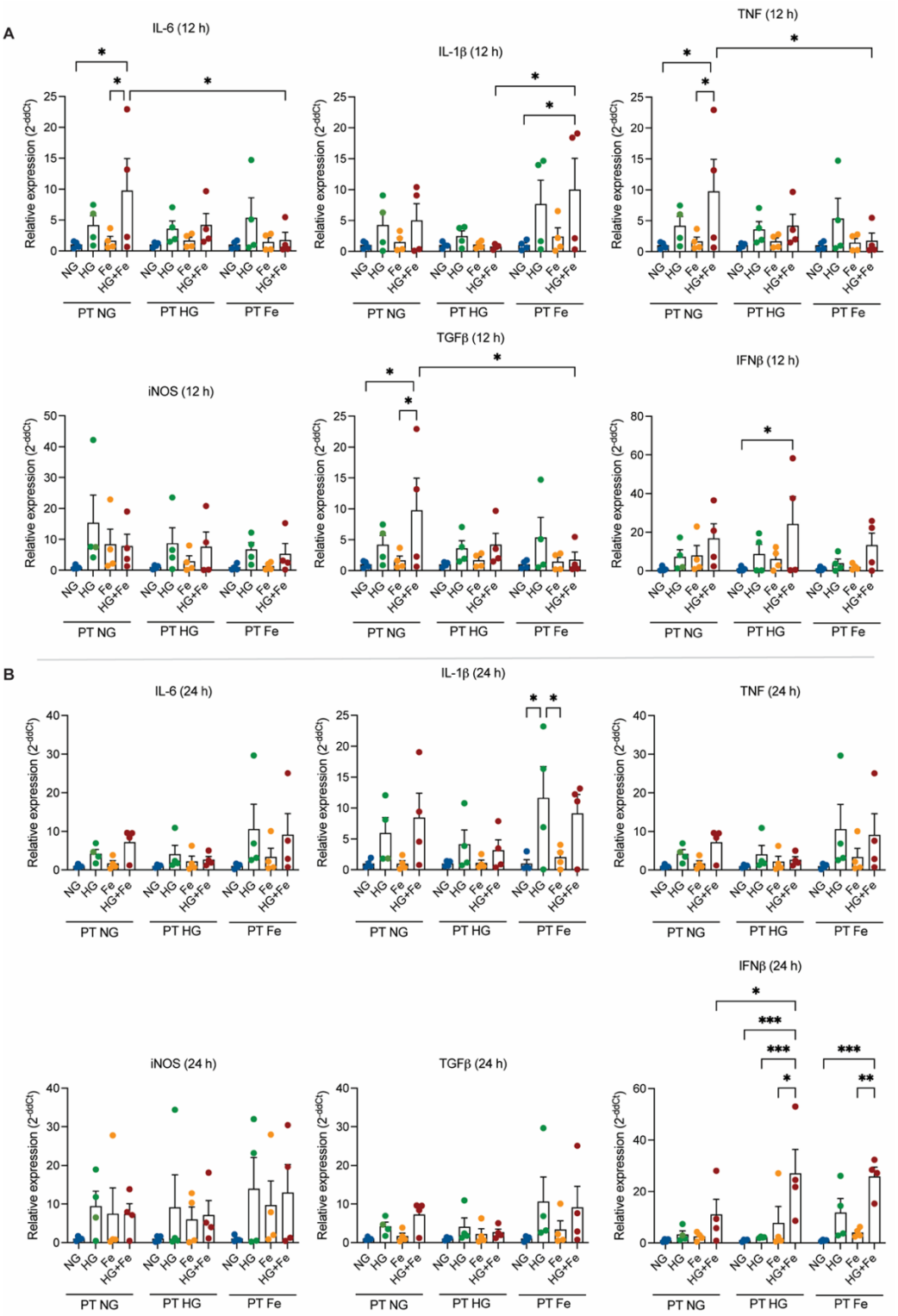

Figure 3.15: Relative gene expression of various pro-inflammatory genes in macrophages. Cells were treated pre-treated (PT) overnight with normal glucose (NG), high glucose (HG) or iron (Fe) followed by 12 (A) and 24 (B) hours of treatment with NG, HG, Fe, and HG+Fe. Values are mean ± s.e.m (n=4). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. PT, pre-treatment; NG, normal glucose; HG, high glucose.

3.3.2 Combining high glucose and iron induces cytokine expression and release in bone marrow-derived macrophages

Given the lack of significance of pre-treating cells with either high glucose or iron, I opted to remove any pre-treatments and use a more physiologically relevant model of bone marrow-derived macrophages (BMDMs). Results from section 3.3.1, while statistically insignificant, showed a trend of an additive or synergistic effect when combining high glucose and iron, regardless of pre-treatment. Thus, subsequent experiments in BMDMs were done with only four treatments; normal glucose (NG), high glucose (HG), iron (50 μM) (Fe) , and high glucose plus iron (HG+Fe) for 6, 12, and 24 hours.

To start, NFκB and IRF3 activation was examined via Western blotting following treatment for 6, 12, 24 hours (Figure 3.4). Under basal conditions, IκB, tightly binds the p65/p50 subunit of NFκB, and upon phosphorylation degrades (leading to lowered total IκB), resulting in phosphorylation and release of p65 to the nucleus. There was significant degradation of IκB, starting at 6 hours (Figure 3.4A,C) when combining high glucose and iron compared to normal glucose, but not individual high glucose or iron treatments. Moreover, there was an increase in phosphorylation of p65 with the combination treatment starting at 6 hours (Figure 3.4A,D). This became statistically significant between the individual treatments of high glucose and iron as well after 12 hours, tapering off to even amounts of phospho-p65 after 24 hours. IRF3 activation was also assessed with the same treatments (Figure 3.4B). Similar to results seen in the raw 264.7 macrophages, there was significant phosphorylation of IRF3 after 6 hours of the combination treatment compared to all others (Figure 3.4E). This returned to baseline after 12 hours and then became apparent again at 24 hours, indicating that combining high glucose and iron was continuously activating IRF3 and potentially leading to downstream production of inflammatory agents. Overall, examination of these two inflammatory pathways revealed that combining high

glucose and iron could be inducing synergistic or additive effect, compared to treating cells with either treatment alone. Next, inflammatory cytokine gene expression and release was assessed.

Gene expression of various inflammatory genes was performed following 6, 12, and 24 hours of treatment as described above (Figure 3.5A). Here, there was significantly elevated gene expression of IL-1β, IL-6, TNF, iNOS, and IFNβ in the HG+Fe group, relative to either treatment alone. Cytokines such as IL-6, iNOS, and IFNβ had increased expression in the combination treatment group, opposed to each treatment alone, particularly at the 12-hour treatment mark. IFNβ remained consistently elevated at each time point compared to the individual treatments, which taken with the Western blotting results, could suggest that these metabolic stressors can induce transcription of interferons and play a role in apoptosis. IL-1β is the end-product of NLRP3 inflammasome activation; a potent pro-inflammatory cytokine that amplifies inflammatory signalling and reinforces activation of NFκB dependent pathways. Here, this cytokine was elevated in the combination treatment group after 12 and 24 hours of treatment, compared to the individual treatments of iron or high glucose. TNF showed a similar trend, except that TNF gene expression in the high glucose plus iron group rose after 6 hours of treatment, mellowed at 12 hours and then rose again after 24 hours.

To observe whether TNF and IL-1β were being produced and eventually released from cells following HG+Fe treatments, ELISAs were conducted for each of these cytokines at each time point (Figure 3.5B-C). There was no release of either of these cytokines after 6 hours of treatment, however the combination treatment induced release of TNF to be significantly higher in comparison to the other treatment groups. IL-1β started to rise after 12 hours with HG+Fe finally inducing a greater effect than each treatment alone after 24 hours. Notably, HG alone significantly increased IL-1β after 24 hours, and TNF after 12 and 24 hours. Iron alone significantly increased

TNF release at both time points, less than high glucose alone, but significant, nonetheless. When considered together with HG+Fe, this indicated a synergistic effect of combining high glucose and iron. Results from Figure 3.5 on gene expression and cytokine release indicated that combining high glucose and iron can induce a synergistic response that leads to NLRP3 inflammasome formation and potential subsequent cell death, explored in the following section.

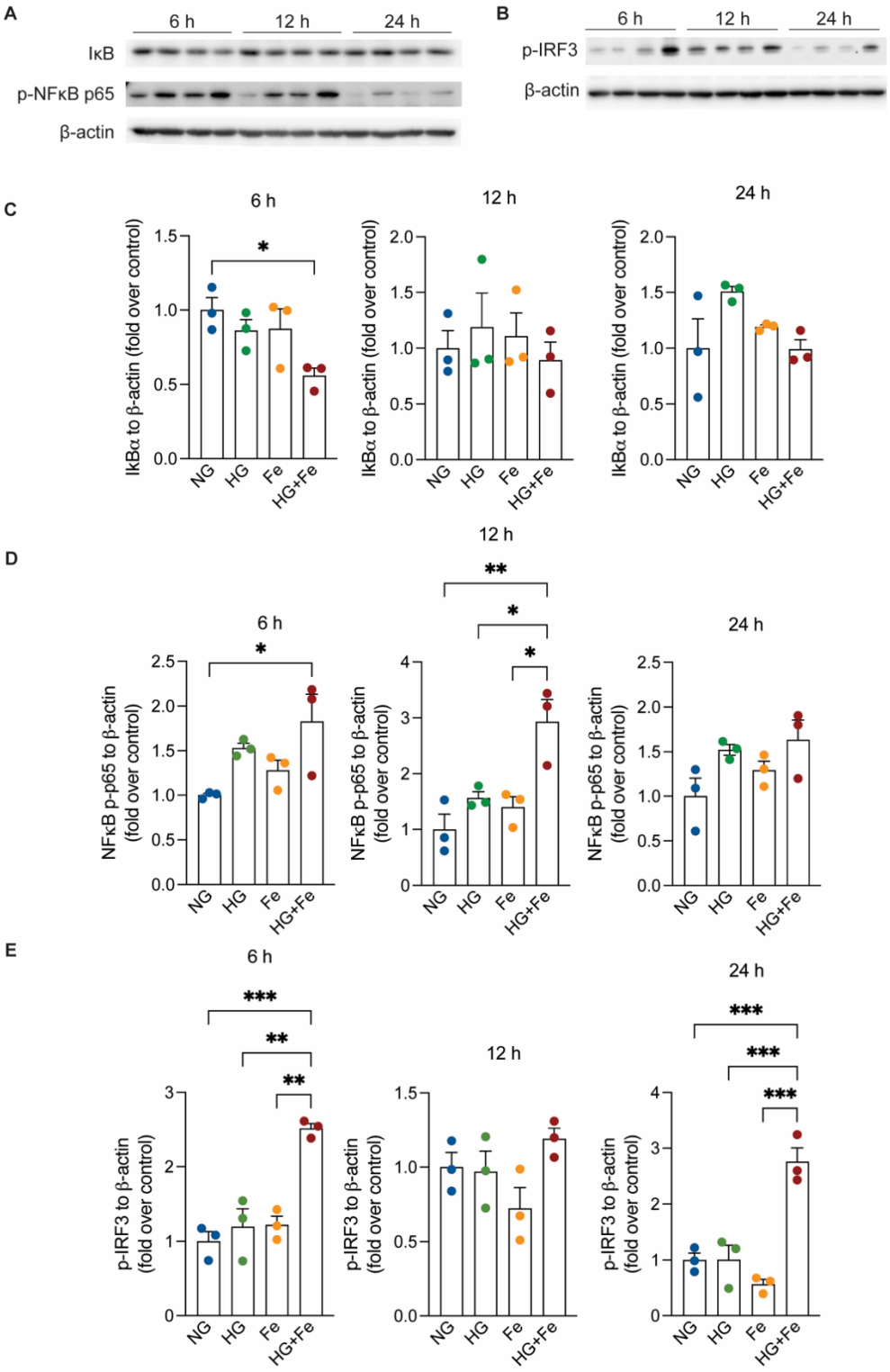

Figure 3.16: Combining high glucose and iron induces inflammatory pathway activation. A) Representative Western blotting of phosphor-NFκB subunit p65, as well as degradation of its negative regulator IκB, quantification of each protein shown in panels C-D. B) Representative Western blotting of phosphor-IRF3 and quantification shown in panel E. Values are mean ± s.e.m (n=3). *P<0.05, **P<0.01, ***P<0.001. NG, normal glucose; HG, high glucose; Fe, iron.

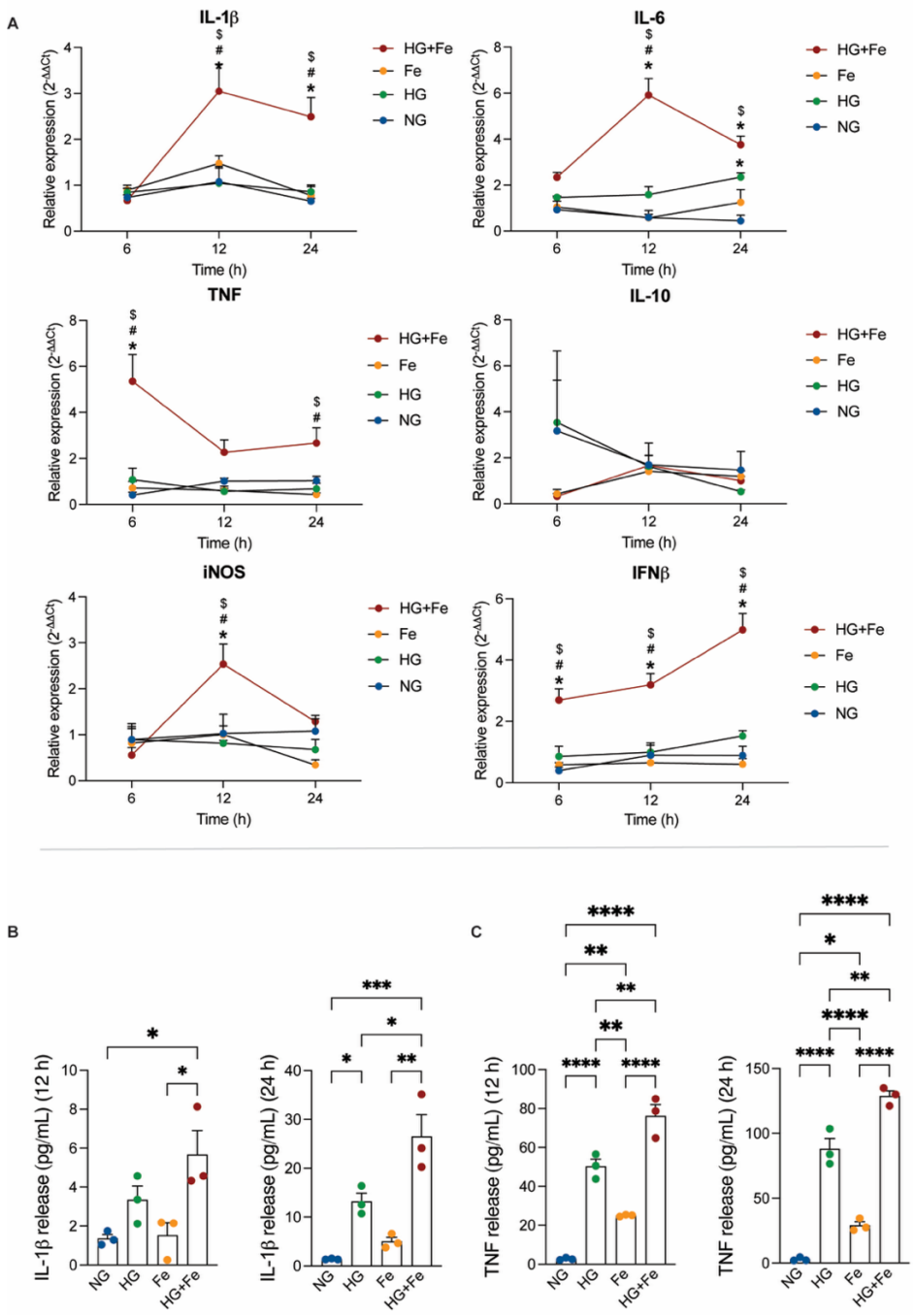

Figure 3.17: Inflammatory gene expression and TNF and IL-1β release from BMDMs treated with high glucose and iron.

