LINKING CELLULAR METABOLISM AND INNATE DEFENCE REGULATOR PEPTIDE FUNCTION

by

Nicole Afacan

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Abstract

Appropriate cellular metabolism is essential to immune cells to survival and their ability to mount effective and appropriate responses to the pathogen and host derived insults they encounter. Malnutrition, caused by nutrient deficiency or excess, can result in significant dysregulation of immune cell activity. Immune cells undergo metabolic reprogramming in response to pathogen and host-derived signals found in their environment. These changes modulate the type of response they will mount. Innate defence regulatory (IDR) peptides, synthetic derivatives of host defence peptides, were developed as anti-infectives that modulate host immune system responses however, much of the mechanisms behind their activity are unknown. In this study, IDR-1018 was shown to modulate glycolytic activity in macrophages, which appeared to be important to its immunomodulatory activity. Activation of the ERK signalling pathway, a major regulator of metabolism and inflammatory responses, by IDR-1018 was found to be a possible mechanism by which IDR-1018 induced both glycolysis and chemokine production. Inhibition of glycolysis using 2-deoxy-d-glucose (2DG) suppressed IDR-1018 induced chemokine production. However, 2DG also suppressed IDR-1018 activity through induction of endoplasmic reticulum stress and the unfolded protein response (UPR), specifically the anti-inflammatory PERK arm of the UPR. The anti-endotoxin activity of IDR-1018 was also found to be associated with modulation of glycolysis. IDR-1018 suppressed lipopolysaccharide (LPS)-induced chemokine and cytokine production, possibly through inhibition of LPS-induced glycolysis. Interestingly, dysregulation of both glycolysis and the UPR by 2DG enhanced the anti-endotoxin activity of IDR-1018, suppressing LPS-induced chemokine and cytokine production. Finally, this study identified a potential new activity for IDRs, the modulation of metabolic pathways dysregulated in response to nutrient excess. Specifically, this study showed that IDR-1018 enhanced HDL-mediated cholesterol efflux from macrophages and smooth muscle cells, two important cellular mediators of atherosclerosis. This may have been a result of IDR-1018 interacting with HDL particles found in serum, facilitating their binding to the plasma membrane of cells. The results presented in this study demonstrated that IDR peptides are potent modulators of both immune cell function and cellular metabolism as well as identified a novel mechanism by which IDR peptides exert their immunomodulatory activity.

Preface

All experimental work was completed in accordance with the guidelines of the University of British Columbia clinical research ethics board (UBC-CREB# H04-70232) and UBC biosafety guidelines.

Chapter 1

Sections reviewing nutritional immunology and overnutrition were published in the following review article: **Afacan N.J.**, Fjell C.D., Hancock R.E.W. 2012. A systems biology approach to nutritional immunology - focus on innate immunity. *Mol Aspects Med* 33:14-25

Sections reviewing host defence peptide and innate defence regulator peptide functions were published in the following book chapter: **Afacan N.J.**, Janot L.M., Hancock R.E.W. "Host defense peptides: Immune modulation and antimicrobial activity in vivo" *Antimicrobial Peptides and Innate Immunity*. Ed. Pieter S. Hiemstra, Sebastian A. J. Zaat. Springer Basel, 2013. 321-358

Chapter 2

Experiments presented in Chapter 2 are included in the following submitted manuscript: **Afacan N.J.**, Hilchie A., Gill E.E., Falsafi R., Hancock R.E.W. Inhibition of glycolysis and induction of the unfolded protein response by 2DG regulates the immunomodulatory activity of the innate defence regulator peptide, IDR-1018. I conducted the majority of the experiments and analysis and wrote the manuscript. Experiments presented in Figures 2.5, 2.7 and 2.8 were completed in collaboration with Dr. Ashley Hilchie. Sample processing for the RNA-Seq study was completed by Reza Falsafi. Initial data processing and analysis of the RNA-Seq data was completed by Dr. Erin Gill but more complete analyses were done by me.

Chapter 3

Experiments presented in Chapter 3 are included in the following manuscript in preparation: **Afacan N.J.**, Gill E.E., Falsafi R., Hancock R.E.W. Inhibition of glycolysis and induction of endoplasmic reticulum stress by 2DG modulates the anti-endotoxin activity of the innate defence regulator peptide, IDR-1018. I conducted the majority of the experiments and analysis as well as wrote the manuscript. Sample processing for the RNA-Seq study was completed by Reza Falsafi. Initial data processing and analysis of the RNA-Seq data was completed by Dr. Erin Gill but

more complete analyses were done by me.

Chapter 4

Experiments presented in Chapter 4 were completed in collaboration with the laboratory of Dr. Gordon Francis, M.D. and are included in the following manuscript in preparation: **Afacan N.J.,** Chan T., Pistolic J., Kong J., Hancock R.E.W., Francis G.A. Innate defence regulator peptide, IDR-1018, enhances HDL-mediated cholesterol efflux from macrophages. I conducted the majority of the experiments and analysis as well as wrote the manuscript. Experiments presented in Figures 4.6-4.10 were completed in collaboration with Teddy Chan.

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2DG- 2-deoxy-d-glucose

ABC- ATP binding cassette

ACAT- Acyl-CoA cholesterol acyltransferase

AKT- protein kinase B

AP-1- activator protein 1

Apo- apolipoprotein

ATF- activating transcription factor

BiP- binding immunoglobulin protein

BSA- bovine serum albumin

CETP- Cholesterol ester transfer protein

CHAC1- ChaC glutathione-specific gamma-glutamylcyclotransferase 1

CHOP- C/EBP homologous protein

CRAMP- cathelicidin-related antimicrobial peptide

DDIT3- DNA-Damage-Inducible Transcript 3 (also known as CHOP)

DLPS- delipidated human serum

ELISA- enzyme-linked immunosorbant assay

ER- endoplasmic reticulum

ERK- extracellular signal-regulated kinase

FAFA- fatty acid free albumin

FBS- fetal bovine serum

FPR2- formyl peptide receptor like-1

GAPDH- glyceraldehyde-3-phosphate dehydrogenase

GEO- Gene Expression Omnibus

GSH- glutathione

hBD- human beta defensin

HDL- high density lipoprotein

HDP-host defence peptide

HIF-1α- hypoxia inducible factor 1α

HSF- human skin fibroblast

IDR- innate defence regulator

IKKε- I-kappa-B kinase epsilon

IL- interleukin

IP-10- interferon γ-inducible protein 10

IRE1α- inositol-requiring enzyme 1α

ISG15- interferon stimulated gene

LC- light chain

LCAT- Lecithin-cholesterol acyltransferase

LDL- low-density lipoprotein

LDLR- low-density lipoprotein receptor

LPS-lipopolysaccharide

M-CSF- macrophage-colony stimulating factor

MAPK- mitogen activated protein kinase

MCP- monocyte chemoattractant protein

MIP- macrophage inflammatory protein

MSH- melanocyte-stimulating hormone

mTOR- mamalian target of rapamycin

NADH- nicotinamide adenine dinucleotide

NADPH- nicotinamide adenine dinucleotide phosphate

NFκB- nuclear factor kB

NLRP- Nod-like receptor protein

NO- nitric oxide

NPC- Niemann-Pick disease

PBMCs- peripheral blood mononuclear cells

PBS phosphate buffered saline

PERK- protein kinase RNA-like endoplasmic reticulum kinase

PFKFB3- 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3

PI3K- phosphatidylinositol-3-kinase

PPAR- peroxisome proliferator-activated receptor

qRT-PCR- quantitative real-time PCR

ROS- reactive oxygen species

SMC- smooth muscle cell

SRB1- scavenger receptor B1

STAT6- signal transducer and activator of transcription 6

TBK1- TANK-binding protein 1

TCA- tricarboxylic acid

TLR- toll like receptor

TNF-α- tumor necrosis factor-α

TRIB3- Tribbles Pseudokinase 3

TUNEL- Terminal deoxynucleotidyl transferase dUTP nick end labeling

UPR- unfolded protein response

WST-1- 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

XBP- X-box binding protein

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Chapter 1: INTRODUCTION

1.1 NUTRITIONAL IMMUNOLOGY- LINKING METABOLISM AND THE IMMUNE RESPONSE

Although metabolism and immunology are often considered to be distinct fields of study, decades of research have made it clear that nutrient availability and metabolism are intimately linked to the host immune response. One of the earliest suggestions for this link came in 1810 when J.F. Menkel observed that undernourished individuals exhibited atrophy of the thymus (1). This was, the seminal observation that launched the field of nutritional immunology, which in the early stages focused on the effect of micro- and macro- nutrient deficiencies on the host immune response.

Beginning in the early 1900s researchers identified vitamins as important micronutrient regulators of the immune system (1). Micronutrients, such as vitamins and minerals, are metabolites are required by cells in low quantities and regulate numerous physiological processes. Deficiencies in various micronutrients are strongly associated with development of immune defects and increased susceptibility to infection (2). For example, zinc deficiency affects one third of the world's population and is a major risk factor for pneumonia, malaria, diarrhea (3) and poor wound healing (4, 5). Zinc deficiency is also associated with increased systemic inflammation, organ damage and disease severity (6-8). Zinc is critical to innate immune cell development and activity, and zinc-deficient conditions impair macrophage phagocytosis and intracellular killing (9, 10). Zinc supplementation in malnourished children and children with *Escherichia coli*-induced diarrhea resulted in reduced phagocytic and fungicidal activity and oxidative burst (10, 11). Conversely, high zinc concentrations have been reported to suppress macrophage chemotaxis, activation, phagocytosis and oxidative burst *in vitro* (12-14). This highlights the fact that nutrients must be present at appropriate levels to ensure proper immune function.

Since the 1960s, research has expanded to macronutrients, which include proteins, carbohydrates and fats (1, 15). Malnutrition due to insufficient intake of macronutrients significantly impairs immune cell function (16). Dietary restriction results in reduced neutrophil trafficking to sites of inflammation, due to reduced integrin expression and chemokine production (17). Interestingly, even brief nutritional replenishment or provision of a single nutrient such as glutamine or arginine is able to restore lost cellular function (17-19). Clinically, malnutrition is associated with more frequent and chronic infections (16, 20) leading to increased

morbidity and hospitalization. Malnourished patents must often undergo nutritional rehabilitation in order to control infections (21). Based on these findings, immunosuppression as a result of malnourishment has become known as nutritionally acquired immunodeficiency. Given that there are an estimated 795 million chronically undernourished people (22), many of whom are likely to develop nutritionally-acquired immunodeficiencies, nutritional immunology remains an important area of research.

1.2 REGULATION OF INNATE IMMUNE CELL FUNCTION THROUGH MODULATION OF CELLULAR METABOLISM

The immune system is bioenergetically demanding. Immune cells specifically require a significant amount of energy and biosynthetic precursors in order to carry out their numerous effector functions that protect the host during infections and maintain homeostasis (23). It is estimated that when quiescent, the immune system requires 1600 kJ/day for leukocyte housekeeping activities. This increases to 2100 kJ/day upon moderate activation of leukocytes, while strong activation can increase it to 3000 kJ/day (24). Immune cells rely on glucose, glutamine and fatty acids, taken up from the microenvironment, for the generation of energy and the biosynthetic precursors required to carry out their effector functions (25-29). These nutrients are metabolized through glycolysis, the tricarboxylic acid (TCA) cycle, oxidative phosphorylation and the pentose phosphate pathway (23, 30). Withdrawal of nutrients or inhibition of these metabolic pathways suppresses immune cell survival and function (31-39). Thus, immune cell survival and activity is tightly coupled to nutrient availability and cellular metabolism.

Under aerobic conditions, oxidative metabolism is the most efficient pathway for generating ATP (23). However, cells can also utilize aerobic glycolysis for energy generation even though it is significantly less efficient (40). Glycolysis, the pentose phosphate pathway and the TCA cycle also provide the metabolic intermediates required for the synthesis of nucleotides, fatty acids and amino acids (41). In mammalian cells, the flux through these pathways is regulated by external signals. With respect to immune cells, these are the same signals that initiate specific immune cell responses, such as growth factors, cytokines and infectious agents. These external signals interact with cellular receptors such as cytokine receptors and Toll-like receptors (TLRs), activating signaling pathways upstream of metabolic regulators such as phosphatidylinositol-3-kinase (PI3K), protein kinase B (AKT) and mammalian target of rapamycin (mTOR) (23, 37, 42). By linking metabolism and immune cell effector responses in

this way, the cell is guaranteed to have the metabolic requirements to carry out its effector functions. Significant research in nutritional immunology now focuses on how the activation of immune cells by these external signals induces metabolic reprogramming, and how this reprogramming regulates cellular activity.

Macrophages and dendritic cells are key effectors of the innate immune response. Classical activation of macrophages (M1) by pathogens and host danger signals results in a pro-inflammatory response that includes the production of pro-inflammatory mediators and the recruitment and activation of various cells, while the activation of dendritic cells leads to the activation of the adaptive immune response (25, 43). The ability to develop this pro-inflammatory phenotype is dependent on metabolic reprogramming. Resting macrophages and dendritic cells predominantly utilize oxidative metabolism, generating ATP from oxidative phosphorylation (30, 37). The activation of macrophages and dendritic cells by pro-inflammatory mediators induces a metabolic shift that upregulates glucose uptake and glycolytic flux while suppressing oxidative phosphorylation (30, 36-38, 44, 45). This metabolic phenotype is reminiscent of the Warburg effect, a term used to describe the metabolic reprogramming that occurs in tumors (40). There are several mechanisms by which this metabolic reprogramming occurs (46). Production of nitric oxide (NO) by inducible nitric oxide synthase (47, 48) suppresses oxidative phosphorylation though nitrosylation of the electron transport chain complexes (49). The PI3K/AKT pathway also appears to be essential for inducing aerobic glycolysis (37). This is partly a result of its ability to activate mTOR, which leads to the activation of the downstream transcription factor, hypoxia inducible factor (HIF)-1α (50, 51). HIF-1α induces the production of several glycolytic genes including 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) (52). PFKFB3 is a glycolytic enzyme that is a major regulator of glycolytic flux through regulation of the synthesis and degradation of the glycolytic intermediate, fructose-2,6-bisphosphate (53). Fructose-2,6-bisphosphate increases glycolytic flux by activating 6-phosphofructo-1-kinase (54). Because the PFKFB3 isoform has the highest kinase:phosphatase activity ratio, resulting in greater fructose-2,6-bisphosphate synthesis, upregulation of PFKFB3 induces a substantial increase glycolytic flux (53). In addition, early (<6 hours) induction of aerobic glycolysis by dendritic cells is dependent upon the activity of TANK-binding protein 1 (TBK1), I-kappa-B kinase epsilon (IKKε) and AKT (36). It is unknown whether this signalling pathway is also utilized by macrophages.

The increase in glycolytic flux is associated with an accumulation of TCA cycle intermediates. Although some glucose does still feed into this pathway, metabolism of glutamine

through glutaminolysis and the γ-aminobutyric acid shunt is largely responsible for sustaining the TCA cycle (38). At least two TCA cycle intermediates have been identified as being essential to pro-inflammatory responses. Succinate stabilizes HIF-1α, inducing the expression of several pro-inflammatory mediators and glycolytic enzymes thus reinforcing the increase in glycolysis (38). Citrate is used in *de novo* generation of fatty acids, which are required for the production of pro-inflammatory lipid mediators as well as the biogenesis of the endoplasmic reticulum (ER) and golgi membranes (36). Membrane biogenesis is essential to meet the increased need for protein production and secretion involved in pro-inflammatory responses. The pentose phosphate pathway, which is driven by glycolysis, is also significantly upregulated in pro-inflammatory macrophages and dendritic cells (36, 38, 45). The metabolites produced by this pathway, ribulose-5-phosphate and nicotinamide adenine dinucleotide phosphate (NADPH), are essential for de novo synthesis of nucleic acids and lipids as well as for maintaining the cellular redox state. Inhibition of this pathway in lipopolysaccharide (LPS)-stimulated dendritic cells prevents lipid accumulation and the expression of pro-inflammatory cytokines (36). In addition to suppressing pro-inflammatory cytokine production, inhibiting the pentose phosphate pathway in macrophages also suppresses M1 polarization (45).

External stimuli also regulate the expression and activation state of different glycolytic enzyme isoforms. This can have a major impact on the polarization of macrophages. Activation of the M2 isoform of the glycolytic enzyme pyruvate kinase can suppress LPS-induced increases to glycolysis and pro-inflammatory mediators while inducing traits associated with the alternatively activated (M2) phenotype. In contrast, the inactive form of pyruvate kinase M2 drives HIF-1α activity, inducing pro-inflammatory responses and enhancing glycolysis (55). It should come as no surprise that inhibition of glycolysis or glutamine metabolism suppresses macrophage and dendritic cell survival, activation, differentiation, and pro-inflammatory responses (30, 36-38, 56-58).

Cytokines such as interleukin (IL)-4 induce the alternative activation of macrophages (M2) resulting in a more anti-inflammatory phenotype that aids in the resolution of parasitic infections, suppresses inflammatory responses and promotes wound healing (25). M2 macrophages have a metabolic phenotype that is similar to that of resting cells but distinct from classically activated (M1) macrophages (30). They predominantly utilize fatty acid oxidation and oxidative metabolism (30, 59). Conversely, inhibition of oxidative phosphorylation prevents the development of the M2 phenotype. Vats et al. (59) found that the metabolic reprogramming associated with the M2 phenotype is dependent on signal transducer and activator of

transcription 6 (STAT6), which induces the expression of genes essential for β-oxidation of fatty acids and mitochondrial oxidation, as well as expression of peroxisome proliferator-activated receptor (PPAR) family members that sustain this metabolic phenotype.

1.3 DISRUPTION OF THE IMMUNE RESPONSE BY NUTRIENT EXCESS

It is now believed that nutrient excess drives the chronic inflammation seen in many metabolic disorders, including type-2 diabetes and atherosclerosis, through the activation of stress responses in the cells of peripheral metabolic tissues. This leads to the production of pro-inflammatory mediators that activate macrophages and promote systemic propagation of the inflammatory response. Acute induction of hyperglycemia in healthy individuals and stimulation of monocytes with high glucose levels *in vitro* induces interleukin (IL)-6, IL-18 and tumor necrosis factor (TNF)-α production (60, 61). Similarly, excess extracellular and intracellular free fatty acids and triglycerides trigger ER stress responses and/or TLR activation (e.g. TLR-2 and -4) in adipocytes and macrophages (62-65). Stimulation of these pathways then activates NF-κB and leads to the production of pro-inflammatory mediators including resistin and monocyte chemoattractant protein (MCP)-1 in adipocytes and interferon γ-inducible protein (IP)-10 in macrophages (65-68).

This link between metabolic disorders and chronic inflammation has also been characterized quite effectively using systems biology techniques, such as meta-analysis of transcriptomic studies. Data sets from multiple experiments examining related phenomena can be integrated and meta-analysis performed to identify genes that behave similarly or differently across the data sets (e.g. (69-73)). If the data sets are from very similar experiments, it allows for the reduction of false positive or negative results within a study. Applying this method to more diverse studies allows for the identification of collections (termed clusters) of genes representing common pathways, ontologies (functional classifications), transcription factor targets, etc., that are commonly or differentially important under varying conditions (e.g. in the presence of an infection or nutritional deprivation) or in multiple pathophysiologies (e.g. comparing asthma or cystic fibrosis as inflammatory lung conditions). From these clusters functional and mechanistic information can be inferred.

A meta-analysis of peripheral blood gene expression profiles of type 2 diabetes, coronary artery disease and their precursor state, metabolic syndrome, was recently completed by comparing data sets from each disease state to control data sets and to each other (69). Metabolic syndrome refers to a constellation of factors, including central obesity, hypertension and

hyperglycemia, which can increase the risk for development of type 2 diabetes, coronary artery disease and stroke. Thus, differences in gene expression between the two disease states and the precursor metabolic syndrome might provide insights into the unique mechanistic features associated with disease progression. The progression from metabolic syndrome to cardiovascular disease was characterized by an increased number of genes associated with macrophage signalling and activation. Conversely, metabolic syndrome and type 2 diabetes were closely related but there was an increase in type 2 diabetes in the expression of genes associated with T-lymphocyte signalling and proliferation.

While the above study demonstrated the utility of meta-analysis, it required the completion of large numbers of microarrays, making studies like these prohibitive. There are several open access repositories, including Array Express and Gene Expression Omnibus (GEO), that house transcriptional expression data from numerous species, conditions and platforms. Meta-analysis tools that can utilize these datasets serve to minimize the requirement of resources for clinically-relevant genomic studies, while maximizing the utility of generated data (72). Gene expression profiles of obesity and type 2 diabetes obtained from multiple experiments (70, 71) were compared using MetaGEX (http://marray.cmdr.ubc.ca/metagex/), a meta-analysis tool developed in our lab. Preliminary analysis of genes differentially expressed in both disease states revealed a large number of dysregulated genes associated with immunity and inflammation, including cytokines/chemokines and their receptors, complement components, interferon-associated molecules and cell surface receptors (**Table 1.1**).