A) Relative gene expression of inflammatory genes via qPCR over a period of 24 hours; IL-1β, IL-6, TNF, IL-10, iNOS, and IFNβ.n=3. *P<0.05 vs NG, # P<0.05 vs HG, $ P<0.05 vs Fe. C) Quantifying TNF release from BMDMs into supernatant following treatment for 12 and 24 hours. D) Quantifying IL-1β release from BMDMs into supernatant following treatment for 12 and 24 hours. Values are mean ± s.e.m n=3. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. NG, normal glucose; HG, high glucose; Fe, iron.

3.3.3 Combining high glucose and iron treatments led to oxidative stress in BMDMs

To assess oxidative stress, flow cytometry was employed to examine reduced glutathione (GSH) and mitochondrial membrane potential using TMRM. GSH is the primary indicator of a cell's redox state, an antioxidant that neutralizes ROS and maintains mitochondrial membrane integrity. Depletion of GSH indicates oxidative stress and mitochondrial dysfunction, which can promote inflammatory signalling. Here, mBBr, a fluorogenic agent that binds to free thiols was used. Upon oxidation, this fluorescence is decreased in intensity and was quantified using flow cytometry. Analyses showed significantly reduced GSH levels in BMDMs treated with HG+Fe (Figure 3.6A). This reduction started at 6 hours, though not statistically significant until 12 and 24 hours, where the combination treatment heavily influenced the oxidative state of these cells. Here, high glucose and iron alone significantly reduced GSH availability in BMDMs compared to the normal glucose control, nevertheless combining the two treatments significantly decreased the number of reduced cells. This showed the ability of these metabolic stressors to induce cellular damage beyond what either treatment could do alone.

TMRM is a fluorogenic dye used to assess mitochondrial function, specifically mitochondrial membrane potential. When cells are healthy and not under any stress, this positively charged dye is retained in the negatively charged mitochondria. Upon cellular distress, mitochondria become less negative and TMRM loses fluorescence intensity. There was significantly reduced polarization of the mitochondria when cells were treated with both high glucose and iron, rather than each stressor alone as early as 6 hours of treatment (Figure 3.6B). This effect was sustained up until 12 hours, tapering off after 24 hours. These results indicated that mitochondrial stress that occurred after 6 hours, could have led to downstream ROS production that led to the oxidative state seen after 12 and 24 hours as indicated by lower reduced cells in

Figure 3.6. Clearly, these data suggest that combining high glucose and iron leads to oxidative stress in BMDMs. Measuring GSH and mitochondrial membrane potential aided in elucidating that the inflammatory activation observed in section 3.3.2 was driven, at least partially, by oxidative stress mechanisms.

3.3.4 Increased cell death and cleaved caspase-3 in BMDMs treated with high glucose and iron

The end-point element to examine was cell death and determining whether the consequences seen in previous sections would ultimately lead to macrophage cell death. To assess this, LDH release was measured from supernatant in BMDMs treated with high glucose and iron (Figure 3.7A). There was a steady increase in cell death across each time point, with the final time point of 24 hours, demonstrating the detrimental effects outlined in the sections above. This result was significantly increased cell death in the combination group, compared to each treatment on its own. This was further corroborated by flow cytometric analysis of a live-dead stain that showed a significant increase in dead cells (Figure 3.7C,E) upon high glucose and iron treatments. Furthermore, cleaved caspase-3, a key marker of apoptosis was also examined, and intracellular cleaved caspase-3 was found to be significantly heightened in high glucose and iron group, opposed to each treatment alone (Figure 3.7B,D). This proposes apoptosis as one mode of cell death that high glucose and iron together could be inducing.

Plainly, combining high glucose and iron in BMDMs induced a marked increase in inflammatory pathway activation, cytokine production, oxidative stress and cell death. This was consistently observed across a variety of assays, begging the question of the exact mechanism through which this is occurring, and whether this would be a finding seen in other immune cells. Results from this study suggest that combining high glucose and iron can lead to ROS and cytokine production, which lead to inflammatory pathway activation and subsequent cell death.

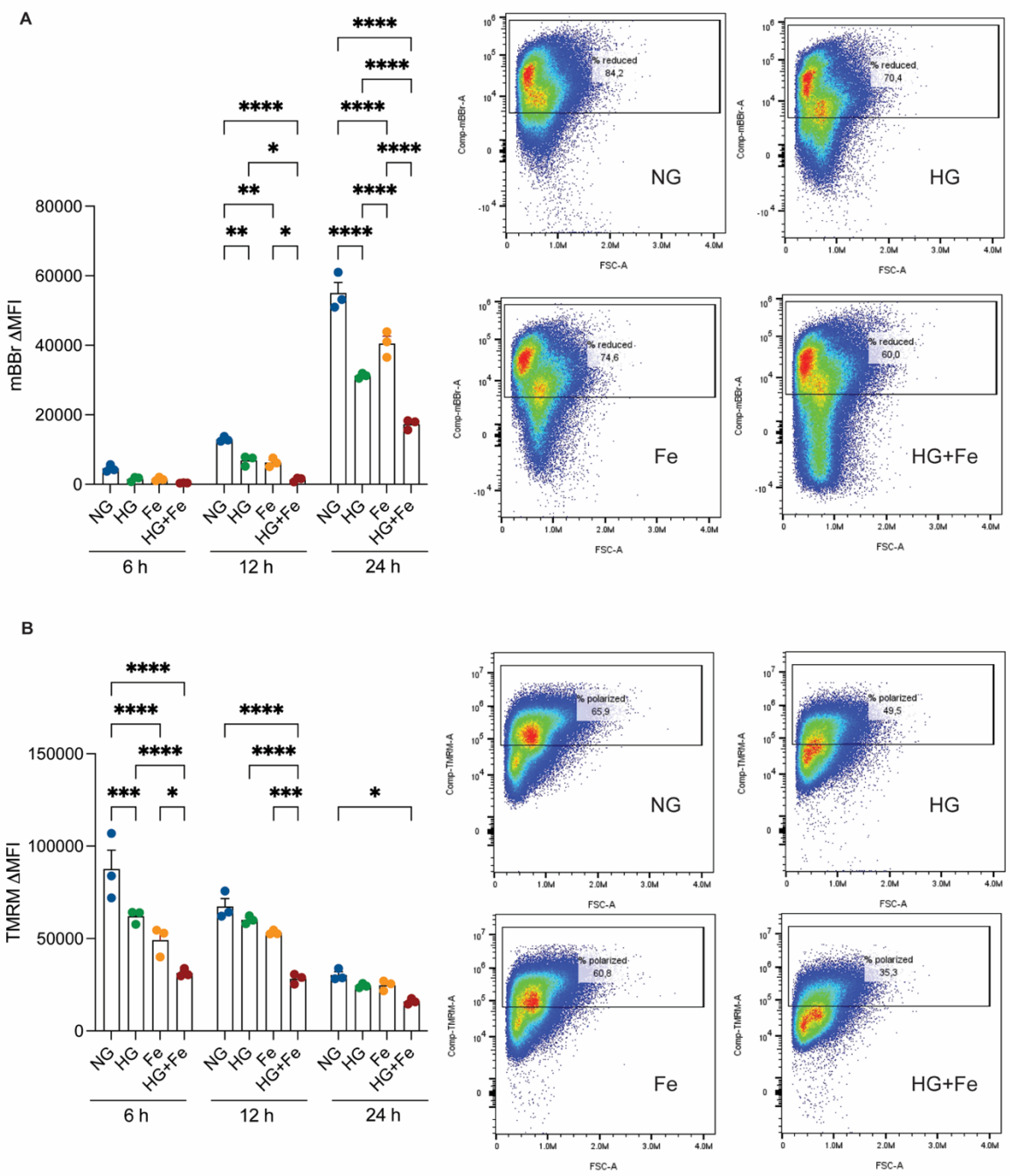

Figure 3.18: Reduced glutathione and polarized mitochondrial membrane potential measured through flow cytometry in BMDMs treated with HG+Fe.

A) MFI of reduced glutathione in BMDMs treated with HG+Fe over a period of 24 hours and associated representative dot plots after 24 hours. B) MFI of TMRM in BMDMs treated with HG+Fe over a period of 12 hours and associated representative dot plots after 24 hours. Values are mean ± s.e.m (n=3). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. NG, normal glucose; HG, high glucose; Fe, iron.

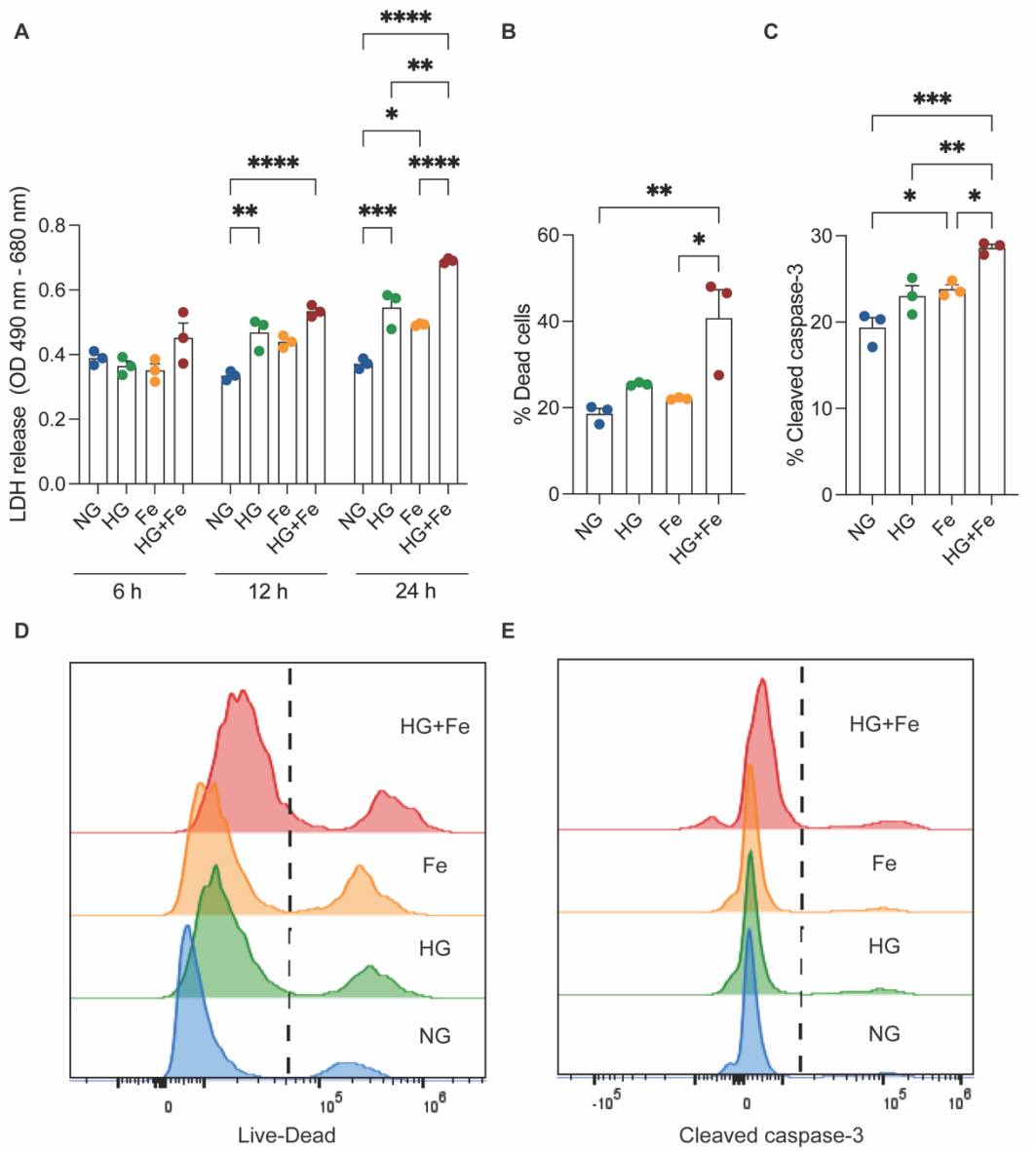

Figure 3.19: Cell death and caspase-3 activation in BMDMs treated with high glucose and iron.

A) Quantified LDH release in BMDMs after 6, 12, and 24 hours of treatment with NG, HG, Fe, and HG+Fe. B) Quantification of flow cytometric analysis of cleaved caspase-3 after 24 hours. C) Quantification of flow cytometric analysis of dead cells via Live-Dead stain after 24 hours. D-E) Associated histograms for cleaved caspase-3 and Live-Dead, respectively. Values are mean ± s.e.m (n=3). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. NG, normal glucose; HG, high glucose; Fe, iron.

3.4 Discussion

Macrophages are key cells for innate and adaptive immunity, playing a pivotal role in the inflammatory response upon tissue infection or injury.[19] Macrophages primarily reside in tissue, possessing an array of diverse and homeostatic functions.[25] In chronic diseases, macrophages orchestrate chronic inflammation that can cause related pathologies and tissue damage. In MetS, elevated glucose or iron levels can induce secretion of pro-inflammatory agents that can exacerbate tissue dysfunction in the heart, liver, pancreas, muscle and adipose tissue.[26,27] Macrophages are heavily influenced by the metabolic state of the tissue they reside in, availability of nutrients and metabolites can alter the tissue microenvironment, which in turn affects macrophage metabolism and function.[26,27] This influence on macrophage metabolism can go on to exacerbate tissue damage and disease progression. Macrophages, like most cells, use glucose as fuel for the cell to produce energy and intermediates for their function.[28] However, plasticity of macrophages allows them to adjust their metabolism based on available nutrients, reverting to other substrates under glucose-deprived states and in a glucose-rich state kick glycolysis into overdrive.[28] When glucose is in abundance, as seen in type 2 diabetes (T2D), macrophages shift to a pro-inflammatory phenotype, feeding into an inflammatory cycle that intensifies metabolic dysfunction.[26–28] Macrophages are biologically adapted for iron handling, playing a vital role in iron recycling via key proteins such as transferrin, ferritin, and ferroportin.[19,29] Macrophages work to prevent iron-mediated toxicity by sequestering iron in ferritin, however when iron overload is occurring, protective mechanisms that prevent iron leakage from macrophages begin to falter.[19,29] Saturation of ferritin stores and suppression of ferroportin can compromise iron sequestration and export. This can further lead to expansion of the labile iron pool, drive oxidative stress and activate inflammatory pathways. What

is unknown currently is specifically how macrophages process free iron and how iron handling intersects with metabolic stress.

This study aimed to examine the intersection between high glucose and iron on deleterious cellular effects in two types of macrophages. In the RAW 264.7 macrophages, pre-treating and essentially "priming cells" to high glucose or iron did not induce significant activation of NFκB, but there was activation of IRF3 when pre-treating with high glucose followed by iron for 6 and 12 hours (Figure 3.2), however this was not corroborated via Western blotting of phospho-IRF3. Furthermore, gene expression analysis (Figure 3.3), did not show significant differences among the pre-treatment groups, though findings indicated that combining high glucose and iron at the same time induced a more robust inflammatory response. In the RAW 264.7 macrophages, there was activation of IRF3 (Figure 3.2A) and increased expression of IFNβ after 24 hours of treatment with high glucose and iron (Figure 3.3B). However, this suggestion is highly context-dependent, a study by Gan *et al.* found that iron treatment suppressed M1-like macrophage cytokine production, but preserved phagocytic its abilities, implying that iron does not inactivate macrophages, but can shift its functional phenotype.[30] Regardless, limited studies have been conducted in this cell line to examine iron metabolism, and even less on how metabolic stress can influence macrophage behaviour.

Results from the RAW 264.7 macrophages led to a conclusion that individual treatments of the macrophages was not sufficient to induce persistent inflammatory pathway activation, but combining them potentially could. Thus, for subsequent experiments, a more physiologically relevant model was employed with BMDMs simultaneously treatment with high glucose and iron. Here, there was significant activation of both NFκB and IRF3 when combining high glucose and iron treatments, followed by pro-inflammatory cytokine gene expression and release (Figures 3.4

and 3.5). Studies examining effects of high glucose and iron are uncommon, though several studies have found that persistent high glucose exposure shift macrophages to a pro-inflammatory phenotype, increasing cytokine gene expression and release.[31–34] In the present study, high glucose alone was inconsistent in raising cytokine gene expression, though there was a marked increase in IL-6 gene expression and TNF and IL-1β release. Iron alone was in a similar situation, but when combining these metabolic stressors, there was a significant increase in cytokine gene expression and release. While high glucose has been well-established as a metabolic stressor that shifts macrophages to a pro-inflammatory phenotype, studies on the influence of iron remain a double-ended conundrum.[35] The variation in studies done examining effects of iron on macrophage phenotype remains heavily dependent on type and concentration of iron, duration of treatment, types of macrophages being used and disease model being examined. In non-alcoholic fatty liver disease (NAFLD), iron was found to disrupt the macrophage polarization balance, leading to inflammation seen in this condition.[36] In THP-1-derived macrophages, iron administration led to increased TNF expression, and treatment with a chelator diminished this, implying iron plays a substantial role in inflammation.[37] On the other hand, iron deficiency has also been implicated in exacerbating inflammation, as seen in alveolar macrophages and adipose tissue-resident macrophages in mice, which was alleviated upon iron supplementation.[38,39] Thus, results from the present study provides insight into the reality that combining these metabolic stressors produces a strong inflammatory response, which far outstrips the effects of these treatments alone.