Table 1.1: Selection of immunologically relevant genes differentially expressed in both obesity and diabetes identified using MetaGEX

Gene ID[a]	Gene name	Obese vs. Lean Control P-value		Diabetes vs. Control P-value		
		Omental Obese GSE15524	Sub-cutaneous Obese GSE15524	Type 1 Diabetes GSE9006	Type 2 Diabetes GSE9006	Diabetes mellitus GSE16415
Cytokine/chemokine and receptors						
3552	Interleukin (IL)1α	–[b]	3.83E-02	2.00E-06	1.45E-03	–
3554	IL1R1 [IL1 receptor, type I]	4.60E-02	–	3.56E-02	1.52E-02	–
7850	IL1R2 [IL1 receptor, type II]	6.29E-03	–	1.37E-03	–	–
3598	IL13RA2 [IL13 receptor α2]	2.06E-02	–	–	4.57E-02	–
6363	Chemokine CCL19	3.98E-03	–	–	–	1.09E-02
3579	Chemokine receptor CXCR2	4.62E-02	–	3.61E-03	–	–
7852	Chemokine receptor CXCR4	1.21E-02	–	–	1.41E-03	–
Complement components and receptors						
708	Complement component binding protein C1QBP	2.95E-05	–	–	5.82E-06	–

Gene ID[a]	Gene name	Obese vs. Lean Control P-value		Diabetes vs. Control P-value		
		Omental Obese GSE15524	Sub-cutaneous Obese GSE15524	Type 1 Diabetes GSE9006	Type 2 Diabetes GSE9006	Diabetes mellitus GSE16415
Complement components and receptors (continued)						
713	Complement component C1QB	2.71E-02	–	–	–	1.40E-02
730	Complement component C7	7.20E-04	–	–	1.40E-02	–
1380	Complement receptor CR2	3.42E-03	–	3.06E-04	–	–
Interferon-associated molecules						
3454	IFNAR1	9.98E-04	–	–	1.69E-02	–
3460	Interferon-γ receptor IFNGR2	3.27E-02	–	3.06E-03	1.24E-03	–
3662	Interferon regulatory factor IRF4	1.13E-02	–	7.88E-03	–	–
10581	Interferon induced IFITM2	6.62E-03	–	9.74E-03	1.67E-02	1.21E-03
10561	Interferon induced IFI44	3.02E-02	–	2.09E-02	1.66E-02	–
10964	Interferon induced IFI44L	2.46E-02	–	2.41E-04	–	–
Cell surface molecules/receptors						
958	CD40, TNF receptor superfamily	2.76E-02	–	–	2.25E-02	–
4481	Macrophage scavenger receptor MSR1	3.21E-02	–	2.40E-02	–	–
3111	HLA-DOA	1.40E-02	–	2.20E-03	–	–
3123	HLA-DRB1	1.38E-02	–	–	–	1.53E-02
3133	HLA-E	1.80E-02	–	3.20E-02	–	–
3135	HLA-G	3.04E-02	–	–	3.09E-03	–
Transcription factors and signaling components						
5465	PPARA [peroxisome proliferator-activated receptor-α]	2.56E-02	–	–	–	4.31E-02
5871	MAP4K2 [MAP kinase kinase kinase kinase]	5.95E-03	–	–	3.11E-02	–
7786	MAP3K12 [MAP kinase kinase kinase]	8.28E-04	–	–	–	3.03E-02
8649	MAPKSP1 [MAPK scaffold protein 1]	1.41E-02	–	–	3.62E-02	–
6772	Transcription factor STAT1	3.21E-02	–	–	3.95E-03	–
7022	Transcription factor TFAP2C	4.56E-04	–	–	–	3.97E-02
10010	TANK [TRAF family member-associated NFKB activator]	4.56E-02	–	–	2.35E-03	–

a. Gene ID indexed NCBI gene: http://www.ncbi.nlm.nih.gov/gene; Data sets indexed in Gene Expression omnibus (GEO): http://www.ncbi.nlm.nih.gov/geo/
b. – signifies not significant (P > 0.05)

In addition, analysis of the over-represented biological processes identified pathways associated with leukocyte/lymphocyte differentiation and activation, initiation of cell signalling, the NF-κB signalling cascade and wound healing. While further work is needed to confirm the significance of these findings, this analysis supports the link between nutrition, metabolic disorders and immunity. Based on these and other studies, it is evident that elevated levels of nutrients such as glucose and lipids may be an underlying cause of the chronic inflammation

observed in the various metabolic disorders.

1.4 HYPERLIPIDEMIA, INFLAMMATION AND ATHEROSCLEROSIS

Cardiovascular disease accounts for approximately 17 million deaths a year. One of the most common underlying causes of cardiovascular disease, atherosclerosis, is promoted by hyperlipidemia (74). Atherosclerosis is an excellent example of how excess nutrients drive aberrant immune cell metabolism and pro-inflammatory responses. During the development of atherosclerosis, apolipoprotein (Apo)B-containing lipoproteins, such as low-density lipoprotein (LDL) particles, are retained in the arterial wall and undergo modifications including oxidation, enzymatic cleavage and aggregation (75). The modified LDL particles then bind to receptors on the surface of endothelial cells, e.g. scavenger receptors, causing endothelial cells to upregulate expression of cell adhesion molecules and chemokines (75, 76). This results in the recruitment of monocytes to the arterial wall, where they differentiate into macrophages, likely as a mechanism to clear the pro-inflammatory oxidized lipoproteins (76).

However, the macrophages begin to contribute to the pathology of atherosclerosis as they accumulate and take up increasing numbers of lipoprotein particles. The macrophages take up excessive amounts of both native and modified forms of lipoproteins through scavenger receptors, macropinocytosis and phagocytosis (75). The substantial increase in intracellular cholesterol as a result of lipoprotein uptake also suppresses the intracellular cholesterol efflux pathway (77). This leads to unwarranted accumulation and storage of cholesterol in macrophages and induction of pro-inflammatory responses. These lipid-laden macrophages, known as foam cells because of the foamy appearance they develop as a result of excessive cholesterol storage, are a major component of atherosclerotic plaques (75).

Excessive extracellular and intracellular cholesterol induces pro-inflammatory responses through a variety of mechanisms. Extracellular cholesterol, in the form of modified LDL can activate TLR2 and TLR4 leading to the activation of transcription factors such as NF-κB and production of pro-inflammatory mediators (78, 79). Activation of TLRs can also suppress cholesterol efflux, exacerbating the accumulation of intracellular cholesterol (80). Intracellular cholesterol crystals have been shown to activate the inflammasome via Nod-like receptor protein (NLRP)3 resulting in the production of IL-1β (81). Intracellular cholesterol can also accumulate in the lipid rafts of the plasma membrane, rendering TLRs hypersensitive to agonists, or in the ER membrane, which can trigger ER stress and eventually apoptosis (82, 83). The apoptotic cells are not properly cleared and undergo secondary necrosis, releasing cellular components that can

also induce pro-inflammatory responses by nearby cells. Production of these pro-inflammatory mediators as well as the accumulation of dead cells and debris recruits more macrophages as well as neutrophils, dendritic cells and T lymphocytes to the site, leading to plaque enlargement (84).

One pathway that can mitigate the effects of excess lipids is the reverse cholesterol transport pathway. This is initiated at the cellular level by efflux of intracellular cholesterol through the ATP binding cassette (ABC) transporters ABCG1 and ABCA1 to mature high-density lipoprotein (HDL) particles and lipid-free or lipid-poor pre-β-HDL, respectively (85). The lipoproteins re-enter the circulation, migrating to the liver. The liver takes up the cholesterol and converts it to bile acids, which are excreted into the intestine in bile (86). Promoting cellular cholesterol efflux has been shown to suppress pro-inflammatory responses in a variety of ways including the inhibition of TLR mediated inflammation by reducing TLR association with lipid rafts (87). Reverse cholesterol transport has become a major therapeutic target for the treatment of atherosclerosis and cardiovascular disease (88). One of the methods by which researchers have attempted to enhance this pathway is through the generation of peptide mimetics of ApoA1, the major protein component and cholesterol acceptor of HDL (89, 90). These peptides were designed based on the physical properties of the apoA1 amphipathic α-helical domains rather than specific amino acid sequences. The result was a collection of peptides consisting of one or more short amphipathic α-helices (91). Like their parent protein, these peptides are capable of stimulating cellular cholesterol efflux and reverse cholesterol transport (89, 90) (**Figure 1.1, over page**). In addition, these peptides were found to be strong immunomodulators both *in vitro* and *in vivo*, modulating inflammatory signalling pathways, chemotaxis and macrophage differentiation, inducing anti-inflammatory responses and suppressing of inflammatory responses induced by bacterial components and in murine models of asthma (92-97).