Both high glucose and iron have been widely recognized as inducers of ROS and subsequent cell death.[40–42] Here we observed consistent results in that both high glucose and iron on their own were able to increase oxidative stress and decrease polarization of the mitochondrial membrane (Figure 3.6), this was significantly heightened when BMDMs were treated

simultaneously with both agents. This falls in line with the inflammatory gene expression and cytokine release data, as oxidative stress induced at the earlier time points could lead to ROS acting as DAMPs to activate inflammatory pathways, ultimately leading to cell death after the final time point of 24 hours. There are several studies that have indicted iron and high glucose as promoters of ROS generation and apoptosis across a variety of cell types, but again, there is a gap in both of these metabolic stressors being investigated in conjunction to one another.[14,43–45]

To summarize, it is evident that combining high glucose and iron induced an inflammatory response, through NFκB and IRF3, leading to increased cytokine expression and release. This was preceded by elevated ROS and succeeded by cell death. This outlines that what is currently known of these metabolic stressors independently could not be capturing the full effect these conditions have on physiology of chronic metabolic disorders that are characterized by persistent inflammation. Studying these mechanisms is inherent to identify key axes involved in progression of MetS to develop appropriate therapeutic strategies.

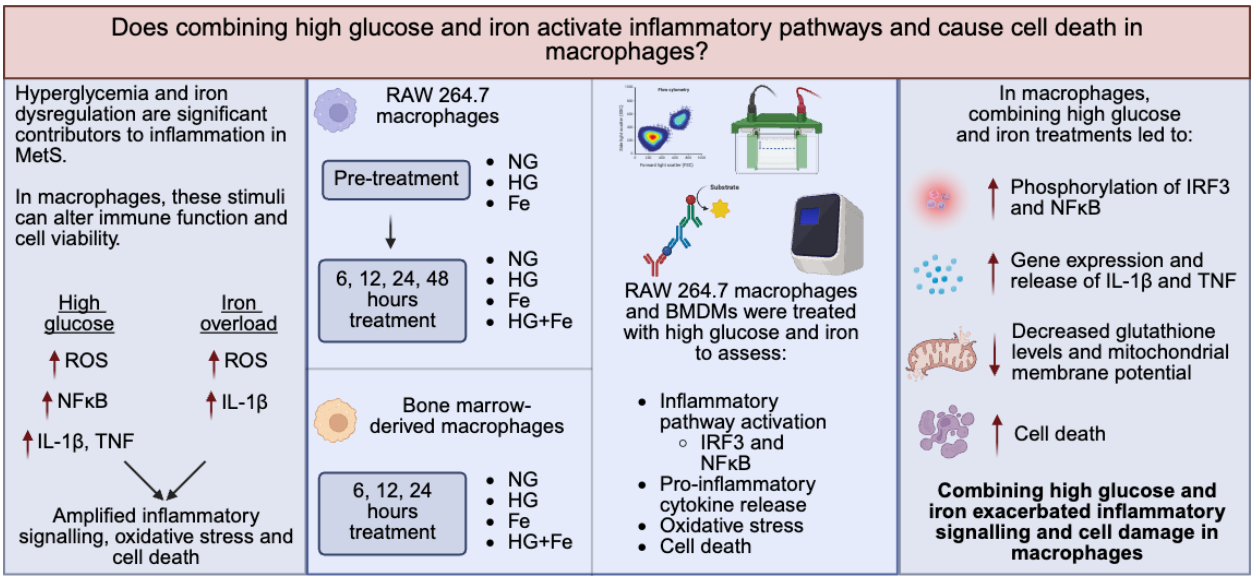

Figure 3.20: Summary schematic of study examining effects of treating macrophages with high glucose and iron.
Image created with BioRender.com.

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Chapter 4 – Immune cell dynamics and cytokine response in PBMCs exposed to high glucose and iron

Author contribution note: I performed all experiments, data analyses, and writing, with guidance from Dr. Ali Abdul-Sater and Dr. Gary Sweeney.

Abstract

Peripheral blood mononuclear cells (PBMCs) are circulating immune cells in the blood, recruited to sites of injury or infection where needed. Patients with metabolic syndrome (MetS) commonly have an altered immune profile compared to healthy individuals, at basal levels and in response to stressors. Glucose and iron are both key elements for immune cell function; however iron overload and high levels of glucose have both been shown to independently induce changes in PBMC populations, inducing oxidative stress and pro-inflammatory cytokine production. In this study, PBMCs were isolated from healthy donors and co-treated with high glucose and iron for 24 hours. Following treatment, flow cytometry was used to examine changes in lymphocytes, monocytes, and NK cells, as well as cell death, cytokine production, and oxidative stress. No significant changes were observed among any of the treatment groups in T cell or monocytes populations, however combining high glucose and iron significantly increased cell death compared to each treatment alone ($P<0.05$). In addition, TNF and IL-1β were produced in classical and intermediate monocytes in the co-treatment group compared to individual treatments ($P<0.05$). This was complemented by a significant increase in oxidative stress and mitochondrial membrane depolarization in both lymphocytes and monocytes. Taken together, it is evident that combining high glucose and iron could lead to exacerbated immune cell dysfunction and inflammation in individuals who are hyperglycemic and have excess iron; as seen in patients with MetS. This amplifies the importance of examining these stressors together, in order to target iron and metabolic pathways that drive immune cell dysfunction in MetS.

4.1 Introduction

In the previous study, there was a significant inflammatory response to combining high glucose and iron treatments in bone marrow-derived macrophages (BMDMs). Increased phosphorylation of NFκB, cytokine gene expression and release, oxidative stress, and cell death were all observed. It was evident that these incredibly relevant metabolic stressors were eliciting an additive inflammatory response. Given the robust response seen in the macrophages, I sought to determine whether these effects on immune cell function would extend to primary human cells; peripheral blood mononuclear cells (PBMCs). PBMCs are immune cells found in blood, critical in the immune response consisting of lymphocytes, monocytes, natural killer (NK) cells, dendritic cells.[1] Most PBMCs remain in a rested state without effector functions until stimulated. T cells account for approximately 60-80% of PBMCs, followed by B cells, NK cells, and monocytes (5-20%), and dendritic cells account for only <1% of PBMCs.[1,2] Composition of PBMCs heavily relies on the physiological state of the donor, as well as nutritional status, hormones levels, and infection/inflammation.[1,2] T cells comprise the largest subset within PBMCs and without stimulation, exist as naïve T cells. Cluster of differentiation (CD) 3 is universally expressed on all T lymphocytes, and they can further be characterized as CD4+ T helper cells or CD8+ cytotoxic T cells.[1,3] B cell populations are involved in antibody production, antigen presentation, and immunological memory.[2,4,5] NK cells mediate cytotoxic activity and rapid innate immune responses, they can also influence dendritic cell maturation through cytokine release.[2,4–6] NK cells are CD3-, and CD56+, which can be divided into two subsets; CD56 bright and CD56 dim NK cells, which have distinct immunoregulatory functions.[2,4–6] Dendritic cells are CD3- and HLA-DR+, acting as professional antigen-presenting cells.[1,3]

The role of immune cells and inflammation has been widely recognized in metabolic syndrome (MetS), affecting multiple organs in a complex interplay of cytokine release, oxidative stress, and metabolic disturbances. PBMCs circulate throughout the body, leaving them exposed to metabolic and inflammatory signals. In fact, some studies have used PBMCs as a biomarker source in MetS, given that these cells reflect internal tissue states and undergo reprogramming upon metabolic changes.[7–9] PBMC metabolism has been found to be altered in various disease conditions including type 2 diabetes (T2D), cardiovascular disease (CVD), steatotic liver disease, and kidney disease.[7,8,10,11] Interest in using PBMCs as markers to assess chronic disease risk has been increasing, PBMCs isolated from minipigs and rats have been used as biomarkers of metabolic risk, and could be used for early diagnosis of MetS.[12,13] Evidently, PBMCs possess the capacity to reflect systemic changes and bioenergetics, including mitochondrial health and respiration, have been examined in chronic diseases.[7,11,14–17] DeConne *et al.* found that cardiometabolic risk factors such as obesity, increased blood glucose and plasma lipids, induced release of pro-inflammatory cytokines and reactive oxygen species (ROS) from circulating PBMCs.[14] Linking this to cholesterol and blood pressure provided insight into how risk factors of MetS can impair immune cell function, which can ultimately affect how these cells respond to infection or injury.[14] A study by Hartman *et al.* revealed that there is a correlation between oxygen consumption, and by extension mitochondrial dysfunction, and production of ROS in PBMCs isolated from diabetic patients.[18] While mitochondrial bioenergetics have been examined in PBMCs in the context of MetS, little is known about how MetS risk factors can affect PBMCs composition and profile.

Hyperglycemia and iron overload (IO) have already been established as risk factors for MetS, inducing detrimental effects in various tissues and cells. However, their effect on activation

and functional response of PBMCs is poorly understood. Hyperglycemia alone has been implicated in PBMCs to induce oxidative stress, impaired mitochondrial respiration, and expression of cytotoxic genes.[19–21] Studies examining IO on PBMCs are scarce, though increased macrophage activation and monocyte scavenging capabilities upon IO have been explored, the effect of iron on PBMC cell subsets and cytokine release has not been characterized.[22–24]

In this study, PBMCs were isolated from healthy donors and treated with high glucose and iron, followed by flow cytometric analysis of immune cell subsets and examination of oxidative stress. I hypothesize that combining these treatments would change frequency of specific populations in PBMCs, leading to an increase in cytokine production, oxidative stress, mitochondrial dysfunction, and cell death. This will provide valuable framework to investigate effects of these metabolic stressors on immune cell profiling, viability, and function.

4.2 Materials and Method

4.2.1 Blood collection and PBMC isolation

Venous blood samples were collected from healthy donors by trained laboratory personnel using standardized venipuncture technique at York University, and samples were delivered to the primary author for downstream processing. Donors were healthy individuals with no known history of chronic inflammatory, metabolic or infectious diseases, and were not taking any medications during time of sampling. Blood was collected from five biologically distinct (4 female, 1 male) donors, with some individuals being sampled on different days.

Following blood draw in 10 mL tubes coated with hepatin, 10 mL of blood was mixed with an equal amount of FACS buffer (2% FBS in -/- PBS). This mixture was carefully pipetted to SepMate tubes (Stemcell Technologies, 85450) lined with 15 mL of Lymphoprep (Stemcell Technologies,18061). Tubes were centrifuged for 10 minutes at 1200xg at room temperature (RT). Following centrifugation, the PBMC later and buffy coat were poured into a fresh 50 mL tube, this solution was topped off with FACS buffer to 45 mL and centrifuged for 8 minutes at 300xg at RT. Supernatant was poured out and 35 mL of FACS was added to the remaining pellet and centrifuged for 8 minutes at 300xg at RT. Supernatant was poured out and the resulting pellet containing PBMCs was resuspended in 1 mL of AIM V media (Thermofisher, 12055091). Cells were counted and plated in a 12-well plate, approximately 500,000 cells were plated in each well. Following plating, cells were rested at 37°C for one hour. After this, they were treated with normal glucose (16 mM D-glucose + 19.5 mM mannitol), high glucose (25 mM D-glucose), iron (50 μM) and high glucose plus iron. For analysis of immune cell markers and intracellular cytokine production, cells were treated for 24 hours. Brefaldin A (Thermofisher, 00-4506-51) was added in the last 4

hours of treatment at a final concentration of 3 μg/mL to collapse the Golgi and prevent cytokine release from cells. For examination of oxidative stress, cells were treated for 6, 12, and 24 hours.

4.2.2 Examining extracellular immune cell markers and intracellular cytokines via flow cytometry

Following 24 hours of treatment, cells were transferred to a 96-well plate and centrifuged at 1200 rpm at 4°C for 2 minutes. Supernatant was collected for downstream assays as mentioned in section 4.2.4 and 4.2.5. Cells were washed with PBS (without Ca^{2+} and Mg^{2+}) and centrifuged at 1200 rpm at 4°C for 2 minutes. Multi-colour wells were resuspended in 50 μL of a live-dead (Thermofisher, L23105) solution with a final dilution of 1:800 in PBS, unstained or single stain wells were resuspend in PBS only. This was followed by incubation for 15 minutes in the dark at RT. Following incubation, cells were centrifuged at 1200 rpm at 4°C for 2 minutes, washed with PBS once, and then washed again with FACS buffer. Next, all wells were resuspended in Fc block at a dilution of 1:25 in PBS and incubated for 15 minutes in the dark at RT. After incubation, cells were washed twice with FACS buffer.

For extracellular staining, multi-colour sample wells were resuspended in a 50 μL multi-stain solution containing Superbright buffer (1:40 in FACS). Table 4.1 outlines the antibodies used, their corresponding fluorophore, and dilution used. Single stain wells were resuspended in a single stain solution containing the appropriate antibody and unstained wells were resuspended in FACS.

Table 4.1: List of antibodies used for extracellular staining in PBMCs

Antibody	Fluorochrome	Dilution	Catalogue #	Manufacturer
CD3	Super Bright 702	1:200	67-0037-42	Thermofisher
CD4	BUV395	1:200	363-0047-42	Thermofisher
CD8	BUV805	1:200	368-0088-42	Thermofisher
CD14	APC-eFluor 780	1:200	47-0149-42	Thermofisher
CD16	Superbright 780	1:100	78-0168-42	Thermofisher
CD56	BV421	1:100	362552	Biolegend
HLA-DR	FITC	1:200	11-9952-42	Thermofisher

Once cells were stained, they were incubated for 25 minutes in the dark at RT. Following incubation, cells were washed twice in FACS buffer and resuspended in 100 µL IC fixation buffer (Thermofisher, 00-8222). The incubation period was for 30 minutes in the dark at RT, the plate was spun at 1200 rpm at 4°C for 2 minutes, decanted, and cells were resuspended in 100 µL of 1X permeabilization buffer (Thermofisher, 00-8333). The permeabilization incubation was overnight in the dark at 4°C. The next day, the plate was spun at 1200 rpm at 4°C for 2 minutes, decanted and multi-colour wells were resuspended in 50 µL intracellular antibody mix in 1X permeabilization buffer which comprised of 2 intracellular cytokines, outlined in Table 4.2. Single stain wells were resuspended in a single stain solution containing the appropriate antibody and unstained wells were resuspended in permeabilization buffer.

Table 5.2: List of antibodies used for intracellular staining in PBMCs

Antibody	Fluorochrome	Dilution	Catalogue #	Manufacturer
TNF	BUV737	1:200	367-7349-41	e-Bioscience
IL-1β	PE	1:200	12-7018-82	Thermofisher

Cells were incubated for 25 minutes in the dark at RT. Following incubation, wells were topped off with 150 µL of 1X permeabilization buffer and spun 1200 rpm at 4°C for 2 minutes. Cells were decanted and washed again twice with 200 µL of 1X permeabilization buffer. Finally, cells were resuspended in 200 uL of 1X permeabilization buffer and ran on the Aurora 5L flow cytometer. Data was unmixed using the Spectroflow software and analyzed on FlowJo.

4.2.2.1 Gating strategy

Figure 1 shows the gating strategy used to evaluate immune cell subsets.

4.2.3 Examining oxidative stress via flow cytometry

To examine ROS production and mitochondrial membrane potential, cells were harvested following treatment for 6, 12, and 24 hours, They were then stained with monobromobimane

(mBBr) (Thermofisher, M1378) at a final concentration 1 μM, 2'7-dichlorofluorescin diacetate (DCFDA) (Sigma-Aldrich, D6883) at a final concentration of 2.5 mM, MitoSox (Thermofisher, 36008) at a final concentration 2.5 μM, and tetramethylrhodamine methyl ester (TMRM) (2.5 nM) for 15 mins at 37°C, protected from light. Following staining, cells were washed in FACS buffer three times and run on the Aurora 5L flow cytometer (Cytek). Data was unmixed to separate fluorescent signals from each dye using the SpectroFlo software (Cytek), and subsequent analyses was performed using the FlowJo software.

4.2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatant from treated cells described in section 4.2.1 was collected. A human IL-1β ELISA kit (Invitrogen, 88-7261-88) and TNF ELISA kit (Invitrogen, 88-7346-88) were used to respectively quantify IL-1β and TNF release into the supernatant from treated cells as per the manufacturers protocol. A standard curve was run with all samples and the equation of the line of best fit was used to quantify protein concentration.

4.2.5 Measuring Cytotoxicity in PBMCs

Supernatant from treated cells described in section 4.2.1 was collected at the end point of 24 hours to examine lactate dehydrogenase (LDH) release from cells using the CyQuant LDH Cytotoxicity Assay kit (Invitrogen, C20301). Supernatant was incubated with the reaction mixture for 30 minutes in a 96-well flat bottom plate at 37°C as per the manufacturers protocol. Following incubation, absorbance was measured at 490 nm and 620 nm. Background signal at 620 nm was subtracted from the 490 nm absorbance for analysis.

4.2.6 Statistical analysis

To compare all experimental treatment conditions, a two-way ANOVA was used to examine the influence of the pre-treating cells with NG, HG, or iron on inflammatory pathway

activation in section X. All other analysis comparing the differences between the four treatment groups (NG, HG, Fe, HG+Fe) was done using a one-way ANOVA followed by Tukey's test for multiple comparisons. All statistical analyses were conducted on GraphPad. v10.5.0.

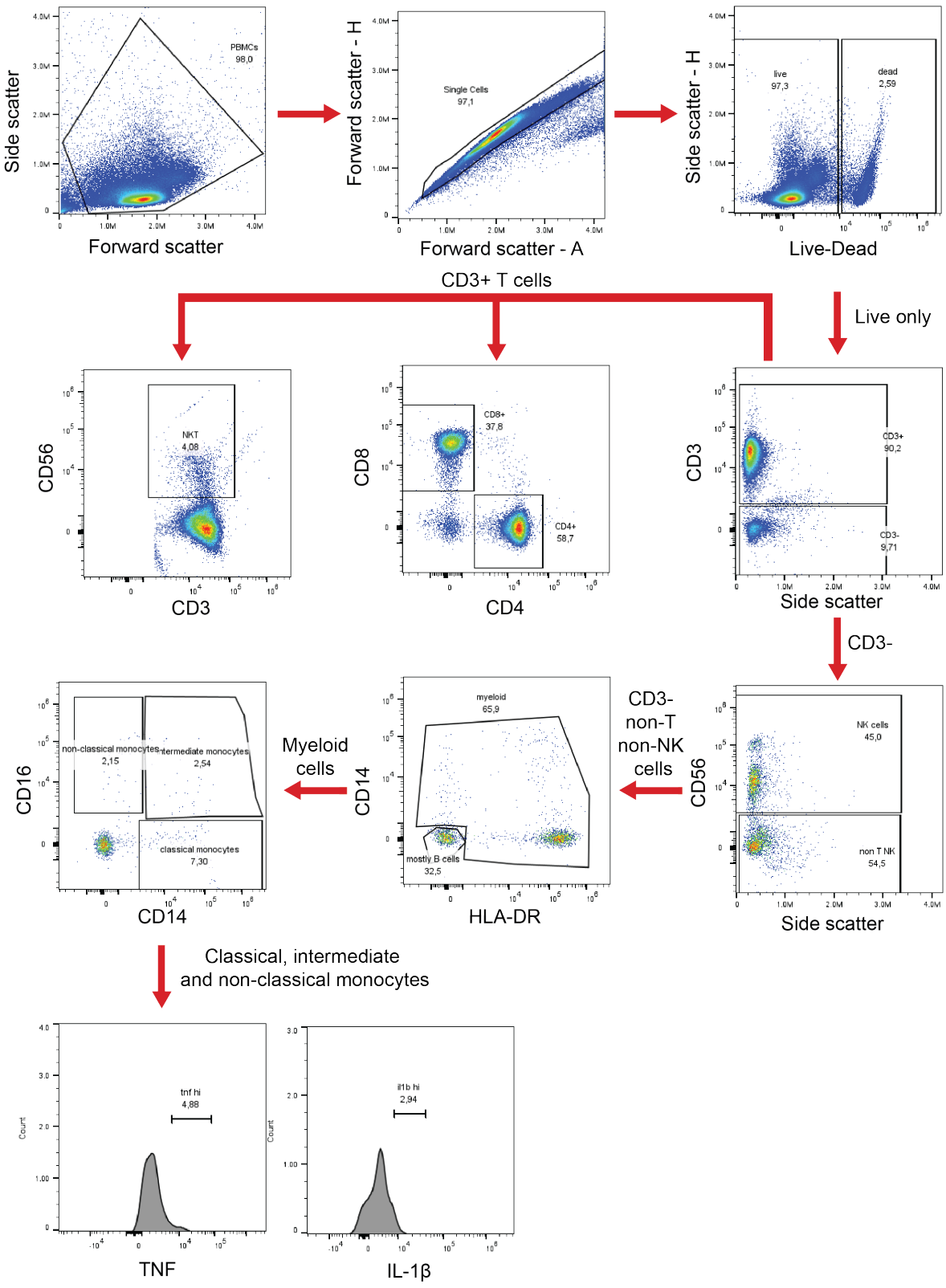


Figure 4.21: Gating strategy to isolate immune cells subsets in PBMCs treated with normal glucose (NG), high glucose (HG), iron (Fe) and HG+Fe.

4.3 Results

4.3.1 Characterizing immune cells subsets in response to high glucose and iron

Characterization of immune cell subsets was done using the gating strategy outlined in Figure 4.1. First, T cells were identified using CD3, CD3+ T cells were then categorized into CD4+ helper T cells or CD8+ cytotoxic T cells. In addition, CD56+ natural killer (NK) T cells were isolated. Figure 4.2 presents T cell subset classification in PBMCs treated with normal glucose, high glucose, iron and high glucose+iron.

T cells comprise most of the population of cells in PBMCs, as evidenced in this study, with T cells making up 65-87% of the total cells. Among these, helper T cells are more abundant than cytotoxic T cells and NKT cells, with NKT cells being least abundant. High glucose and iron treatments did not influence a significant shift in helper T cells or cytotoxic T cell populations. NKT cells on the other hand, were not influenced by high glucose or iron treatments alone, but together they induced a significant increase in CD56 bright NKT cells. This presents aptitude of these treatments in increasing total count and, potentially, activation of NKT cells.

Next, CD3- cells, which include B cells, monocytes, dendritic cells, and NK cells were examined. Figure 4.3 shows classification of NK cells, non-T non-NK cells, and monocytes upon high glucose and iron treatments. Combining these two treatments caused a significant increase in NK cells compared to the normal glucose control, but not compared to the individual treatments of each stressor. This indicated that the effects of combining high glucose and iron were not statistically different than treating with high glucose or iron alone. There was no significant difference among the non-T non-NK cells, which includes B cells, monocytes, and dendritic cells. Monocytes can be categorized as classical, intermediate, and non-classical. Among these, there was a decrease in the number of classical monocytes upon treatment with high glucose and iron,

while number of intermediate and non-classical monocytes remained relatively stable across all treatments.

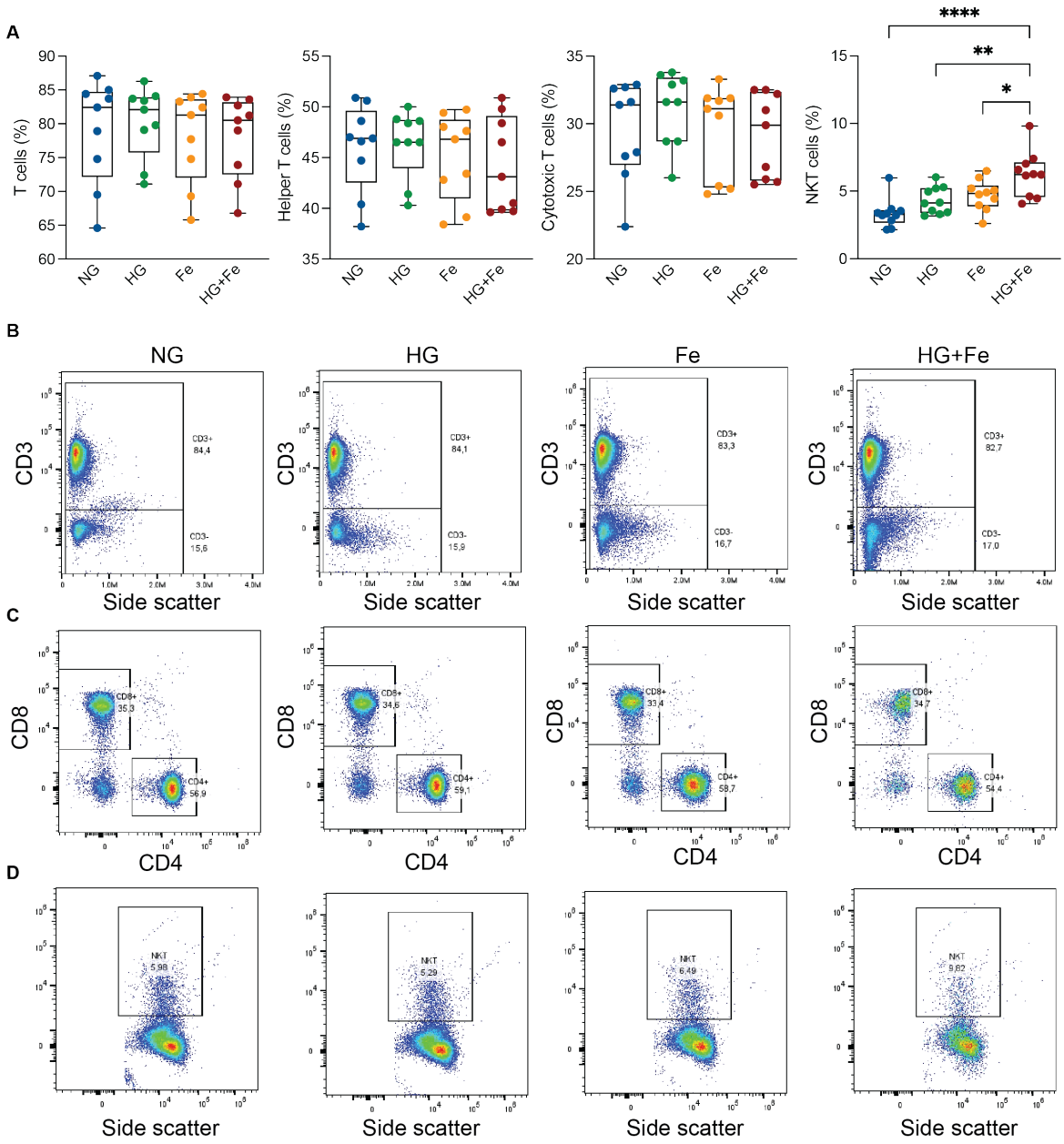


Figure 4.22: Characterizing T cell subsets using flow cytometry in PBMCs treated with high glucose and iron.

A) Quantification of different T cell subsets in PBMCs, including % of total T cells, helper T cells, cytotoxic T cells, and NK T cells. B) Representative dot plots of CD3+ and CD3- cells. C) Representative dot plots of CD4+ and CD8+ T cells. D) Representative dot plots of CD56+ NK T cells. Values are mean ± s.e.m (n=9-10), outliers excluded. *P<0.05, **P<0.01, ****P<0.0001. NG, normal glucose; HG, high glucose; Fe, iron.

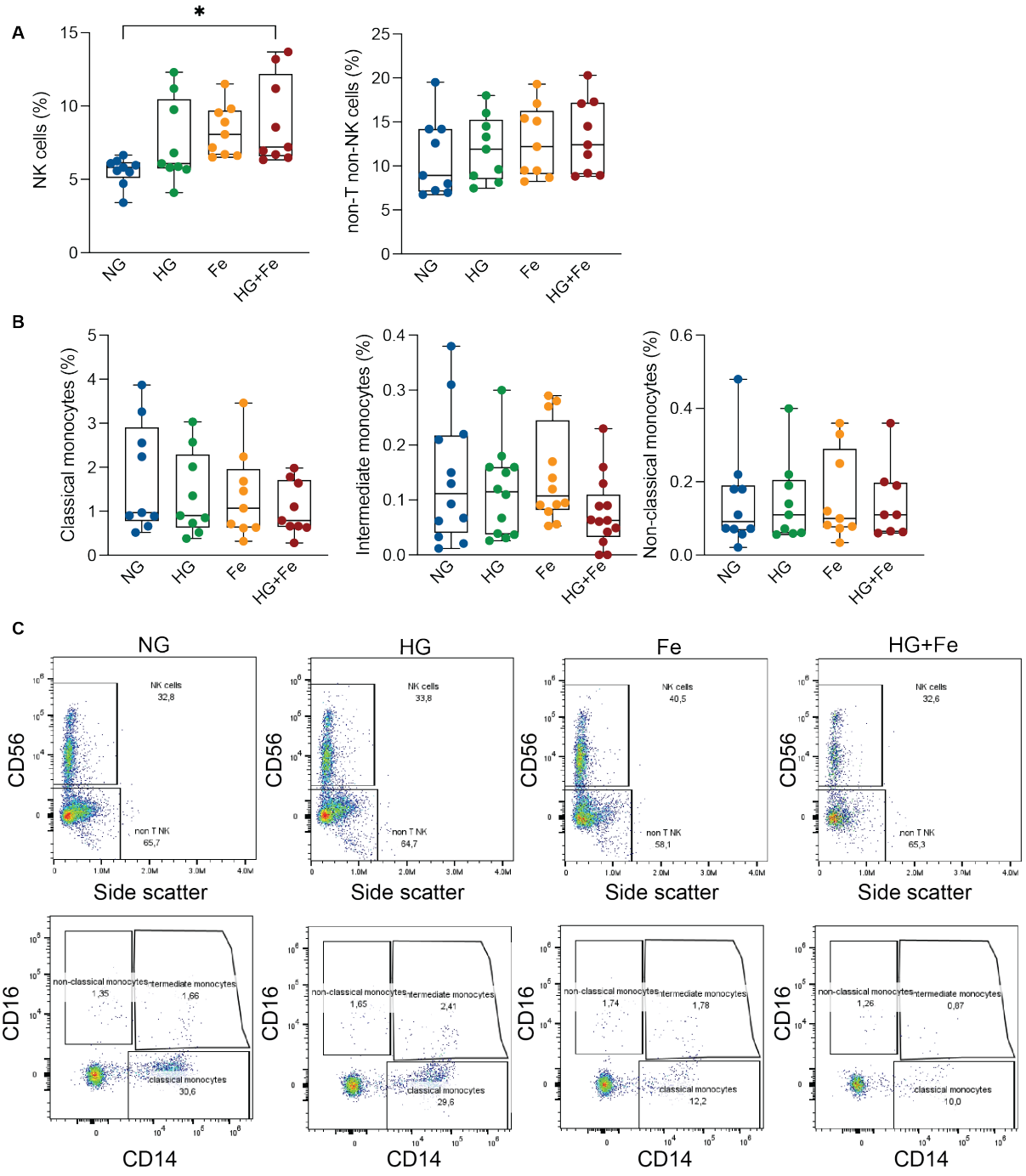


Figure 4.23: Classification of NK cells, non-T non-NK cells, and monocytes in PBMCs treated with high glucose and iron.

A) Quantification of NK cells and non-T non-NK cells. B) Quantification of classical, intermediate and non-classical monocytes in the myeloid cell population. C) Representative dot plots of NK cells, non-T non-NK cells, and monocytes. Values are mean ± s.e.m (n=9-10), outliers excluded. *P<0.05. NG, normal glucose; HG, high glucose; Fe, iron.

4.3.2 Examining cell death and intracellular cytokine production in response to high glucose and iron

Next, cell death and intracellular cytokine production was examined. Combining high glucose and iron led to a significant rise in cell death than either treatment on its own (Figure 4.4). Intracellular TNF and IL-1β production was examined and quantified according to each monocyte subtype (Figure 4.5). Here, there was significant increase of TNF in classical and intermediate monocytes when combining high glucose and iron, beyond the significant production each treatment elicited on its own. IL-1β production was also significantly elevated when combining the two stressors, and this was observed in each monocyte type. Interestingly, iron alone caused significant increase in TNF in classical monocytes, but high glucose alone induced significant IL-1β production in classical and intermediate monocytes. This suggests co-treatments could be providing an additive or synergistic effect, with distinct pathways of monocyte activation on their own that could converge when combining the two treatments. Notably, the fact remains that combining the two treatments amplifies pro-inflammatory cytokine production in monocytes subtypes.