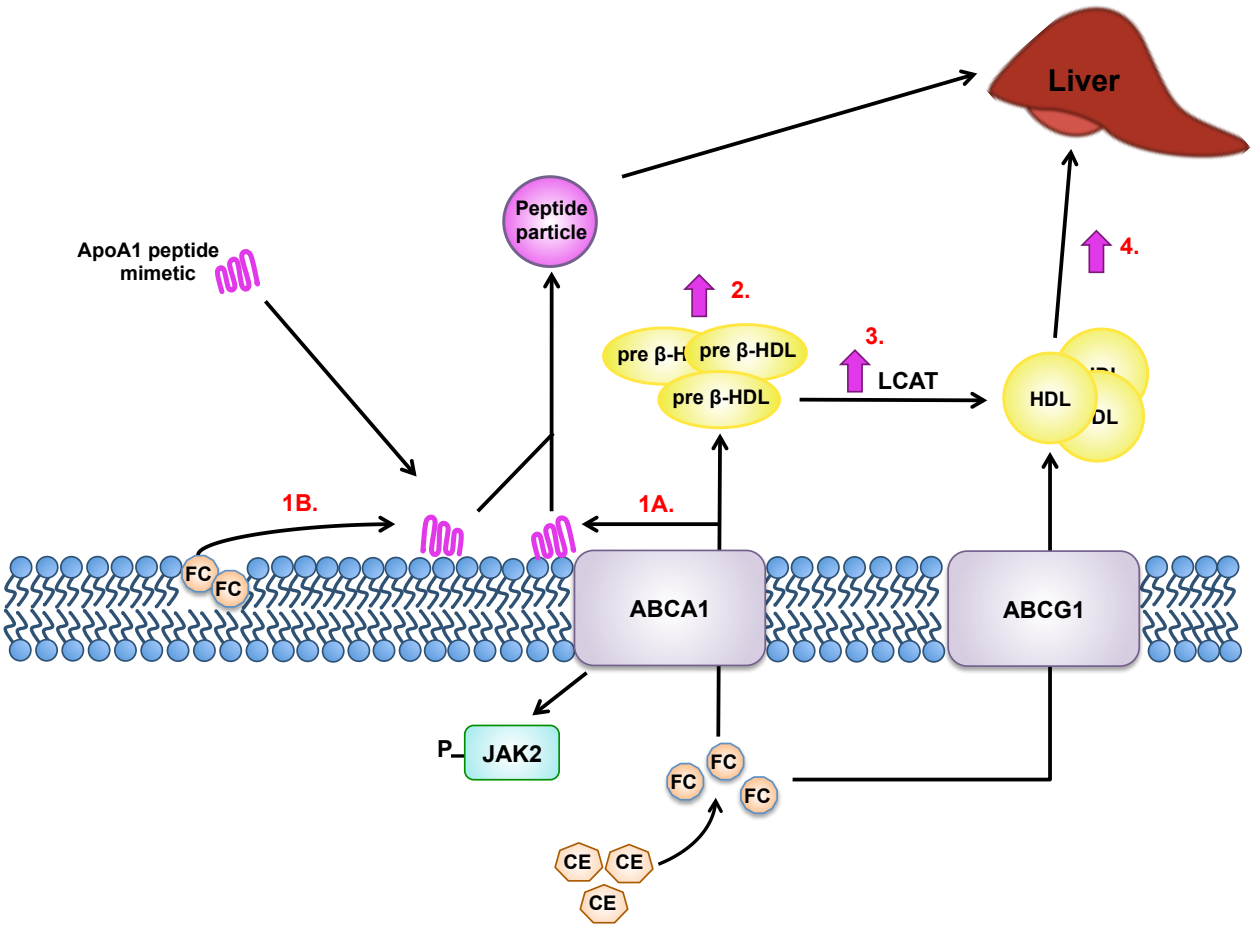

Figure 1.1: Proposed mechanisms for induction of cholesterol efflux and reverse cholesterol transport by ApoA1 peptide mimetics. Multiple mechanisms have been proposed for ApoA1 peptide mimetic mediated cholesterol efflux and reverse cholesterol transport. ApoA1 peptide mimetics can induce cholesterol (and phospholipid) efflux directly through binding to and microsolublization of the plasma membrane of cells. This can occur in an ABCA1-dependent (1A) or independent (1B) manner, both of which depend on peptides possessing high lipid affinity. The peptide/lipid particles produced are transported to and taken up by the liver. Cholesterol efflux through the ABCA1-dependent pathway requires activation of JAK2 by the peptide mimetics. In addition, ApoA1 peptide mimetics enhance cholesterol efflux by binding to and stabilizing the rapidly degraded ABCA1. ApoA1 peptide mimetics can also improve cholesterol efflux and reverse cholesterol efflux indirectly by increasing pre β-HDL levels (2), enhancing LCAT activity and HDL maturation (3), and promoting HDL-mediated cholesterol delivery to the liver (4).

1.5 ENDOPLASMIC RETICULUM STRESS AND THE UNFOLDED PROTEIN RESPONSE

Another important cellular pathway linked to both cellular metabolism and the innate immune response is the unfolded protein response (UPR) (98, 99). The main function of the UPR is to alleviate ER stress induced by the accumulation of misfolded proteins, by promoting

proper protein folding, inhibiting translation of proteins and, if necessary, activating protein degradation pathways (100, 101). Three stress-sensing signalling pathways make up the UPR defined by the transmembrane sensors that activate them, namely the activating transcription factor (ATF)-6, protein kinase RNA-like endoplasmic reticulum kinase (PERK) and inositol-requiring enzyme 1α (IRE1α) pathways (100). The relationship between ER stress, the UPR, metabolism and inflammation is extremely complex. As mentioned above, ER stress and the UPR are upregulated in many metabolic disorders (102). Upregulation of ER stress leads to leptin and insulin resistance observed in type-2 diabetes (62, 63). In the case of insulin resistance, this is mediated in part by hyperactivation of c-Jun N-terminal kinase (JNK) by the IRE1α pathway. Since JNK is also a major regulator of inflammatory responses, it is possible that activation of ER stress is an underlying mechanism behind the chronic inflammation observed in metabolic disorders like obesity and type-2 diabetes (62). Activation of ER stress has also been shown to promote the development of foam cells through the upregulation of the scavenger receptor, CD36 (103). Erbay et al. (104) showed that inhibition of ER stress in macrophages exposed to high levels of toxic lipids, such as oxidized LDL, is protective against the development of atherosclerosis. Once again, these studies indicate that ER stress is a major underlying pathway in the development of metabolic disorders. However, activation of ER stress has also been reported to suppress LPS-induced production of pro-inflammatory cytokines, prevent macrophage differentiation and promote apoptosis (105). This may be due in part to ER stress-mediated suppression of glucose uptake and glycolysis, both of which are essential for immune cells to mount an effective pro-inflammatory response (23, 106).

All three arms of the UPR have been shown to directly regulate the pro-inflammatory responses of immune cells (99). The IRE1α pathway, via activation of the transcription factor X-box binding protein (XBP)-1 is a positive regulator of TLR-driven inflammatory responses in macrophages (107, 108). XBP-1 binds to the promoter region of several pro-inflammatory mediators, including IL-6, inducing their transcription (108). In contrast, the PERK pathway appears to be a negative regulator of inflammation since it is specifically inhibited by TLR signalling while exogenous activation of PERK pathway components inhibits TLR-driven inflammatory responses (101, 109, 110). Activation of the PERK pathway has also been found to be protective in a mouse model of the auto-immune disease, multiple sclerosis (111). Although there is a clear link between the pathways of the UPR and inflammation, the reason for this strong connection remains to be fully elucidated and is an area of active research.

1.6 SMALL MOLECULE REGULATORS OF THE IMMUNE RESPONSE - HOST DEFENCE PEPTIDES

In order to mount an effective immune response that is appropriate for the particular insult encountered, cells of the immune system rely on external signals to induce metabolic reprogramming and activate their effector responses (23). Many of the host-derived signals that have been studied are growth factors and cytokines. However, as mentioned above, small molecules such as ApoA1 peptide mimetics are also capable of regulating immune cell metabolism and activity. There has been significant interest in the ability of host peptides and their synthetic derivatives to manifest pleiotropic activities associated with either metabolism or the immune system, and regulate both processes.

Host defence peptides (HDPs) are found in almost all complex organisms and are a major component of the human innate immune system. HDPs are generally between 12 and 50 amino acids in length, of which 2-9 residues are positively charged and approximately 50% are hydrophobic. This unique make-up allows HDPs to fold into amphipathic secondary structures with both hydrophilic and hydrophobic patches (112). Classification of HDPs is difficult owing to their enormous sequence diversity, however folded peptides fall into one of four structural groups: β-sheets (for example human α- and β-defensins), α-helical (for example the human cathelicidin LL-37), extended structures (for example bovine indolicidin) and looped peptides (for example bovine bactenecin) (113, 114). Expression of HDPs is co-ordinately regulated at both the transcriptional and translational level, allowing multiple HDPs to be produced at a single body site. Gene expression and protein secretion of HDPs depends on the species, tissue and cell type and stage of differentiation. In mammals, numerous cell types including neutrophils, monocytes, T- and B-lymphocytes and epithelial cells produce both cathelicidins and defensins. Expression and secretion of an HDP can be constitutive or induced by specific stimuli such as pro-inflammatory cytokines or microbial agents (115). For example, human keratinocytes constitutively express the β-defensin hBD-1, but hBD-2 and hBD-4 are induced by stimulation of Toll-like receptors (TLR) or pro-inflammatory cytokines (116). The expression of the human cathelicidin, LL-37, in leukocytes and epithelial cells is induced by inflammatory stimuli, and histone modification and vitamin D both play an important role in this process (117-121). In addition to vitamin D, recent evidence suggests that cathelicidin expression is regulated by the transcription factor Hypoxia Inducible Factor-1α (HIF-1α) (122-125).

Once translated, HDPs exist in an inactive pro-form that can be stored at high

concentrations in intracellular granules. Human neutrophils store β-defensins and LL-37 within their granules, releasing them into phagosomes or secreting them extracellularly upon activation, e.g. at the site of infections. HDPs must be proteolytically cleaved to release the mature, biologically active C-terminal domain (126, 127).

HDPs were classically associated with direct antimicrobial killing, however this activity is significantly suppressed under *in vivo* conditions such as high salt concentrations and the presence of negatively charged serum components like glucosaminoglycans and apolipoproteins (128, 129). In contrast, the pleiotropic immunomodulatory activities of HDPs (**Figure 1.2)** appear to be unaffected by high salt concentrations and serum components (130, 131). HDPs recruit immune effector cells to sites of inflammation. Certain human α- and β-defensins and LL-37 can act as chemotactic agents for various cells including monocytes, macrophages, neutrophils and/or T cells. This is mediated in part by their ability to stimulate production of chemokines and cytokines such as IP-10, MCP-1, macrophage inflammatory protein (MIP)-3α, IL-6, and IL-10 by a variety of cells including keratinocytes and monocytes although direct chemoattractant ability is known for some HDP (132-136).

HDPs also suppress pro-inflammatory responses induced by TLR activation of neutrophils and monocytes (137-139). Neutrophils stimulated with LPS or Gram-negative bacteria in the presence of LL-37 exhibit reduced secretion of TNF-α, IL-1β, IL-6, and IL-8 (140). The reduction in TNF-α is partly due to modulation of downstream signaling pathways and partly due to binding of LL-37 to both LPS and CD14 blocking activation through TLR4 (141-144). Interestingly, while LL-37 and the α-defensin, HNP-1, inhibit *N. meningitidis* lipo-oligosaccharide-stimulated reactive nitrogen species production by macrophages, they enhance endotoxin-induced reactive oxygen species (ROS) production (140, 145-147), consistent with pathway modulation.