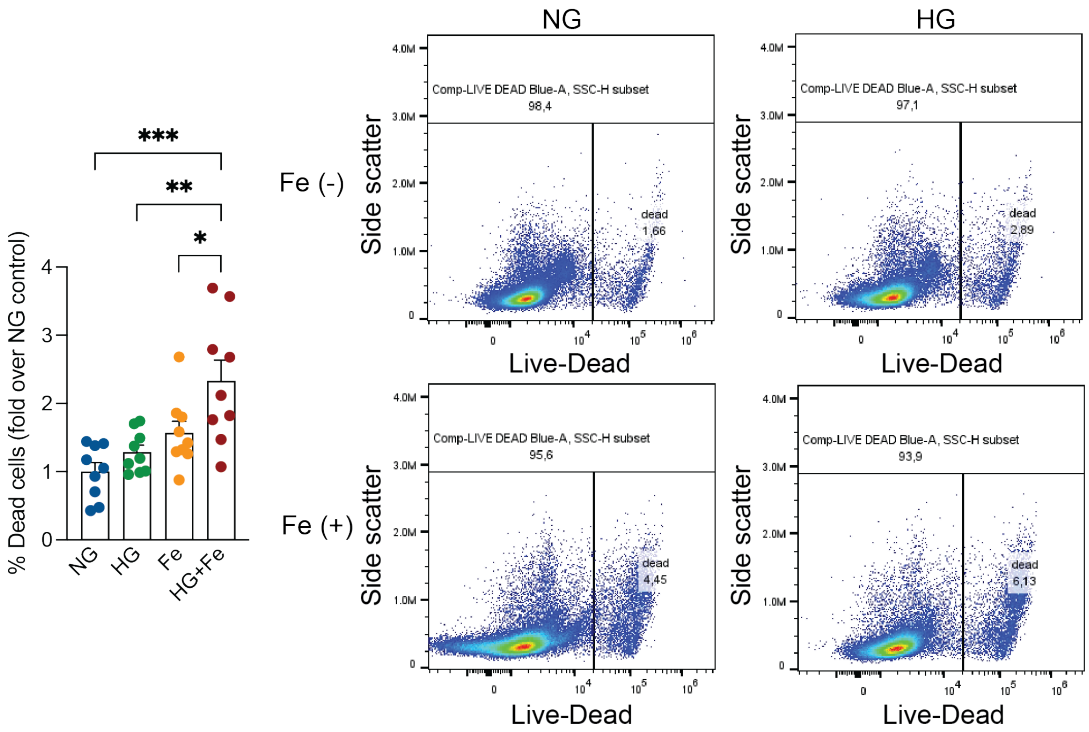


Figure 4.24: Cell death in PBMCs treated with high glucose and iron.
Quantification of cell death in PBMCs using Live-Dead blue via flow cytometry, and representative dot plots. Values are mean ± s.e.m (n=9-10), outliers excluded. *P<0.05, **P<0.01, ***P<0.001. NG, normal glucose; HG, high glucose; Fe, iron.

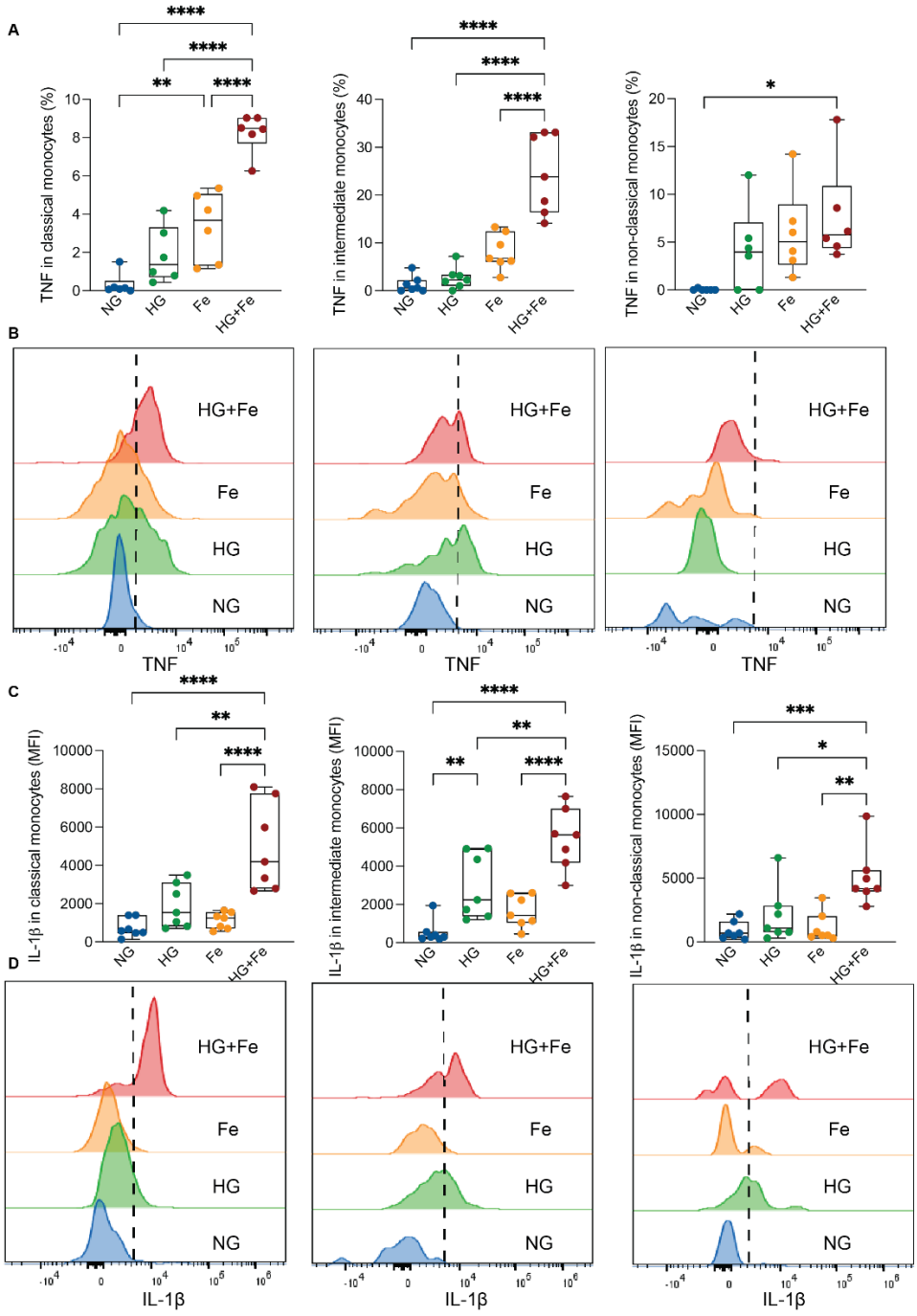


Figure 4.25: Intracellular cytokine production assessed via flow cytometry in monocyte subtypes upon treatment with high glucose and iron.

A) Quantification TNF in classical, intermediate, and non-classical monocytes. B) Representative histograms of TNF in classical, intermediate and non-classical monocytes. C) Quantification IL-1β in classical, intermediate, and non-classical monocytes. D) Representative histograms of IL-1β in classical, intermediate and non-classical monocytes. Values are mean ± s.e.m (n=6-7), outliers excluded. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. NG, normal glucose; HG, high glucose; Fe, iron.

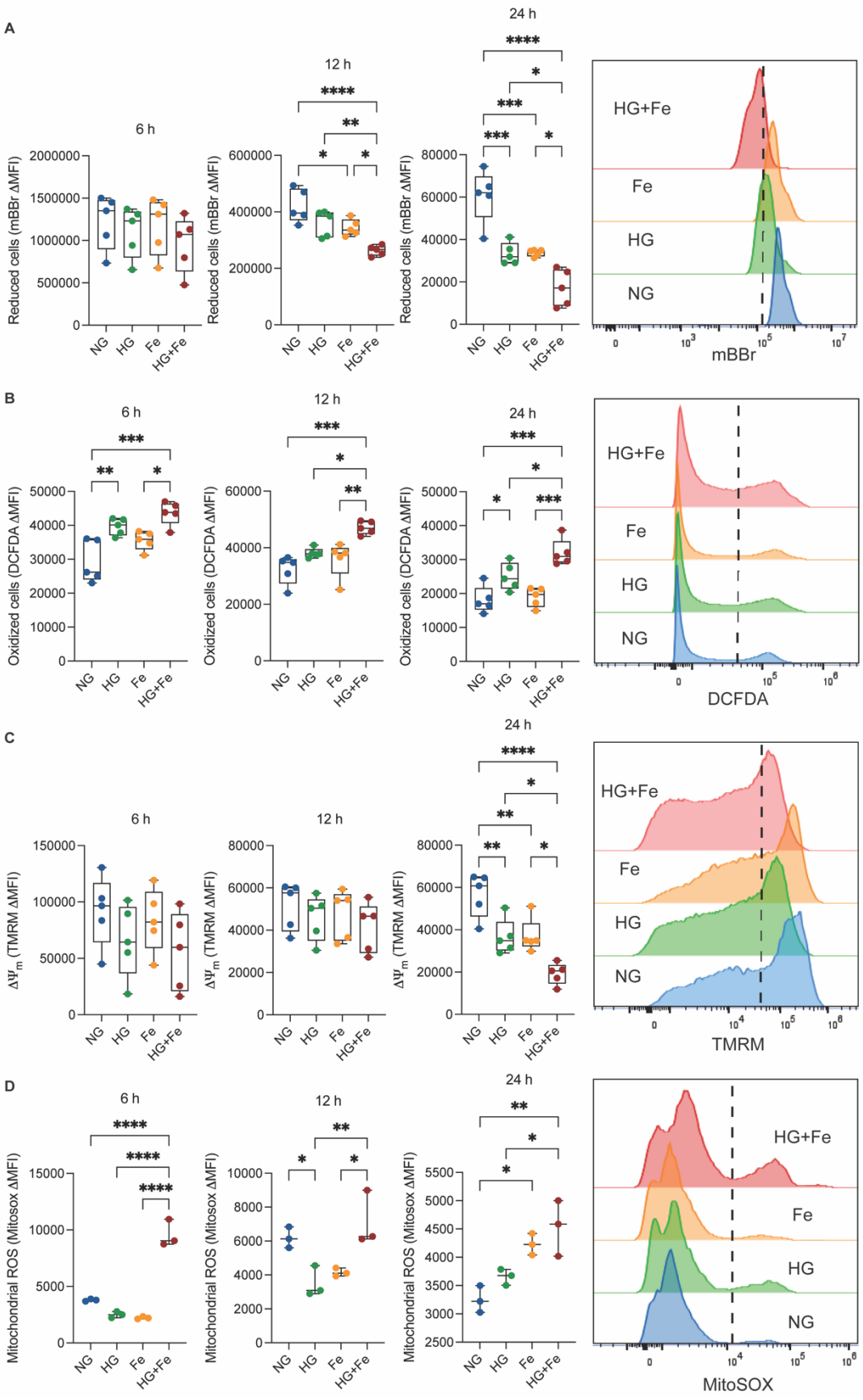
4.3.3 Examining ROS production and mitochondrial dysfunction in PBMCs treated with high glucose and iron

In addition to immune cell characterization and cytokine expression in PBMCs, ROS production was also examined as a mode of influencing immune cell dynamics and cell death upon high glucose and iron treatments. PBMCs were treated accordingly for 6, 12, and 24 hours to assess general ROS and mitochondrial ROS production via flow cytometry. mBBr and DCFDA were used to examine general ROS, while TMRM and Mitosox Red were used to examine mitochondrial membrane potential ($\Delta\Psi m$) and mitochondrial ROS, respectively. To elucidate differences among immune cell subsets, ROS production was measured in lymphocytes, monocytes, as well as PBMCs as a whole.

Figure 4.6 depicts ROS production in lymphocytes when treated with high glucose and iron for 6, 12, and 24 hours. After 12 hours of treatment with high glucose and iron, there was a significant decline in ΔMFI of mBBr, an indicator of cellular reduced thiols. This was sustained to the 24-hour mark, with both high glucose and iron significantly decreasing the number of reduced cells independently. However, the co-treatments undoubtedly had the most pronounced effect on cellular redox, significantly reducing GSH compared to all treatments. This is further corroborated by a significant increase in DCFDA, a dye that binds to general cellular ROS, when combining high glucose and iron at each time point. Evidently, there was sustained ROS production over the course of 24 hours when combining high glucose and iron, which was not observed with either treatment alone. Additionally, mitochondrial function and mitochondrial superoxide production was measured to provide insight into an organelle-specific effect of these metabolic stressors (Figure 4.6C-D). While mitochondrial superoxide production was increased at each time point in the co-treatment group, mitochondrial membrane potential measured by TMRM was only impacted after 24 hours, as shown by significant depolarization of the mitochondrial membrane.

As such, ROS accumulation could be occurring independent of immediate or sustained mitochondrial membrane potential depolarization. In addition, production of general ROS could be leading to impaired mitochondrial function and superoxide release.

In monocytes (Figure 4.7), the number of reduced cells upon high glucose and iron treatments significantly decreased beginning at 6 hours, though this was not significant when considering the high glucose and iron treatments alone. After 24 hours of co-treatment, there was a significant decrease in reduced cells, however this is only significant compared to iron alone, suggesting that high glucose is independently responsible for the reduced state of cells. Conversely, when examining general ROS production using DCFDA, there was a significant increase in the high glucose+iron group, compared to each treatment alone (Figure 4.7B), suggesting that the combination treatment amplifies intracellular ROS levels. When considering mitochondrial function, there is a steady downward trend of mitochondrial membrane polarization after 6 and 12 hours, finally becoming statistically significant after 24 hours of co-treatment. Similar to lymphocytes, there was an increase in mitochondrial superoxides after each time point when combining high glucose and iron, compared to each treatment alone. This again implies that prolonged ROS production could be leading to mitochondrial membrane depolarization, and subsequent cell death. Overall, in both lymphocytes and monocytes, high glucose can drive ROS production and impair mitochondrial function on its own, the noteworthy observation here is that this effect is further exacerbated when adding iron to the treatment. This is something iron cannot accomplish independently, indicating that combining these metabolic stressors elicits cellular damage of a greater magnitude.