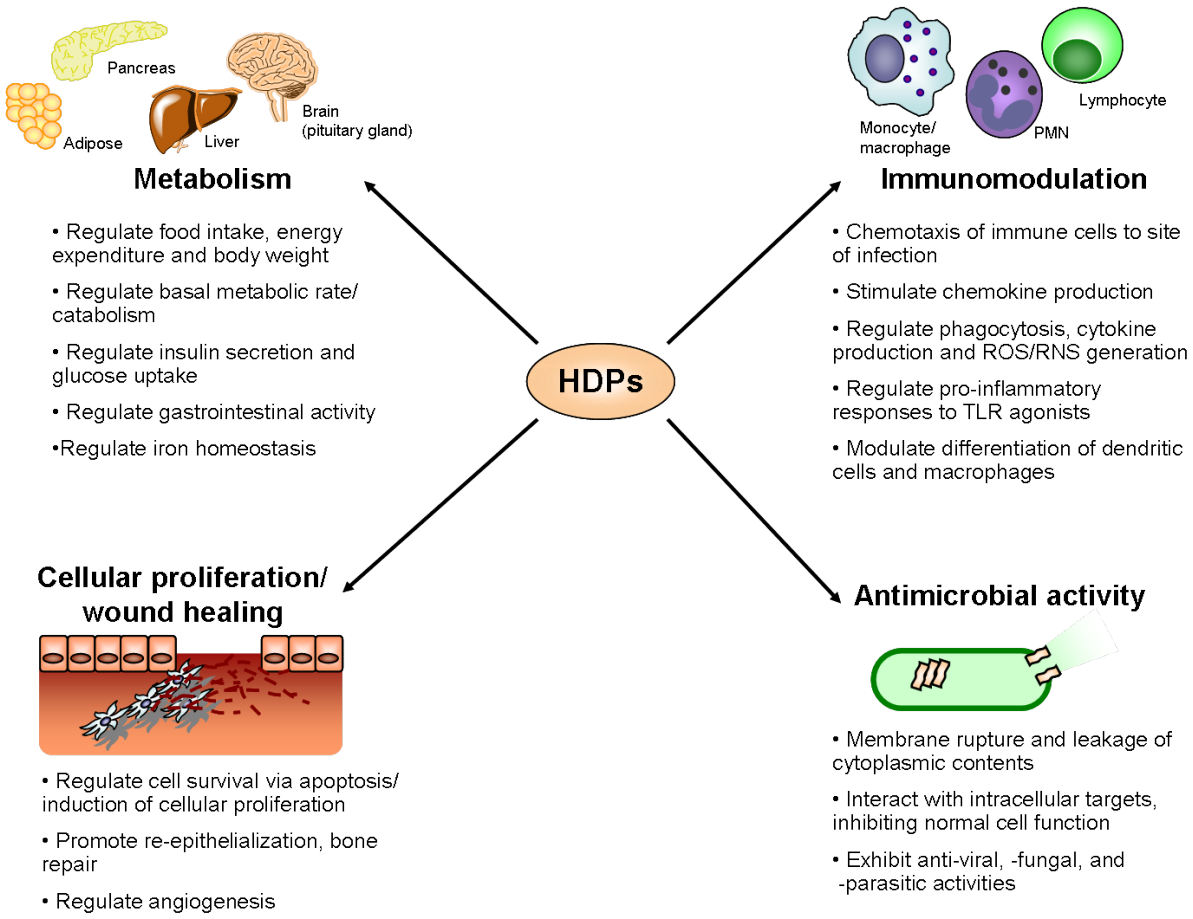

Figure 1.2: The biological functions of host defence peptides

In addition to the activities described above, several novel immunomodulatory functions have been attributed to HDPs. Cathelicidins and defensins significantly enhance wound repair and angiogenesis (148, 149). Many of the studies on wound repair and angiogenesis have focused on the cathelicidins human LL-37 and porcine PR-39, which are highly expressed in wound beds and are involved in re-epithelialization and neovascularization through distinct mechanisms (150). LL-37 induces keratinocyte proliferation and migration by inducing the release of active epidermal growth factor receptor ligands (151, 152). PR-39 induces expression of syndecans, cell surface heparin sulfate proteoglycans that regulate cellular proliferation and migration in response to heparin-binding growth factors (153). LL-37 directly activates formyl peptide receptor like-1 (FPR2) on endothelial cells, while PR-39 induces endothelial cells to express vascular endothelial growth factor and its receptor through stabilization and activation of the transcription factor HIF-1α. The actions of LL-37 and PR-39 both result in cellular proliferation and formation of vascular structures (154, 155).

The role of defensins in wound healing and angiogenesis *in vivo* is being increasingly appreciated. In animal models of wound healing, up-regulation of hBD-2 and -3 expression in

the wound bed is associated with accelerated wound closure (148, 149). *In vitro* studies on intestinal wound healing suggest that hBD-2 induces migration of intestinal epithelial cells by signalling through the chemokine receptor CCR6, preventing apoptosis and up-regulating expression of mucins 2 and 3, which are essential to the protective barrier between epithelial cells and the intestinal lumen (156, 157). hBD-2, -3 and -4 have also been shown to act in a similar manner to LL-37, inducing keratinocyte migration and proliferation, through activation of epidermal growth factor receptor and production cytokines/chemokines (136). *In vitro,* hBD-2 is also capable of inducing endothelial cell proliferation, migration and vascular structure formation, three processes essential to angiogenesis, although the underlying mechanisms and its relevance *in vivo* remains unclear (158). α-Defensins have the opposite effect on neo-vascularization, preventing *in vivo* neo-vascularization in the chicken chorioallantoic membrane assay. *In vitro* they were found to inhibit vascular endothelial growth factor-induced migration and adhesion of endothelial cells to fibronectin, as well as their proliferation and survival (159). In the murine model of hypoxia-induced retinal angiogenesis, local and systemic administration of α-defensins was able to reduce neo-vascularization by over 40% (160). This property of α-defensins is therefore being studied as a possible treatment for pathologic retinal neo-vascularization, such as that observed in diabetes.

Cationic peptide hormones and neuropeptides also exhibit extensive immunomodulatory activities and should be considered members of the HDP family of peptides (**Table 1.2**). For example, α-melanocyte-stimulating hormone (α-MSH), a short, cationic α-helical neuropeptide which regulates melatonin deposition and metabolism also possesses direct antimicrobial activity as well as significant immunomodulatory activity both *in vivo* and *in vitro* (161). This peptide has exhibited antifungal and antibacterial activity (162) even in the presence of physiological salt concentrations (163). With respect to its immunomodulatory activity, α-MSH suppresses chemotaxis and the production of pro-inflammatory cytokines, lipid mediators and reactive oxygen and nitrogen species as well as upregulates production of anti-inflammatory cytokines and modulates T lymphocytes activity (161, 164, 165).

Table 1.2: Cationic peptide hormones and neuropeptides that regulate metabolism and exhibit immunomodulatory activity

Cationic Peptide Hormones & Neuro-peptides	Metabolic Regulation	Immunomodulatory Activity	Refs
Leptin	• Decreases food intake; • Increases basal metabolism/catabolism under nutrient rich conditions	• Activates macrophages: promotes phagocytosis and leukotriene B4, nitric oxide and pro-inflammatory cytokine production; • Increases chemotaxis of neutrophils and lymphocytes; • Promotes Th-1 responses	(166) (167) (168)
Ghrelin	• Energy homeostasis and weight regulation • Stimulates appetite/food intake • Decreases insulin secretion • Promotes gastrointestinal motility	• Anti-inflammatory; • Inhibits pro-inflammatory cytokine release; • Protective in many inflammatory disease models	(169)
Vasoactive Intestinal Peptide (Pituitary adenylate cyclase-activating polypeptide -27 and -38)	• Stimulates insulin and glucagon secretion • Regulates energy and lipid metabolism as well as body weight • Regulate gastrointestinal function	• Cytoprotective • Extensive modulation of innate and adaptive immune effector cell functions • Promotes mast cell degranulation and pro-inflammatory cytokine production • Reduce cell recruitment • Inhibits production of pro-inflammatory cytokines/chemokines and ROS/RNS by macrophages • Promotes anti-inflammatory cytokine production • Promote Th-2 responses	(170) (171) (172)
Hepcidin	• Iron homeostasis • Inhibits intestinal iron absorption • Promotes iron sequesterization in macrophages/liver	• Expression is induced by inflammatory stimuli • Diminishes iron availability for bacteria and tumors • Inflammation-induced hypoferremia leads to anemia • Type II acute-phase protein	(173)

Cationic Peptide Hormones & Neuro-peptides	Metabolic Regulation	Immunomodulatory Activity	Refs
Adreno-medullin (natriuretic peptide)	• Natriuretic/diuretic • Decreases salt appetite • Inhibits insulin secretion • Increases circulating glucose levels	• Vasodilator • Inhibits production of pro-inflammatory cytokines/chemokines • Promotes anti-inflammatory cytokine production • Induces regulatory T lymphocytes	(174) (175) (176)
Cortistatin-17/ Somato-statin	• Inhibit insulin and Ghrelin secretion	• Inhibits production of pro-inflammatory cytokines/chemokines • Promotes anti-inflammatory cytokine production • Induces regulatory T lymphocytes	(177) (176)
MSH α- and γ-	• Reduces food intake • Increases basal metabolic rate	• Cytoprotective • Inhibits production of pro-inflammatory cytokines, lipid mediators and ROS/RNS • Suppresses chemotaxis • Enhances anti-inflammatory activity of leukocytes • Activates T regulatory lymphocytes	(178)
Substance P	• Regulates adipose tissue responses • Inhibits insulin-mediated glucose uptake by adipocytes	• Modulates inflammatory responses in monocytes/macrophages via differential activation of neurokinin 1 receptor isoforms • Proliferative effect on cells of myeloid lineage • Modulation of chemokine-induced responses in monocytes	(179) (180) (181)

Given their extensive activities (**Figure. 1.2**), HDPs have become attractive candidates for the development of novel therapeutics for a variety of inflammatory conditions such as infection, autoimmune disease and cancer. Innate defence regulator (IDR) peptides, synthetic derivatives of HDPs were developed with more potent immunomodulatory activity in an effort to generate new therapeutics that modulate the immune system.

1.7 INNATE DEFENCE REGULATOR (IDR) PEPTIDES

IDR peptides have been developed using a variety of rational and semi-random drug design techniques (182). Many of the peptides developed not only have improved immunomodulatory activity, but also exhibit less toxicity than HDPs. The amino acid sequences of natural peptides are used as templates and subjected to amino acid substitutions, sequence

scrambling and truncation in order to develop large libraries of novel peptide sequences. For example, synthetic derivatives of α-MSH were generated through peptide truncation in order to identify the minimum sequence required for antimicrobial and immunomodulatory activity. The C-terminal tripeptide, KPV was identified as the mediator of the antimicrobial and immunomodulatory activities of α-MSH (163, 183). Synthetic derivatives of α-MSH, based on this tripeptide exhibit even greater antimicrobial and immunomodulatory activity than the parent peptide both *in vitro* and *in vivo* (184-187). As a result of their potent anti-infective activities, α-MSH and its synthetic derivatives have become attractive therapeutic candidates for the treatment of numerous inflammatory conditions associated with the brain and peripheral organs (161). Quantitative structure activity relationship modeling is also used to design peptides with the structural characteristics required for specific activities (182, 188). Regardless of the method used to generate the synthetic peptides, all the peptides generated must be screened *in vitro* to determine whether they possess immunomodulatory activity, and so, to what degree. Screening for immunomodulatory activity is difficult because of the number of different cell types the peptides act on and the variety of responses they elicit. Therefore, specific markers of activity have to be used such as the ability to induce chemokine production and ability to suppress TLR agonist-induced pro-inflammatory cytokine production (141, 142, 188). This is an iterative process since the synthetic peptides with enhanced immunomodulatory activity compared to their parent peptide are used as templates to generate new peptide libraries that may contain even stronger immunomodulators.

Several synthetic derivatives of the bovine cathelicidin bactenecin, developed using this method, have proven to be even more potent immunomodulators *in vitro* and *in vivo*. IDR-1, a 13-amino acid peptide that lacks antimicrobial activity is protective in multiple murine models of infection including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus* and *Salmonella enterica* serovar Typhimurium (189). This is attributed to its ability to modulate immune responses, inducing the production of chemokines to attract monocytes/macrophages to the site of infection while suppressing other pro-inflammatory responses. IDR-1002, a 12-amino acid peptide, is a more potent immunomodulator than IDR-1. Much like IDR-1, IDR-1002 is protective in a murine *S. aureus* infection model. However, a substantially lower dose is needed to confer protection compared to IDR-1 (190). The protective effect of IDR-1002 was also associated with its ability to induce chemokine production, through modulation of multiple signalling pathways, and to recruit monocytes and neutrophils to the site of infection (190). Further *in vitro* studies revealed that IDR-1002 enhanced monocyte migration

through a variety of mechanisms including, induction of chemokine production and enhancement of chemokine receptor expression as well as promotion of monocyte adhesion to the extracellular matrix component, fibronectin through activation of β1-integrin (L. Madera, PhD thesis, UBC, 2012) (191).

Finally, IDR-1018, another 12-amino acid peptide derived from bactenecin, has received a significant amount of attention because of it is the most potent peptide developed to date and has pleiotropic activities, including anti-infective and anti-inflammatory activities and anti-biofilm activity (192). As shown for IDR-1 and IDR-1002, IDR-1018 induces chemokine production, suppresses pro-inflammatory responses induced by pathogens and provides protection in a wide range of *in vivo* models of infection, inflammation and wound healing (192-196). In addition, IDR-1018 has been shown to rescue dysfunctional autophagy, and modulate macrophage differentiation as well as promote wound healing in part through inducing cellular proliferation and metabolism (197-199).

Based on the research described above, it is clear that HDPs and IDR peptides as well as peptide hormones and neuropeptides, are capable of regulating the immune response. Although, very little work has been done on the ability of HDPs and IDRs to integrate metabolism and the innate immune response, the results of several studies have hinted at a link between immunomodulatory peptides and the regulation of metabolism.

1.8 HDPs AND IDR PEPTIDES- POTENTIAL REGULATORS OF METABOLISM

Of the immunomodulatory peptides discussed, the most is known about how cationic peptide hormones and neuropeptides regulate metabolism since these peptides were originally identified as regulators of metabolism (**Table 1.2**). For example, α-MSH reduces food intake while increasing basal metabolic rate. Ghrelin, a peptide hormone that exhibits anti-inflammatory properties, including inhibition of pro-inflammatory cytokine production, was originally identified as a regulator of energy homeostasis, stimulator of food intake and suppressor of insulin secretion (169).

Significantly less is known about the modulation of metabolism by classic HDPs and their synthetic derivatives, IDRs. Radek et al. (200) demonstrated a link between HDPs and the endocrine system, a major regulator of metabolism, finding that the cholinergic component of the endocrine system regulates the tissue levels of the murine cathelicidin, cathelicidin-related antimicrobial peptide (CRAMP). The human α-defensin, HNP-1 inhibits hepatic gluconeogenesis and glycogenolysis thereby reducing blood glucose levels; an activity

independent of insulin (201). In addition, HDPs and IDRs modulate the production of several pro-inflammatory molecules such as TNF-α and IL-6 (202) that are also associated with obesity, adipocyte lipolysis and insulin resistance (168). This provides a potential mechanism by which HDPs and IDRs might regulate metabolism within an organism however, significantly more research is required.

There is also evidence that HDPs and IDRs might play a role in metabolism at the cellular level. The peptide IDR-1018 has been shown to significantly enhance metabolism of primary human fibroblasts in a dose dependent manner (199). LL-37 is taken up by macrophages and binds to the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (203). A similar interaction was also observed between the synthetic peptide, IDR-1 and GAPDH. The binding of LL-37 and IDR-1 to GAPDH causes a mild, though insignificant, suppressive effect on GAPDH enzymatic activity. Interestingly, siRNA knock down of GAPDH suppresses peptide-induced chemokine gene expression and protein production. The suppression of peptide activity is due to a loss of p38 mitogen activated protein kinase (MAPK) signalling, which is essential to LL-37 induced cellular responses like chemokine production (138). It is also possible that the suppression of peptide activity was partly a result of a decrease in glycolytic flux, a consequence of silencing GAPDH (204, 205), although LPS-induced responses were unaffected. Previous studies have shown that the inhibition of glycolysis inhibits TLR agonist induced pro-inflammatory responses such as cytokine synthesis and secretion (36-38, 58). However, knock down of GAPDH appeared to have no effect on TLR agonist induced transcriptional responses or protein production nor did it alter cellular proliferation (203). Thus, it remains unclear whether inhibition of glycolysis might have played a role in the immunomodulatory activities of LL-37 and IDR-1. In fact, although there is evidence that HDPs and IDRs can modulate metabolism and that metabolism might be important to their immunomodulatory activity, the nature of this relationship remains to be elucidated and is the focus of this thesis.

1.9 HYPOTHESIS AND RESEARCH GOALS

HDPs and IDRs exhibit potent and pleiotropic immunomodulatory activities (202) and *in vivo* studies utilizing IDRs suggest that monocytes and macrophages play a principal role in peptide-mediated immunomodulation (189, 190). Although a significant amount of research has been done on the mechanisms underlying these activities, much remains to be elucidated. There have been some hints that HDP and IDR immunomodulatory activity may be linked to cellular metabolism (199, 203). The activity of macrophages is dependent on their ability to metabolize

glucose, glutamine and fatty acids, which is regulated through metabolic reprogramming induced by external stimuli such as growth factors, TLR agonists, and cytokines (23). Stimulation of macrophages with pro-inflammatory mediators, such as bacterial lipopolysaccharide induces a shift in metabolism from oxidative phosphorylation towards aerobic glycolysis (30, 38). This shift is essential for mounting an effective inflammatory response. Therefore, I **hypothesized** that like TLR agonists and cytokines, IDR-1018 exploits certain nutrients and induces alterations to cellular metabolism in order to exert its immunomodulatory activities on macrophages. This project utilized a particularly potent immunomodulatory peptide, IDR-1018 to (i) determine the ability of IDRs to alter cellular glucose metabolism using human macrophages as a model system and (ii) assess whether active glucose metabolism is required for IDR peptides to carry out their immunomodulatory activity, utilizing the glycolysis inhibitor 2-deoxy-D-glucose (2DG).

Malnutrition as a result of nutrient excess drives the chronic inflammation observed in many metabolic disorders, including atherosclerosis. One of the hallmarks of atherosclerosis is the excessive accumulation of lipids and immune cells, which leads to inflammation and plaque formation (206). Lipid-laden macrophages and smooth muscle cells are major mediators of this lipid accumulation and inflammation (75, 207). HDL and its main protein component and cholesterol acceptor, ApoA1, can mitigate these effects through induction of cellular cholesterol efflux and reverse cholesterol transport (85). Thus ApoA1 peptide mimetics have been developed as a potential new therapeutics that enhance cholesterol efflux from macrophages and promote reverse cholesterol transport (89, 90). These peptides also possess pleiotropic immunomodulatory activities (92, 94-97). HDPs and IDRs share many of the physical and immunomodulatory properties of ApoA1 mimetic peptides (202). Therefore, I **hypothesized** that like apoA1 and its peptide mimetics, HDP and IDR peptides can promote cholesterol efflux from cells. To address this, the ability of IDR-1018 to promote intracellular cholesterol mobilization and efflux from cholesterol-loaded macrophages and smooth muscle cells was assessed.

Chapter 2: INHIBITION OF GLYCOLYSIS AND INDUCTION OF THE UNFOLDED PROTEIN RESPONSE BY 2DG REGULATES THE IMMUNOMODULATORY ACTIVITY OF IDR-1018

2.1 INTRODUCTION

Cationic HDPs are a critical component of the innate immune system. Originally thought to act by direct antimicrobial killing, many of these peptides have now been characterized as broad immune modulators exhibiting both pro- and anti-inflammatory properties (115, 208), and it has been suggested that this might be their primary biological function (184). Synthetic derivatives of these peptides, IDRs were generated by our laboratory with the goal of improving the key immunomodulatory activities exhibited by their parent peptides, to enable the development of a new class of anti-infectives that enhance/support the immune system rather than acting directly against the pathogen both *in vitro* and *in vivo (202)*. One of the most potent IDRs developed, IDR-1018, is capable of stimulating chemokine production, suppressing pathogen-induced pro-inflammatory responses, regulating macrophage differentiation, and providing protection in several *in vivo* infection models (193-196, 198, 199). While a significant amount of research has been done on the activity of HDPs and IDRs, the mechanisms behind their activity appear to be quite complex and have not been fully elucidated.

The close association between the innate immune system and nutrient metabolism has only been appreciated in recent years. Cells of the innate immune system, such as macrophages, rely on glucose, glutamine and fatty acid metabolism to generate energy and the metabolites required for macromolecule biosynthesis, as well as to regulate their response to infectious agents (25-27). Interestingly, metabolism by immune cells is regulated by external signals such as cytokines and TLR agonists (25). Substantial research has focused on how glucose metabolism by macrophages and dendritic cells is altered upon immune activation, and what affect this has on cellular responses. Activation by pro-inflammatory cytokines and TLR agonists results in elevated glucose uptake, glycolysis, pentose phosphate pathway activity, and TCA cycle anapleurosis with a concomitant decrease in oxidative phosphorylation (36-38, 55). Conversely, the ability of these cells to effectively respond to pro-inflammatory stimuli is suppressed by perturbations to these pathways (36-38, 55). Thus, the modulation of cellular metabolism is considered a major driver of immune cell activity.

Cellular metabolism and the innate immune response are also connected via the unfolded protein response (UPR) (98, 99). The UPR is made up of three stress-sensing arms, namely ATF-

6, PERK and IRE1α pathways (100), which are activated in response to the accumulation of misfolded proteins in the ER. Activation of the UPR alleviates the ER stress induced by the misfolded proteins or, if that is not possible, promotes apoptosis (100, 101). Two of the arms of the UPR have also been linked to the modulation of immune cell responses (99). Activation of the transcription factor XBP-1, through the IRE1α pathway is required for sustained TLR-driven inflammatory responses in macrophages (107, 108). In contrast, exogenous activation of the PERK pathway suppresses TLR-driven inflammatory responses (101, 110). Although it is evident that the UPR and inflammation are connected, a lot remains to be discovered.