A
6 h
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24 h
Reduced cells (mBBr ΔMFI)
NG
HG
Fe
HG+Fe
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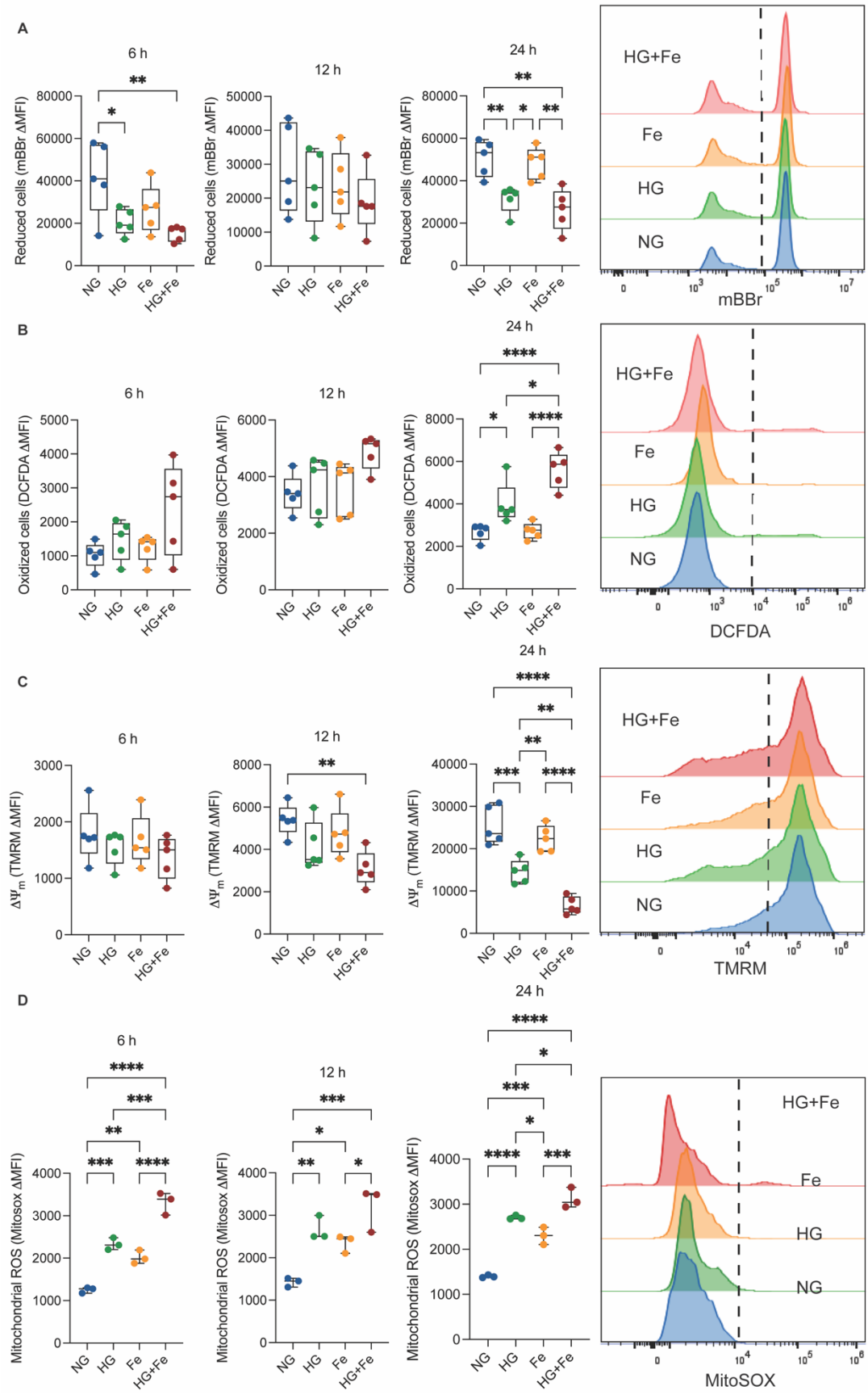
Oxidized cells (DCFDA ΔMFI)
DCFDA
C
ΔΨm (TMRM ΔMFI)
TMRM
D
Mitochondrial ROS (Mitosox ΔMFI)
MitoSOX

Figure 4.26: Examining ROS production and mitochondrial membrane potential in lymphocytes treated with high glucose and iron over a period of 24 hours.
A) Quantification of ΔMFI of mBBr, a general indicator of cellular redox state, after 6, 12, and 24 hours of treatment, and representative histogram. B) Quantification of ΔMFI of DCFDA, indicator of general ROS, after 6, 12, and 24 hours of treatment, and representative histogram. C) Quantification of ΔMFI of TMRM, indicator of mitochondrial membrane potential, after 6, 12, and 24 hours of treatment, and representative histogram. D) Quantification of ΔMFI of MitoSOX Red, indicator of mitochondrial superoxides, after 6, 12, and 24 hours of treatment, and representative histogram. Values are mean ± s.e.m (n=3-5). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. NG, normal glucose; HG, high glucose; Fe, iron.

Figure 4.8 depicts quantification of ROS data in PBMCs overall. Given that the data is expressed as ΔMFI, there is large variability given that lymphocytes and monocytes will express different levels of fluorescence of each dye. Even so, looking at PBMCs as a whole there is significant increase in general ROS (Figure 4.8B) after 24 hours, and TMRM and MitoSOX Red display a similar trend as seen in lymphocytes and monocytes individually, that being mitochondrial superoxide production significantly increased at each time point when co-treating cells with high glucose and iron, finally impacting mitochondrial membrane potential after 24 hours.

It is evident that while immune cell subsets didn't dramatically shift upon high glucose and iron treatments, these metabolic stressors were still able to induce cell death, pro-inflammatory cytokine production, and ROS generation. While the immune cell landscape remains unaltered, high glucose and iron can exert profound functional effects on PBMC subsets, which can alter immune cell responses and promote a pro-inflammatory environment.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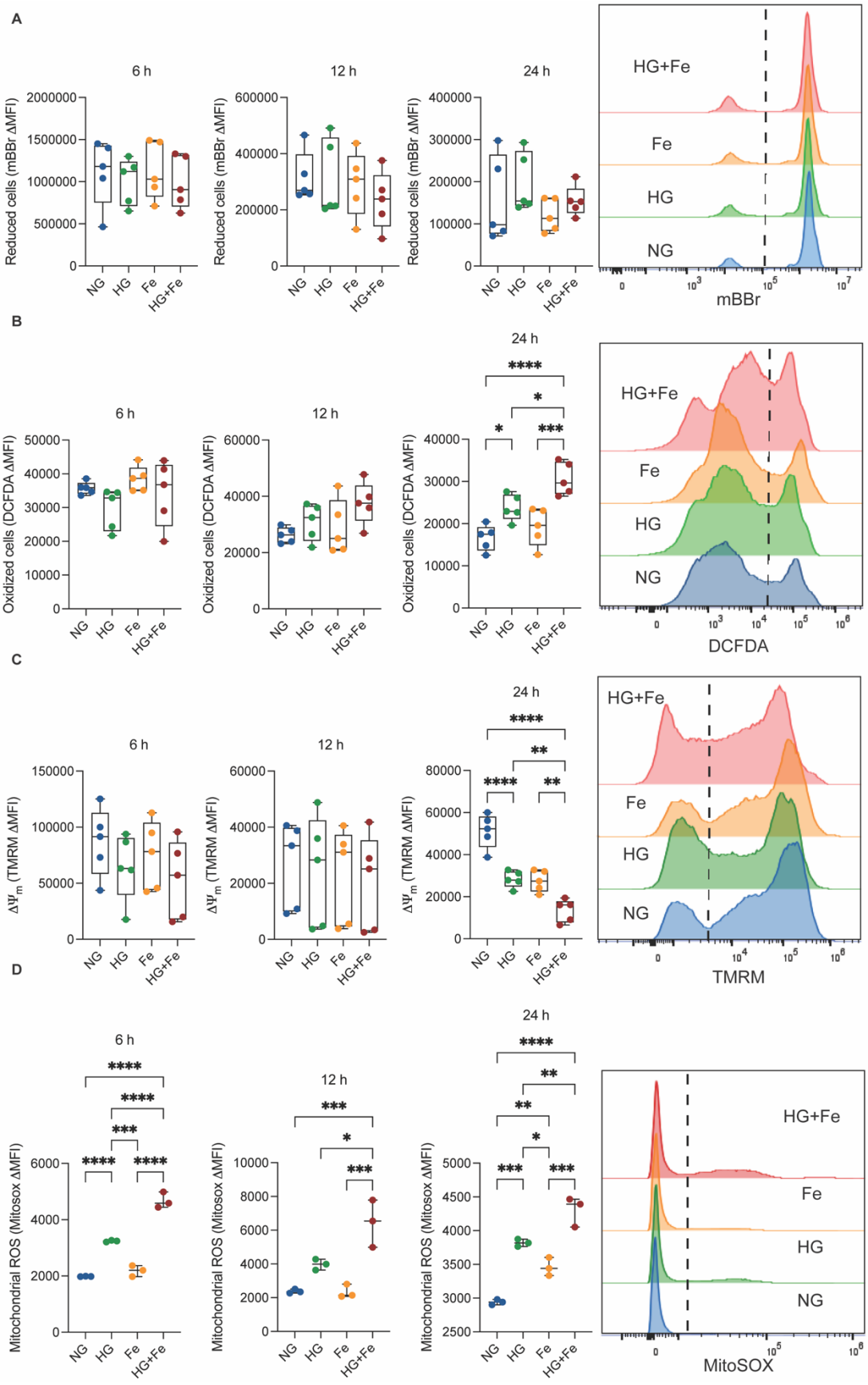
Oxidized cells (DCFDA ΔMFI)
DCFDA
C
ΔΨm (TMRM ΔMFI)
TMRM
D
Mitochondrial ROS (Mitosox ΔMFI)
MitoSOX

Figure 4.27: Examining ROS production and mitochondrial membrane potential in monocytes treated with high glucose and iron over a period of 24 hours.
A) Quantification of ΔMFI of mBBr, a general indicator of cellular redox state, after 6, 12, and 24 hours of treatment, and representative histogram. B) Quantification of ΔMFI of DCFDA, indicator of general ROS, after 6, 12, and 24 hours of treatment, and representative histogram. C) Quantification of ΔMFI of TMRM, indicator of mitochondrial membrane potential, after 6, 12, and 24 hours of treatment, and representative histogram. D) Quantification of ΔMFI of MitoSOX Red, indicator of mitochondrial superoxides, after 6, 12, and 24 hours of treatment, and representative histogram. Values are mean ± s.e.m (n=3-5). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. NG, normal glucose; HG, high glucose; Fe, iron.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Reduced cells (mBBr ΔMFI)
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Oxidized cells (DCFDA ΔMFI)
DCFDA
C
ΔΨm (TMRM ΔMFI)
TMRM
D
Mitochondrial ROS (Mitosox ΔMFI)
MitoSOX

Figure 4.28: Examining ROS production and mitochondrial membrane potential in PBMCs treated with high glucose and iron over a period of 24 hours.
A) Quantification of ΔMFI of mBBr, a general indicator of cellular redox state, after 6, 12, and 24 hours of treatment, and representative histogram. B) Quantification of ΔMFI of DCFDA, indicator of general ROS, after 6, 12, and 24 hours of treatment, and representative histogram. C) Quantification of ΔMFI of TMRM, indicator of mitochondrial membrane potential, after 6, 12, and 24 hours of treatment, and representative histogram. D) Quantification of ΔMFI of MitoSOX Red, indicator of mitochondrial superoxides, after 6, 12, and 24 hours of treatment, and representative histogram. Values are mean ± s.e.m (n=3-5). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. NG, normal glucose; HG, high glucose; Fe, iron.

4.4 Discussion

Studies examining the effects of high glucose or iron overload on immune cell profiles remain scarce. Given that inflammation is a focal point of metabolic disease, this is a key knowledge gap that limits this axis as a potential biomarker or therapeutic target. There are virtually no studies examining immune cell profiles combining both of these stressors, which is alarming given their physiological relevance. Hyperglycemia, a key hallmark of diabetes, has been strongly linked to inflammation in diabetes, having adverse results on disease progression and complications.[25–29] Hu *et al.* investigated type I interferon signalling in monocytes, concluding that hyperglycemia influenced this signalling pathway and promoted the production of pro-inflammatory cytokines and chemokines during microbial infection.[30] Other studies have also established that diabetic patients are at higher risk for inflammation and infection, suggesting that key immune cells are defective or possess altered activity influences by hyperglycemia.[31–37] Iron overload (IO) has been studied in immune cells, revealing that IO can modify immune cell activity and function in PBMCs and monocytes/macrophages.[23,38,39] Excess iron has been found to impair monocyte differentiation in macrophages, induce expression of genes relating to oxidative stress and inflammation in PBMCs, weaken phagocytic abilities of monocytes, and change immune cell distribution.[23,38,39] Consequences of IO are rooted in oxidative stress, IO has revealed itself as a potent inducer of ROS in not only immune cells, but also hepatocytes, skeletal muscle cells, and endothelial cells.[22,40,41]

In this study, high glucose and iron treatments were combined in PBMCs over 24 hours to examine shifts in immune cell distribution, cytokine production, and oxidative stress. No significant changes were found among T cell populations when comparing the four treatment groups. This was unexpected, as hyperglycemia has been proven to promote changes in T cell

populations in different disease models.[42–44] A single cell analysis of human PBMCs in diabetic and non-diabetic patients found diabetic patients had altered T cell subsets, with higher cytotoxicity and expansion of CD4 and CD8 T cells.[44] An interesting study on T cell senescence reported that pre-diabetic patients with elevated blood glucose levels had increased senescent CD8 T cells and pro-inflammatory cytokine expression.[43] Patients with diabetes have skewed T cell balance as demonstrated in various pieces of literature, however these studies embark on looking deeply into naïve, memory, cytotoxic and effector T cells and their activation.[45,46] A study on effects of IO on PBMCs revealed that T cell populations were altered, observing decrease CD4+ helper T cells, increased cytotoxic CD8+ T cells.[38] Another study on CD8+ T cells from patients with endometriosis found that excess iron has detrimental effects on T cell function, impairing their ability to localize at sites of injury.[47] In this study, only total numbers of T cell subsets were examined and no shift was observed in these populations. While this is in contrast with some of the literature, there are few studies that examine effects of metabolic stressors on cell distributions in PBMC, with most focusing on a specific subtypes, rather than total counts.

Interestingly, combining high glucose and iron treatments increased total NKT cell counts, which was not accomplished by either treatment alone. NKT cells are T cells with NK-like behaviour and make up a small proportion of the total T cell population.[4] Thus, the findings that other T cell subsets are unchanged when combining high glucose and iron is intriguing. NKT cells are metabolically primed cells that rapidly respond to stress, and their enrichment in this context could be due to altered mitochondrial and oxidative stress induced by combining high glucose and iron. Interestingly, a study by Adamski et al. found that NKT-like cells were depleted in diabetic patients compared to healthy controls, leaving patients potentially susceptible to infections.[48] This is in contrast to what was observed in this study when combining high glucose and iron, however

this discrepancy can be explained by the fundamental differences between acute metabolic stress *in vitro* and chronic diabetic immune remodelling. This renders studies examining NKT cell dynamics of vital importance, to improve the vast hole that currently exists in this area of study.

Monocyte subpopulations remained unchanged among the treatment groups, though there was a distinct decrease in classical and intermediate monocytes when combining high glucose and iron. Additionally, total monocyte numbers were depleted when combining these two treatments, suggesting that the combined stressors were causing cell death in monocytes. Here, combining high glucose and iron treatment significantly increased cell death in total PBMCs, and given that only the monocyte population was changed, it's possible that these metabolic stressors are causing monocytes to be more susceptible to cell death.[49,50] This left the remaining monocytes in a hyper-inflammatory state, producing significantly more TNF and IL-1β in each subtype. Data in this study suggests that combining high glucose and iron was significantly inducing NLRP3 inflammasome activation, as indicated by increased IL-1β in all monocyte types. This could also consequently amplify TNF production, which is significantly amplified in classical and intermediate monocytes. There are numerous studies that have reported increased pro-inflammatory cytokine expression in diabetic patients, released from both lymphocytes, NK cells, and monocytes.[51–55] On the other hand, deleterious effects of excess iron are heavily associated with oxidative stress and mitochondrial dysfunction, more so than high glucose on its own.[23,56–59] In immune cells, studies have demonstrated that IO induces ferroptosis in CD8+ T cells, and impairs macrophage differentiation due to oxidative stress in monocytes.[23,47] ROS production elevated by high glucose and IO individually can also serve as DAMPs that activate immune cells and give rise to pro-inflammatory cytokine production.[60–65] As such, there is significant evidence on the role of ROS in inflammatory signalling with high glucose and IO independently, but not in

conjunction with one another. Given the results from this study indicating that there was elevated pro-inflammatory cytokine formation, oxidative stress and mitochondrial dysfunction upon combining high glucose and IO in PBMCs was investigated as well.

Oxidative stress and mitochondrial function were examined in lymphocytes, monocytes, and PBMCs when combining high glucose and iron treatments after 6, 12, and 24 hours via flow cytometry. In lymphocytes, there was a significant increase in oxidative stress after 12 hours of treatment with high glucose and iron, which was sustained for 24 hours, evidenced by a decrease in reduced thiols and increase in ROS species (Figure 4.6). Additionally, there was significant release of mitochondrial superoxides at every time point, which eventually led to mitochondrial membrane depolarization after 24 hours, indicative of mitochondrial dysfunction at this final time point. Similar findings were observed in monocytes (Figure 4.7), and when looking at PBMCs all together (Figure 4.8), there is slight variation. Given the difference in MFI of lymphocytes and monocytes can skew overall fluorescence of PBMCs, which is why looking at these populations separately is more practical. Even so, PBMCs demonstrated increased oxidative stress and decreased mitochondrial membrane polarization when combing high glucose and iron, relative to each treatment alone. It is critical to note that generation of ROS, cytokine production, and mitochondrial dysfunction in lymphocytes and monocytes were exacerbated when combing high glucose and iron, in a manner largely consistent with an additive effect. The individual effects of the treatment were consistent with literature findings as mentioned previously, however the effect of the co-treatments is a novel conclusion.[60–65] It is interesting to note that while there were no changes in T cell populations, lymphocytes were still undergoing oxidative stress. Since oxidative stress was prominent after 12 hours of treatment, it is possible that a longer treatment time may have induced a shift in T cell distribution and viability. In the literature, PBMCs that underwent

changes in T cell distribution and activation, were isolated from diabetic patients who are in a chronic state of hyperglycemia.[42–44] Thus, the acute 24-hour model used in this study may not have been sufficient to induce changes in T cell populations. In monocytes, the significant rise in ROS could have acted as DAMPs to promote the pro-inflammatory cytokine production observed in this study. Studies have clearly demonstrated that monocytes, which are highly inflammatory and short-lived cells, are incredibly susceptible to oxidative stress which can drive cytokine release.[51,55,60]

In summary, this study revealed that combining high glucose and iron treatment significantly induced cell death, oxidative stress, mitochondrial dysfunction, and pro-inflammatory cytokine production in PBMCs. This effect was generally an additive one, suggesting that the combination of these two stressors, as commonly seen in patients with MetS, is greater than either of the two independently. This highlights the needs to target both hyperglycemia and iron dysregulation in strategies aiming to reduce immune cell dysfunction and inflammation in MetS.

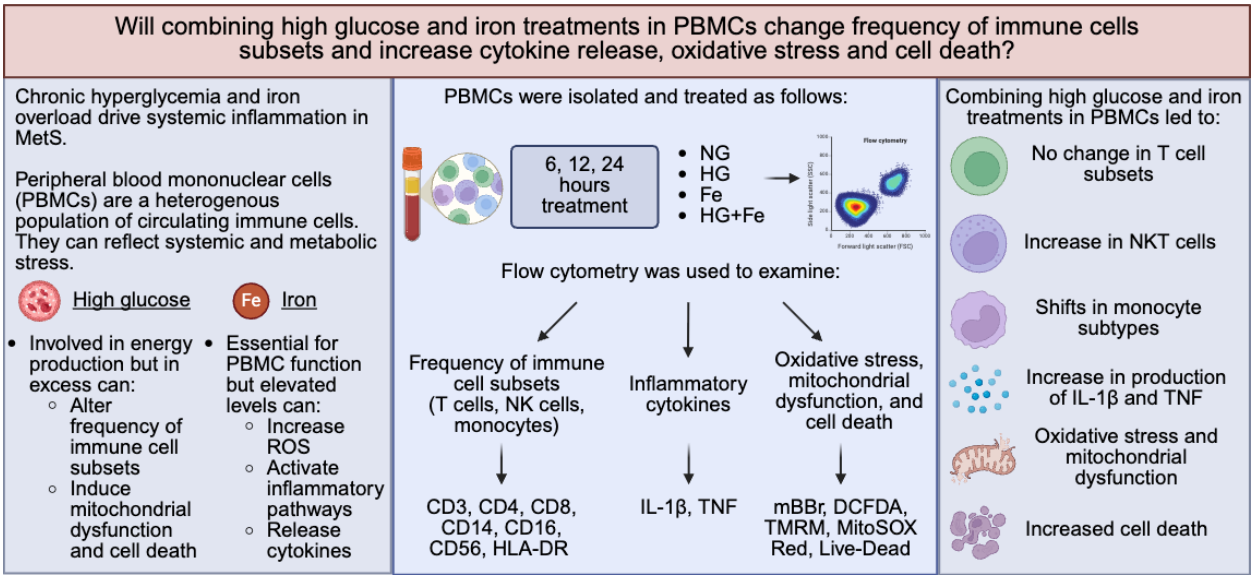

Figure 4.29: Summary schematic of study examining effects of treating PBMCs with high glucose and iron.
Image created with BioRender.com.

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Chapter 5 – Conclusions and Future Directions

5.1 Summary

Hyperglycemia is a hallmark feature of metabolic syndrome (MetS), and the root of many complications in the development of metabolic and immune-related dysfunction.***30*** Hyperglycemia inducing cell death, oxidative stress, mitochondrial dysfunction and inflammatory signalling has been well-established in numerous tissues and cell types.[1–4] My hypothesis was that the highly relevant metabolic stressors of high glucose and iron overload drive inflammatory dysfunction by promoting oxidative stress, cell death, and impaired cellular homeostasis. During my PhD studies, I embarked on examining various inflammatory axes, immune cell populations, cell death, and a potential therapeutic target that could attenuate metabolic stress-induced inflammation. Immune cells undergo various changes when exposed to metabolic stressors, a process that warrants further investigation given the importance of inflammation in cardiovascular outcomes in MetS. In this chapter, I will consolidate the arguments from each study to come to a unified conclusion on the findings of this dissertation.

5.1.1 Study #1 – The adiponectin mimetic peptide ALY688 mitigates high glucose-induced cell death via an autophagy-dependent mechanism

This project aimed to examine the detrimental effects of high glucose in inducing cell death, and its associated mechanisms in the heart *in vitro* and *in vivo*. More importantly, this study assessed the ability of an adiponectin mimetic peptide, ALY688, in reducing cell death and inflammation. Briefly, H9c2 rat cardiomyoblasts were pre-treated with ALY688 (300 nM) overnight, followed by 24 hours of treatment with high glucose (with and without ALY688). Cell death was examined via Western blotting, flow cytometry and confocal microscopy, revealing that ALY688 carries significant potential in attenuating high glucose-induced cell death. Probing further into the mechanistic aspect, it was revealed that ALY688 was doing this in an autophagy-

dependent manner. This ties into the inflammatory aspect of this study where macrophages treated with conditioned media containing DAMPs secreted by the H9c2 cells, potentially as an outcome of impaired autophagy. RAW 264.7 macrophages were found to have decreased high glucose-induced inflammatory signalling when treated with ALY688. In an STZ moue model, similar findings were made in that hearts from STZ-treated mice had increased caspase-3 and inflammatory genes were significantly upregulated. This emphasized the key role inflammation in modulating cell health under metabolic stress, and that macrophages are particularly susceptible to high glucose-induced damage. This is the novel finding from this study which propelled me into further investigating the impact of metabolic stressors on immune cells as done in studies #2 and #3.

While this study provided valuable insight into mechanisms of adiponectin action, there are limitations to acknowledge. The use of H9c2 cardiomyoblasts does not fully encapsulate the phenotype, metabolic profile, and contractile abilities of cardiomyocytes. As a result of this, effects of high glucose and regulation of autophagy and inflammation may differ in more physiologically relevant models. Additionally, use of Atg7 KO cells to examine autophagy-deficiency may lead to activation of other pathways, such as apoptosis, ER stress responses, and mitochondrial dysfunction as a compensatory mechanism. This can make it difficult to discern effects of high glucose vs the knockout of Atg7. Key signalling pathways such as AMPK and mTOR were also not examined, which would have provided a link between adiponectin signalling and regulation of autophagy downstream of AdipoR1/2 receptors. Lastly, the use of STZ-induced diabetic mouse model primarily reflects the type 1 diabetes-like β-cell loss that does not represent the metabolic and inflammatory state of cells in MetS patients who could suffer cardiovascular-related complications. In this model, hyperglycemia is the dominant upstream stressor, however the

cardiovascular pathology in MetS is driven by additional factors such as altered lipid profile, obesity, and iron dysregulation. This multifactorial metabolic environment is what contributes to mitochondrial dysfunction, impaired autophagy, and accumulation of DAMPs. As such, inflammatory and autophagy-related responses observed reflect glucose-driven activation, when in reality, detrimental effects leading to MetS go beyond that singular factor. A better experimental system would incorporate an additional trait seen in MetS patients, such as iron overload, to create a more relevant metabolic environment. This would be an improved representation of the features of MetS that drive persistent inflammation and oxidative stress, beyond what hyperglycemia accomplishes alone.

5.1.2 Study #2 – Investigating combined effects of high glucose and iron on inflammatory signalling and cytokine release in macrophages

Given the key role of inflammatory processes found in study #1 upon high glucose treatment, I sought to create a model that encompasses metabolic stressors simultaneously. MetS is not only driven by cardiomyocyte dysfunction, but chronic-low grade inflammation and dysregulated immune cell activation are key characteristics as well. Chronic inflammation is initiated by innate immune cells, particularly macrophages as they directly contribute to tissue inflammation through activation of transcription factors and cytokine production. While iron overload is not a diagnostic criterion of MetS, elevated ferritin and iron dysregulation are common features of MetS. This was the rationale for combining high glucose and iron treatments in macrophages for this project.

In this project, two types of macrophages were used; RAW 264.7 macrophages and bone marrow-derived macrophages (BMDMs). Data from the raw macrophages showed significant activation of the IRF3 pathway, but not NFκB, though there was significant variation in this data.

Gene expression analysis of inflammatory cytokines found significant upregulation of IFNβ, TNF, and TGFβ, however results from the ELISA showed no significant release of TNF or IL-1β. This was unexpected given I was hypothesizing that combining high glucose and iron could be the necessary stimulus to activate NLRP3. Due to the inconsistent inflammatory response to high glucose and iron in the RAW macrophages, a more physiologically relevant model of BMDMs was used. Here, when combining high glucose and iron treatments, significant cell death, substantial TNF or IL-1β gene expression and release, and oxidative stress and mitochondrial dysfunction were reported. This was a novel finding that confirmed that effects of multiple metabolic stressors can be greater than the effects of just one, emphasizing that converging metabolic stressors can amplify inflammatory signalling. This highlights the multifactorial nature of MetS, where multiple metabolic inputs can drive cellular dysfunction. Overall, this study demonstrated the need to examine these stressors together to determine the pathways to target for therapeutic intervention.

Limitations for this study started with using RAW 264.7 macrophages, which are functionally deficient in a key component of the NLRP3 inflammasome; Apoptosis-associated Speck-like protein containing CARD (ASC). Given that a large part of my hypothesis was dependent on NLRP3 inflammasome activation, this was a problem and part of the reason BMDMs were used for the remainder of the study. Even so, using the RAW 264.7 macrophages was useful in determining that IRF3 pathway activation was not to be dismissed. Originally, only NFκB was to be examined, as an upstream transcription factor for pro-inflammatory cytokine production. Therefore, it was interesting that high glucose and iron together could induce IRF3. Aside from using RAW 264.7 macrophages, another limitation of this study was that functionality was not tested to elucidate the upstream mechanisms driving the inflammatory response observed. These

include measuring phagocytic ability of macrophages, which is a key function of this cell type, and functional ROS assays to determine whether ROS is driving downstream inflammation and cell death. As a final point, different modes of cell death were not discerned. Determining whether apoptosis, ferroptosis, and necrosis are driving cell death in macrophages when exposed to high glucose and iron would determine how cells are dying. This is essential to identify which upstream mediators are involved in the context of metabolic stress.

5.1.3 Study #3 – Immune cell dynamics and cytokine response in PBMCs exposed to high glucose and iron

The third project of this dissertation was centred around using PBMCs to examine immune cell distribution, cytokine production, and ROS generation upon co-treatment with high glucose and iron. PBMCs were isolated from healthy donors and similar to study#2, were exposed to high glucose and iron for 24 hours. Following treatment, flow cytometry was used to examine any shifts in immune cell populations. Here, I discovered that T cell subtypes remained unchanged across each treatment group, but interestingly there was a reduction in classical monocytes and increase in cell death. In addition, there was significant TNF and IL-1β production in almost all monocyte classes. Taken together, this could imply that monocytes were killed upon co-treatment with high glucose and iron, but the remaining monocytes left were highly inflammatory in secreting these cytokines. Oxidative stress and mitochondrial dysfunction were also investigated and interestingly, both lymphocytes and monocytes displayed similar trends in that ROS generation and mitochondrial dysfunction increased when compounding high glucose and iron treatments. This created a comprehensive look at what is happening to the monocytes when combining high glucose and iron treatments, and it is consistent with data from BMDMs in study #2. Evidently, monocytes are incredibly susceptible to an increase in ROS, cell death, and secretion of inflammatory agents. On the other hand, while there was significant ROS production and mitochondrial dysfunction in

lymphocytes, no changes were observed in T cell distribution. This could mean that while T cells were undergoing oxidative stress, the intensity was insufficient to alter any changes in their distribution and activation. Perhaps a longer exposure time would have elicited measurable changes in T cell numbers and subtypes. Overall, findings from this study were consistent with study #2, indicating that combining these metabolic stressors renders them significant contributors to systemic inflammation.

The main limitation of this study was the selected markers only allowed for characterization of major immune cell subsets. These markers do not provide any information on functional responses, including proliferation, effector/memory differentiation, and additional cytokine and transcription factor analysis. Subtle shifts in effector vs memory T cells, cytotoxic potential of NK cells or monocyte polarization may have been missed in this panel. Second, while oxidative stress was examined, it is uncertain whether the magnitude or duration of metabolic stress induced pronounced shifts in T cell or NK cell function, distribution and proliferation. This would have shed light on whether ROS was translating to biologically significant changes in immune cell behaviour. Finally, functional assays on how combining high glucose and iron can drive immune cell activation was limited. While cytokines were analyzed, metabolic flux assays would have provided mechanistic insight into how these metabolic stressors drive ROS and inflammatory signalling, ultimately converging at cell death.

Summary of Key Findings		
Study #1	**Study #2**	**Study #3**
The adiponectin mimetic peptide ALY688 mitigates high glucose-induced cell death via an autophagy-dependent mechanism • High glucose significantly induced cell death and inflammation in H9c2 cardiomyoblasts. The adiponectin mimetic, ALY688, was able to reverse this. • High glucose significantly impaired autophagy, which ALY688 was able to restore. • Protective effects of ALY688 were not observed in an autophagy-deficient cell line. • In STZ mice, ALY688 was able to significantly reduce active caspase-3 and inflammatory gene expression.	**Investigating combined effects of high glucose and iron on inflammatory signalling and cytokine release in macrophages** • Two types of macrophage cell lines were used to examine effects of combining high glucose and iron treatments. • In RAW 264.7 macrophages, pre-treating cells with high glucose or iron followed by the opposing treatment did not significantly cause activation of NFκB or expression of inflammatory genes. • Combining high glucose and iron in BMDMs significantly increased cell death, oxidative stress, and inflammatory cytokine gene expression and release.	**Immune cell dynamics and cytokine response in PBMCs exposed to high glucose and iron** • PBMCs from healthy donors were isolated and treated with high glucose and iron. • Examination of immune cell subsets revealed no change in frequency of T cells, but a significant increase in NKT cells was observed. • Combining treatments showed a trend of decreasing classical and intermediate monocytes • Co-treatment with high glucose and iron led to significant oxidative stress and mitochondrial dysfunction • Increased cell death and production of IL-1β and TNF in PBMCs treated with high glucose and iron.

Figure 31.1: Summary of key findings of each study.
Image created with BioRender.com.

5.2 Future directions

Given the limitations mentioned in the previous section, future studies could work on filling the gaps these studies could not address. In the first study, given the robust response and protective effects by ALY688 observed, using a more physiologically relevant cell line, such as AC-16, or neonatal/adult ventricular cardiomyocytes from rats or mice would be useful. This is essential to prove the consistency of the model and the ability of ALY688 to attenuate cellular damage and death. These models would allow for examination of metabolic stress responses in a context that more closely resembles the adult human heart, including contractility, electrophysiology and cell signalling. Additionally, since deletion of Atg7 could activate

compensatory pathways (ER stress, mitochondrial stress, ubiquitin-proteosome system), pharmacological strategies inhibiting autophagy or autophagy signalling pathways would help better understand cardiomyocyte survival in metabolic stress. The use of inhibitors would be acute, reversible and avoid triggering compensatory responses induced by knocking out Atg7. Using transient or inducible genetic approaches, such as siRNA or shRNA would also aid in avoiding chronic stress pathways that come with a complete knockout. In addition, functional assays such as mitochondrial flux, ROS production over time, and contractility measurements would further clarify how ALY688 can rescue the heart from the detrimental effects induced by hyperglycemia. Another essential addition to this project would be a more robust mouse model. Rather than using STZ to completely annihilate β-cells, combining a low dose of STZ and high-fat diet in mice would be a more accurate representation of hyperglycemia in MetS patients. To elaborate on the immune cell activation, PBMCs could be isolated from the blood and the heart itself could be perfused, digested, and stained with pertinent markers for flow cytometric analysis. Thus, we could distinguish between immune cell populations in circulation, tissue-resident, and those that are recruited. Major immune cell populations (T cells, B cells, NK cells, and macrophages/monocytes), activation markers (MHC-II, CD86/80, CD69/25), and intracellular cytokines could be investigated. This would paint an extremely detailed picture into the immune cell behaviour and inflammation induced by hyperglycemia, and the potential for ALY688 to resolve it. Beyond that, using echocardiography to assess systolic function, ventricular wall thickness and chamber dimensions would be useful in studying heart function.