The goal of the studies presented in this Chapter was to determine if immunomodulatory peptides are capable of altering glucose metabolism and, if so, whether this alteration was essential to their immunomodulatory activity. In this study, 2-deoxy-D-glucose (2DG), a glucose analog that inhibits glycolysis, was employed as a tool. Here I showed that IDR-1018 enhanced glucose metabolism, and that this enhancement was important for the immunomodulatory activity of this peptide. In addition it was demonstrated that 2DG modulated IDR-1018 activity both through inhibition of glycolysis and activation of the UPR, a combination not identified in previous immunological studies.

2.2 MATERIALS AND METHODS

2.2.1 Reagents

The peptide IDR-1018 (VRLIVAVRIWRR-NH_2) was synthesized using solid phase Fmoc chemistry and purified to >95% using reverse phase HPLC (CPC Scientific, Sunnvale, CA). 2-Deoxy-D-glucose (2DG) and sodium oxamate were purchased from Sigma Aldrich (St. Louis MO). Tunicamycin, was purchased from EMD Millipore (ON, Canada). Salubrinal was provided as a generous gift from Dr. Stuart Turvey.

2.2.2 Generation of human monocyte-derived macrophages

Human monocyte-derived macrophages were generated as described previously (198). Briefly, peripheral blood mononuclear cells (PBMCs) were seeded at a concentration of $2x10^6$ cells/ml in serum free RPMI 1640 medium for 45 minutes. Medium was then gently removed and fresh RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), was added. Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere for 24 hours after which, the medium was replaced with RPMI 1640 supplemented with 10% FBS and 10 ng/ml of macrophage-colony stimulating factor (M-CSF) (Research Diagnostic Inc, Concord, MA). Cells were incubated as described above for 5 days. On the sixth day, the cells were gently washed to

remove non-adherent cells, and the media was replaced with fresh RPMI 1640 supplemented with 10% FBS and 10 ng/ml of M-CSF. On day seven, cells were washed to remove any remaining non-adherent cells at which point they were ready for experimental use.

2.2.3 Metabolism assays

A modified form of the WST-1 cell proliferation assay (Roche) was used to screen for peptides with the ability to alter cellular metabolism. PBMCs were seeded at $1x10^5$ cells/well in a 96 well plate and 10 µl of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) was added. Cells were incubated for 2 hours to at 37°C and 5% CO_2 after which the absorbance at 450 and 900nm was measured (0 hr measurement). The cells were then stimulated with 50 µg/ml IDR-1018 and incubated at 37°C and 5% CO_2 for a further 4 hours with absorbance measurements (450 nm and 900 nm) made every 30 minutes. Treatments were completed in triplicate for each experiment. The results were plotted as the average change in OD of five independent experiments.

To measure glycolysis, macrophages were stimulated with 20 µg/ml of IDR-1018 for 4 hours. Supernatants were harvested and lactate measured using a colorimetric lactate assay kit (Trinity Biotech, Bray Ireland) as per the manufacturer's instructions. Results are the average of six independent experiments. Intracellular ATP was measured using the Celltiter-Glo 2.0 luminescent cell viability assay (Promega, Madison WI) according to the manufacturer's instructions with minor modifications. Briefly, macrophages were cultured in 48 well plates in the presence or absence of 20 mM 2DG for five minutes then stimulated with 5, 20, or 50 µg/ml of IDR-1018 for 24 hours. The cells were lysed in the same plate using the kit reagent, lysates were transferred to a black walled clear bottom 96-well plate, and luminescence was measured. Data were reported as percentage of the unstimulated control. Results are the average of five independent experiments.

Intracellular glutathione (GSH) was measured as described previously (209). Briefly, macrophages were pretreated with 5 or 20 mM of 2DG for 5 minutes then stimulated with 5, 10, or 20 µg/ml of IDR-1018. After a 16-hour incubation, cells were detached from the plate via scraping in 300 µl of assay buffer (100 mM sodium phosphate, 5 mM EDTA, pH 7.5) and counted. Cells were lysed via incubation at -80°C after which, 50µl of each sample was transferred to a 96 well plate for the assay. To measure GSH, 50 µl of reaction buffer (1.5 mM DTNB, 0.5 mM NADPH in assay buffer) was added to the samples followed by 25 µl of glutathione reductase (1 U/ml). Assay buffer was used as a blank and GSH alone was used to create a standard curve (0-10 nM). The reaction was incubated for 5 min then the OD_{405} was

measured. The GSH concentration was determined for each sample by comparison with the standard curve, and normalized to cell numbers.

Intracellular reactive oxygen species (ROS) production was assayed using the fluorescent stain, H_2DCFDA (Molecular Probes, Eugene OR) as described previously (193). Briefly, macrophages were pretreated with 20 mM 2DG for 5 minutes then stimulated with 20 μg/ml IDR-1018 for 4 hrs. Macrophages were washed and incubated for a further 30 minutes in medium supplemented with 1 μM H_2DCFDA. The cells were then washed with PBS and detached from the wells of the tissue culture plate using gentle scraping. Fluorescence was assessed using flow cytometry.

2.2.4 RNA-Seq sample preparation and analysis

RNA-Seq was performed using the Illumina Genome Analyzer IIx platform as described previously, with some modifications (198). Macrophages were pretreated or not with 20 mM 2DG for 5 minutes then stimulated or not with 20 μg/ml IDR-1018 for four hours. The Qiagen RNeasy Isolation Kit (Qiagen, Valencia CA) was used to extract RNA from cell lysates. The RNA concentration, integrity and purity were assessed using the RNA Nano Kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). The Illumina Truseq (Illumina catalogue number FC-122-1002) RNA library preparation kit was used to prepare libraries for sequencing. Briefly, mRNA, purified from 1 μg of total RNA using poly-dT beads, was used to synthesize cDNA. This was followed by end repair, 3' end adenylation and ligation of adaptors containing unique barcodes. Finally, DNA containing the adaptor molecules was amplified using PCR then quantified. Cluster generation was carried out on a CBOT instrument followed by RNA sequencing on a GAIIx instrument (Illumina, San Diego CA), performed as a single end run of 64 nucleotides. Raw data was demultiplexed and converted to FASTQ files using CASAVA. Bowtie 2 (210) and TopHat2 (211) were then used to align the reads to the Ensembl GRCh37.74 reference genome after which SAMtools (212) was used to sort and index the bam and sam files. Read count tables were generated using htseq-count. Differential gene expression analysis was performed using edgeR (213) using a design formula that corrected for donor variability. Bioinformatic analysis of the data sets was carried out using two systems biology tools developed in our laboratory, the InnateDB database (214) (http://www.innatedb.ca), used to identify biological pathways that were overrepresented amongst the differentially expressed genes, and NetworkAnalyst (72) (http://www.networkanalyst.ca/NetworkAnalyst/), used to generate a protein:protein interaction network for differentially expressed genes. The cut-off for

differential expression was defined as a fold change greater than or equal to +/- 1.5 and a false discovery rate corrected *p* value less than or equal to 0.05.

2.2.5 Enzyme-linked Immunosorbent Assay (ELISA)

Tissue culture supernatants were stored at -20°C. After thawing, cytokine levels were measured by ELISA, using antibodies for macrophage inflammatory protein (MIP)-1α, IL-8 (BioSource-Life Technologies, Carlsbad CA), monocyte chemoattractant protein (MCP)-3, interferon γ-inducible protein (IP)-10 (R & D Systems, Minneapolis MN), and MCP-1 (eBioscience, San Diego CA), according to manufacturer's instructions. Results were presented as average fold change relative to unstimulated control so as to reduce the significant donor-to-donor variability with respect to absolute concentrations.

2.2.6 Immunoblotting analysis

Following treatment, macrophages were lysed with NP-40 buffer (150 mM NaCl, 1% Nonidet-40, and 50 mM pH 8.0 Tris-HCl) containing phosphatase and protease inhibitor cocktails (Sigma Aldrich). Total protein concentration was quantified using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL). Equal amounts of protein were used for each experiment. Samples were run on a 12% SDS-PAGE gel then transferred onto an Amersham polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Little Chalfont UK). Blots were blocked using 5% skim milk, to prevent non-specific binding, then incubated with primary antibody against β-actin, phospho-JNK, phospho-ERK, total JNK, total ERK, BiP, or LC3B (all from Cell Signaling Technology, Danvers MA). Following incubation with primary antibody, blots were washed and probed with either horseradish peroxidase-conjugated anti-mouse (R&D Systems) or anti-rabbit (Cell Signaling Technology) IgG secondary antibody. Finally, blots were washed and developed using Sigma ECL (Sigma Aldrich) or Millipore Luminata Classico ECL (Millipore, Billerica MA) and film exposure (GE Healthcare). Quantitative densitometry was performed using ImageJ software. Results were normalized to β-actin protein expression.

2.2.7 TUNEL staining

DNA fragmentation, a marker of apoptosis, was assessed using the Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay as described previously (215). Briefly, macrophages were cultured in Lab-Tek 8-well chamber slides (Thermo Fisher Scientific). Cells were pretreated with 20 mM 2DG for 5 minutes then stimulated with 5 or 20 μg/ml biotinylated IDR-1018 for 16 hours. Methanol-free formaldehyde

at 4% (Thermo Fisher Scientific) was used to fix the cells. The slides were washed with PBS then incubated on ice while cells were permeabilized using 0.1% Triton X-100 in 0.1% sodium citrate. Following permeabilization, slides were washed with PBS, dried and stained for 1 hour (at 37°C in 5% CO_2) with the reaction mixture provided in the TUNEL assay kit (Roche diagnostics, Laval QC). Finally, stained slides were washed with phosphate buffered saline (PBS), then coverslips were mounted using Permount (Fisher Scientific) and the slides were imaged (200X). Quantification of TUNEL staining was performed using ImageJ software and normalized to cell number.

2.2.8 Statistical analysis

Data was analyzed using Graphpad Prism software, version 6. A Paired student t-test was used to analyze lactate production by macrophages stimulated with IDR-1018. One-way ANOVA was used to analyze the ELISA results from macrophages stimulated with IDR-1018 with or without 2DG. Two-way ANOVA was used for all other experiments. In all cases, $p < 0.05$ was considered statistically significant.

2.3 RESULTS

2.3.1 IDR-1018 induced an increase in metabolism by PBMCs

Several synthetic IDR peptides generated in our laboratory were screened for their ability to enhance glucose metabolism in PBMCs, by using a modified WST-1 cell viability assay that measures glycolysis and TCA cycle dependent (NADH) and NADPH production (216, 217). PBMCs stimulated with 50 μg/ml IDR-1018 significantly increased the metabolic activity of cells compared to untreated controls (**Figure 2.1**). This increase occurred as early as 1.5 hours post stimulation and continued to be evident for up to 4 hours. This result was consistent with the results of a previous study that found that IDR-1018 was capable of significantly enhancing the metabolism of primary human fibroblasts (199).