Study #2 was largely a pilot study to elucidate whether combining high glucose and iron would even render a significant change in inflammation and cell death. To enhance translational relevant, a human monocyte-derived macrophage cell line would be beneficial to employ. Studying

functional consequences of inflammatory activation induced by high glucose and iron would bring a more complete understanding of how these stressors alter macrophage behaviour and function. These include live-cell ROS flux measurements, phagocytic uptake assays, and metabolic flux assays. Additionally, the use of cell death inhibitors (apoptosis, ferroptosis, pyroptosis) would distinguish which upstream pathways to target that are linking metabolic stress, ROS, inflammation leading to cell death. Another mechanistic experiment I would suggest would involve the use of MCC950, an inhibitor of NLRP3. Given that my hypothesis was that combining high glucose and iron would complete full activation of NLRP3 and lead to pyroptosis, this experiment would be vital in differentiating inflammasome-dependent or independent mechanisms of cell death and inflammation. Future studies investigating the contribution of NLRP3 and specific inflammatory pathways upon high glucose and iron treatments would aid in identifying the pathways to mitigate inflammation in MetS.

Future work on study #3 should definitely incorporate activation markers to capture functional responses, differentiation, and maturation status. These include T cell differentiation and cytotoxicity, NK cell cytotoxicity, and monocyte polarization. It remains unclear whether the co-treatment was sufficient to induce functional changed in proliferation, subset distribution or effector function. Additional cytokine analysis would also be useful in elucidating the upstream pathways these cytokines are emerging from. An interesting and more relevant proposal for this project would be to obtain PBMCs from diseased patients (who have chronically elevated glucose and iron levels and are MetS patients), and corresponding healthy donors to examine immune cell distribution and cytokine levels. Integrating a humanized model would be a translatable and relevant addition to examine immune cell profiles. Alongside it, using PBMCs mimicking the immune cell distribution seen in these patients using high glucose and iron treatments could open

the door to creating a model that could be used for mechanistic studies on inflammatory signalling pathways. To tie it all together, the aforementioned experiments for studies #2 and #3 could also use ALY688 as a therapeutic intervention. Given the strong evidence supporting its anti-inflammatory and anti-apoptotic potential from study #1, it would be interesting to elucidate whether ALY688 can attenuate high glucose- and iron-induced cell death and inflammation simultaneously. To elaborate on the mechanistic aspect of studies #2 and 3, examining upstream regulators and downstream effectors of NFκB and IRF3 could identify converging pathways that mediate the effects of metabolic stressors. For example, AMPK, mTOR, FoxO transcription factors and inflammasome components are all linked to oxidative stress, cell death and inflammation and warrant further investigation. These studies would bolster targeted therapies to attenuate inflammation and organ injury seen in MetS.

While it is of vital importance to examine cell-type specific models, exploring intercellular interactions would also be a viable consideration for the future. Of course, MetS is a complex, multi-organ condition that involves interactions between multiple cell types, including endothelial cells, adipocytes, and hepatocytes. Co-culture systems or organoid models could be employed to study paracrine signalling, cytokine crosstalk and interactions between the immune system and cardiomyocytes during metabolic stress.

5.2.1 Potential translation and therapeutic application

It is crucial to address the interplay of co-occurring metabolic stressors beyond what I have established *in vitro*. In mice, I would propose injecting them with a low dose of STZ (25-50 mg/kg), iron dextran at a dose considered to be iron overload (10-50 mg/kg) combined with a high fat diet over a period of 4-8 weeks (along with single stimulus controls). This novel model would accurately mimic key features of MetS including hyperglycemia, iron dysregulation, altered lipid

profiles and obesity. This would allow mechanistic insight into oxidative stress, inflammation and cell death in relevant organs including the heart, muscle, liver, and pancreas. In addition, PBMCs could be isolated and tissue resident immune cells could be examined via flow cytometry to discern changes in immune cell profiles and cytokine production. Beyond that, oxidative stress and mitochondrial function could be examined in immune cells and different organs. This would provide cohesive insight into the inflammatory and oxidative state of immune cells and metabolic organs upon exposure to multiple metabolic stressors. For example, I would expect mice fed a high fat diet and injected with STZ and iron dextran to have a significantly altered immune cell profile. I would expect the PBMCs to have significantly increased activation of cytotoxic T cells and NK cells, as well as production of pro-inflammatory cytokines. In the heart, I would expect to see an increase in recruited macrophages that are expressing a pro-inflammatory phenotype and secreting cytokines.

Once the effects on inflammation, oxidative stress and cell death have been established to be deleterious in mice, I would introduce ALY688 as a potential therapeutic. Given its evident potency at resolving inflammation and restoring autophagy as mechanisms of improving cell viability, I would expect it to exert a similar protective effect in this scenario. ALY688 presents itself as a multi-faceted drug candidate that can target multiple damaging cellular processes, including reducing NFB activation, promoting AMPK, and suppressing NADPH oxidase. While ALY688 has strong potential as a candidate to address multiple cellular dysfunctions underlying MetS, rigorous studies are required to elucidate whether these effects reproducible across pathways that contribute to disease pathology.

5.3 Conclusion

To bring these chapters to a close, these studies collectively demonstrated that metabolic stressors can significantly induce cell death, inflammation, and oxidative stress in cardiomyoblasts and immune cells. I explored a therapeutic intervention that was successful in reducing high glucose-induced cell damage in cardiomyoblasts using cellular and mouse models. On the other hand, I also was able to prove that combining metabolic stressors of high glucose and iron significantly compounded the cellular damage that either stimulus could induce on its own. This emphasizes the importance of studying multiple co-existing metabolic stressors, opposed to single stressor studies, which do not capture the complex environment that exists in MetS and related cardiovascular outcomes. Further studies using physiologically relevant models that target signalling pathways that these stressors share are essential to develop therapeutic interventions that can mitigate cellular and, by extension, organ dysfunction. Ultimately, continuing to study the multiple axes that underlie MetS will aid in alleviating its role as a growing global health burden.

5.4 References

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Appendix A – Supplementary data

This section will include supplementary data from study #1 and study #2.

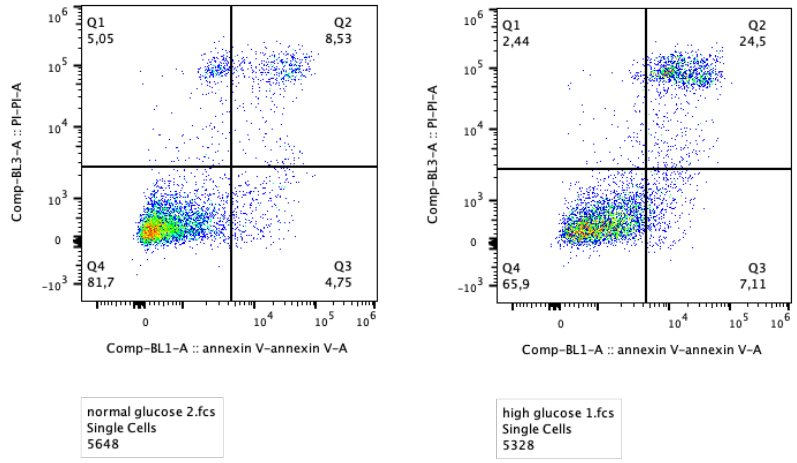

Figure A32: Flow cytometric analysis of early and late apoptotic H9c2 rat cardiomyoblasts treated with high glucose.
A) Dot plot of H9c2 cells treated with normal glucose for 24 hours. B) Dot plot of H9c2 cells treated with high glucose for 24 hours. C) Quantification of % viable cells.

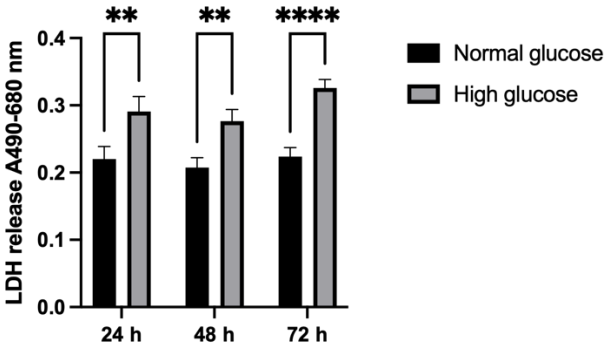

Figure A33: LDH release in H9c2 cells following high glucose treatment over the course of 24 hours.

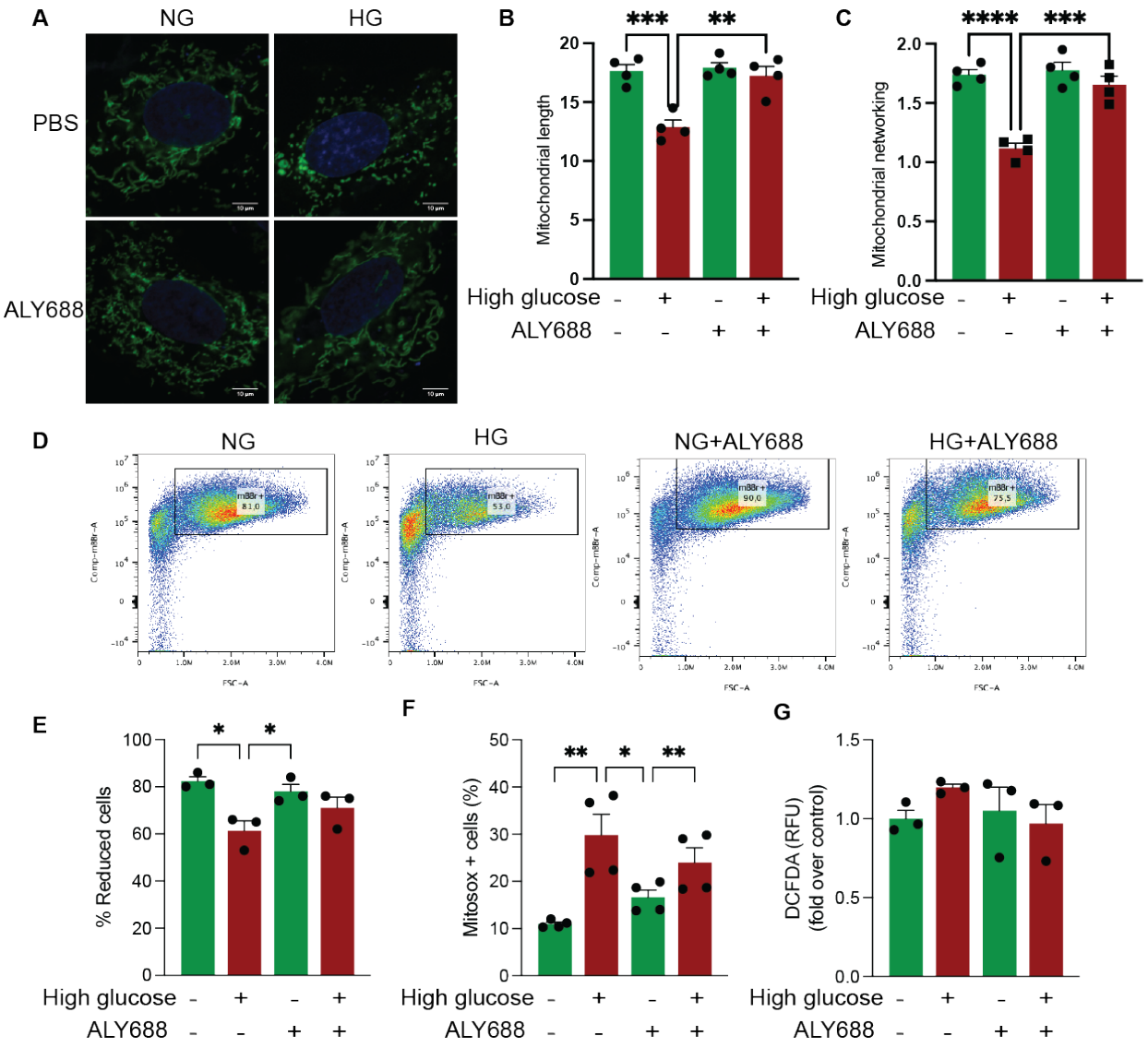

Figure A34: Examining oxidative stress in H9c2 cardiomyoblasts treated with high glucose and ALY688.

A) Representative confocal imaging of H9c2 cells treated with normal glucose and high glucose ± ALY688 for 24 hours and stained with MitoTracker Green. B) Quantification of mitochondrial length in H9c2 cells treated with normal glucose and high glucose ± ALY688. C) Quantification of mitochondrial networking in H9c2 cells treated with normal glucose and high glucose ± ALY688. D) Representative dot plots of H9c2 cells treated with normal glucose and high glucose ± ALY688 for 24 hours and stained with mBBr. E) Quantification of % reduced cells. F) Quantification of % MitoSOX+ cells. G) Quantification of % DCFDA+ cells, fold over control.

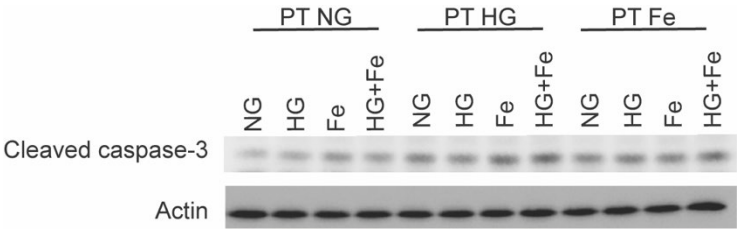

Figure A35: Western blotting of cleaved caspase-3 in H9c2 cells treated with high glucose and iron.

Western blotting of cleaved caspase-3 in H9c2 cells treated with normal glucose (NG), high glucose (HG), iron (Fe), and HG+Fe for 24 hours.

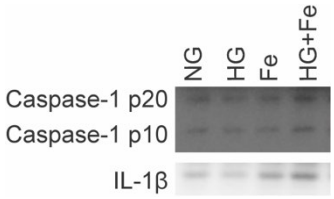

Figure A36: Western blotting of cleaved caspase-1 and IL-1β released into supernatant from BMDMs treated with high glucose and iron.

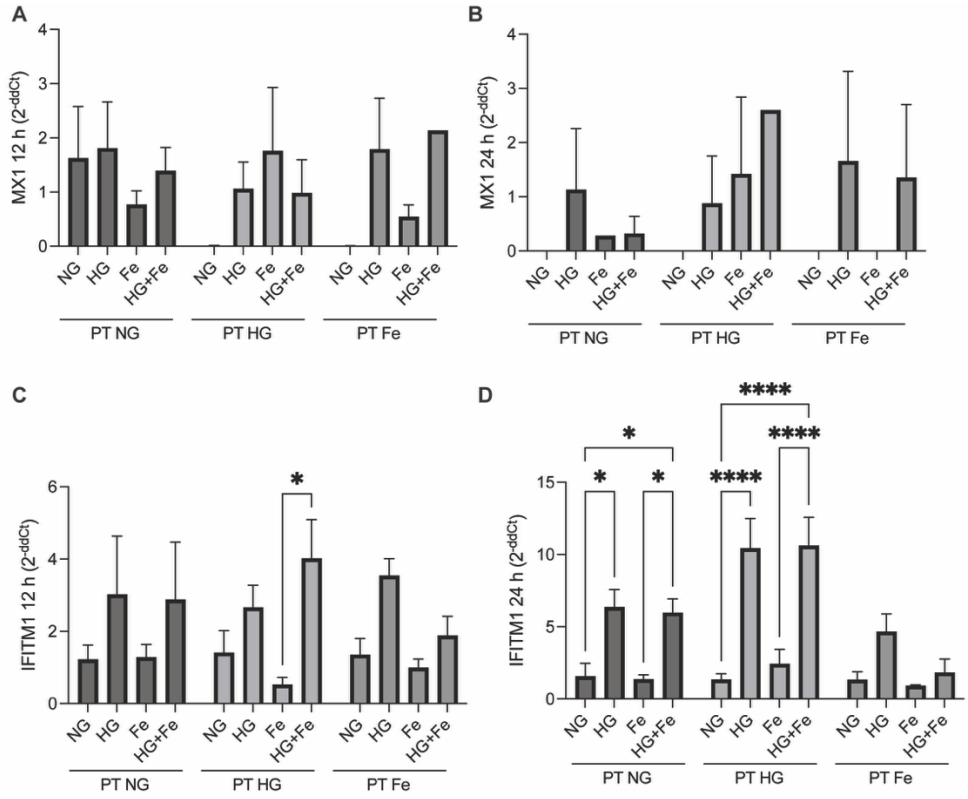

Figure A37: Relative gene expression of MX1 and IFITM1 in RAW 264.7 macrophages after 12 and 24 hours.

A) Gene expression of MX1 after 12 hours of treatment. B) Gene expression of MX1 after 24 hours of treatment. C) Gene expression of IFITM11 after 12 hours of treatment. D) Gene expression of IFITM1 after 24 hours of treatment. PT, pre-treatment, normal glucose, NG; high glucose, HG; iron, Fe.