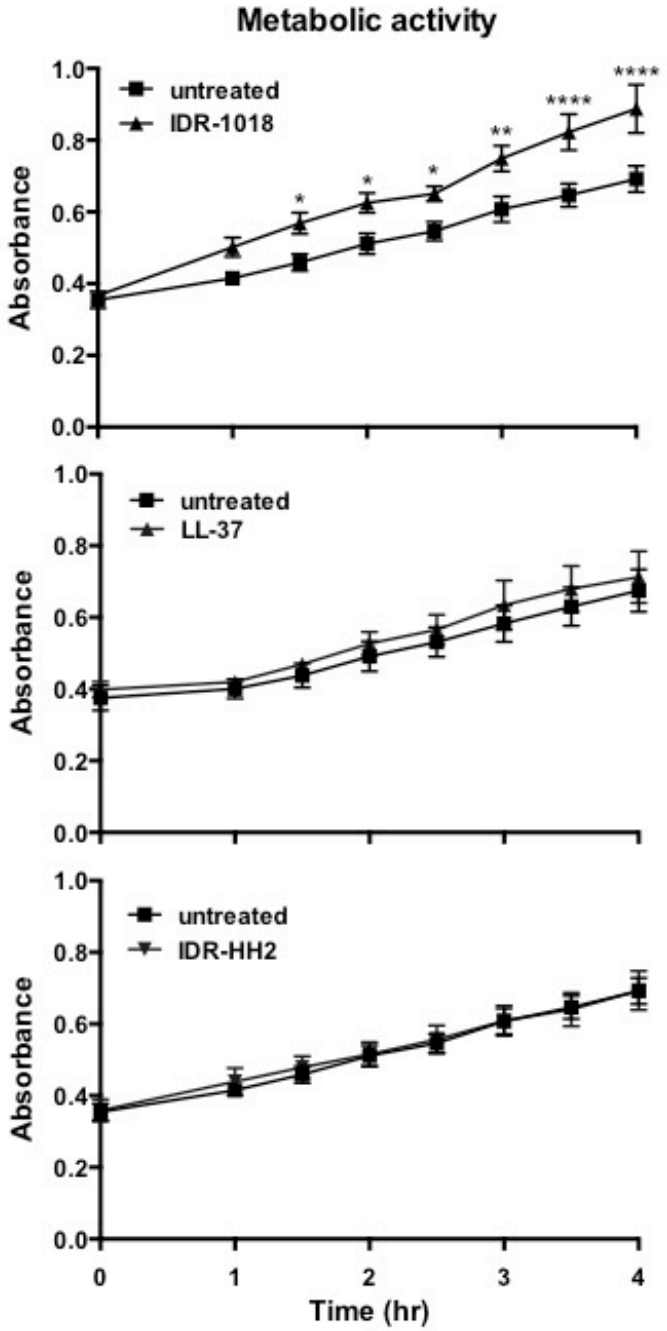

Figure 2.1: IDR-1018 enhanced glucose metabolism by PBMCs. The tetrazolium salt, WST-1 was added to cultured PBMCs for 2 hours then cells were stimulated with 50 μg/ml of IDR-1018. Absorbance was read every 30 minutes for 4 hours. Five independent experiments were completed, each in triplicate, with error bars representing SEM. Data was compared using two-way ANOVA ****p<0.0001, ** p <0.01 and * p <0.05.

Several other peptides tested, including the HDP LL-37 and the sequence-related synthetic peptide IDR-HH2, had no effect on metabolism in PBMCs. Previous studies found that WST-1 reduction is enhanced by inhibiting mitochondrial respiration, while inhibition of glucose uptake or glycolysis suppressed its reduction (216, 217). This inferred that IDR-1018 was

enhancing glycolysis, inhibiting mitochondrial respiration or a combination of the two.

2.3.2 IDR-1018 induced glycolysis, which was important to its immunomodulatory activity

To further characterize the impact of IDR-1018 on glucose metabolism we focused on monocyte-derived macrophages, the major immune cell types through which IDRs act (189, 190). Specifically, we sought to confirm that, as is the case for many TLR agonists (36-38), activation of macrophages by IDR-1018 induced glycolysis. Thus, extracellular lactate produced by macrophages was measured after four hours of stimulation with 20 μg/ml of IDR-1018. IDR-1018 treatment led to significantly increased extracellular lactate levels indicating that the peptide induced glycolysis by cells (**Figure 2.2**).

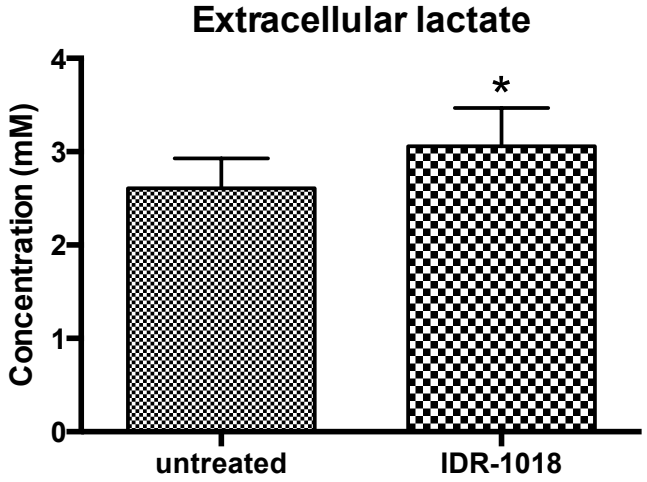

Figure 2.2: IDR-1018 enhanced glycolysis by macrophages. Macrophages were stimulated with 20 μg/ml of IDR-1018 for 4 hours. Lactate levels in the media were assessed using a colorimetric assay (Trinity biotech). Results are from 6 independent experiments, with error bars representing SEM. Data was compared using a paired Student's t-test, * $p < 0.05$.

Induction of glycolysis by TLR agonists is a recently established mechanism by which inflammatory responses, such as cytokine production, are regulated in both macrophages and dendritic cells (36-38, 55). Thus to determine if the increase in glycolysis induced by IDR-1018 was important to its immunomodulatory activity, macrophages were stimulated with IDR-1018 in the presence of 2DG, which blocks glycolysis by inhibiting hexokinase II and phosphoglucose isomerase (218). A hallmark of IDR immunomodulatory activity is the ability to induce chemokine production by a variety of cell types, including macrophages (190, 193, 195, 198). IDR-1018 induced MCP-1, IL-8 and MIP-1α production was significantly decreased by the addition of 20 mM 2DG. Interestingly, 2DG had no effect on IP-10 production (**Figure. 2.3**).

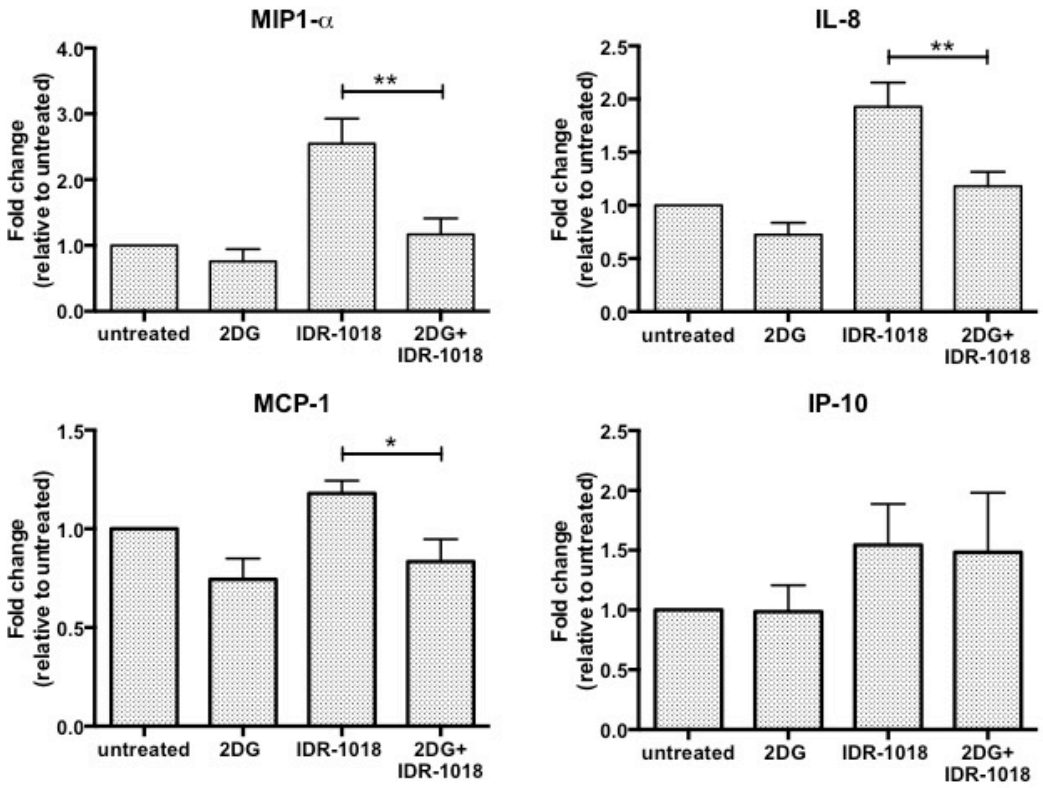

Figure 2.3: Glucose metabolism was important to IDR-1018-induced production of certain chemokines by macrophages. Macrophages were untreated or pretreated with 20 mM of 2DG for 5 min followed by stimulation, or not, with 20 μg/ml IDR-1018 for 4 hours. Supernatants were harvested and MIP-1α, IL-8, MCP-1, and IP-10 production was assayed via ELISA. At least 5 independent experiments were completed, with error bars representing SEM. Data was compared using one way ANOVA $^{**}p < 0.01$ and $^{*}p < 0.05$.

It was possible that the suppression by 2DG of IDR-1018 induced chemokine production might have stemmed from the depletion of ATP generated via glycolysis, since the activation of cells involves substantial gene expression and protein production, two energy intensive processes (219). However, IDR-1018 treatment did not significantly alter ATP levels at any of the concentrations tested, when the level of intracellular ATP was measured in macrophages stimulated with IDR-1018 alone or in the presence of 2DG (**Figure 2.4**). In contrast, treatment of cells with 2DG significantly reduced ATP levels by 45-65% under all conditions tested.

Thus, while IDR-1018 had no effect per se on ATP levels, the substantial decrease of ATP observed in 2DG treated cells was consistent with the possibility that the maintenance of cellular energy stores through active glycolysis is important for IDR-1018 activity. In other studies it has been shown that the depletion of glycolytic or mitochondrial ATP has no effect on TLR-induced cytokine production by dendritic cells (36). Nevertheless it was unclear whether

inhibition of glycolysis by 2DG affected IDR-1018 function through depletion of ATP or through indirect suppression of metabolic pathways downstream of glycolysis (220).

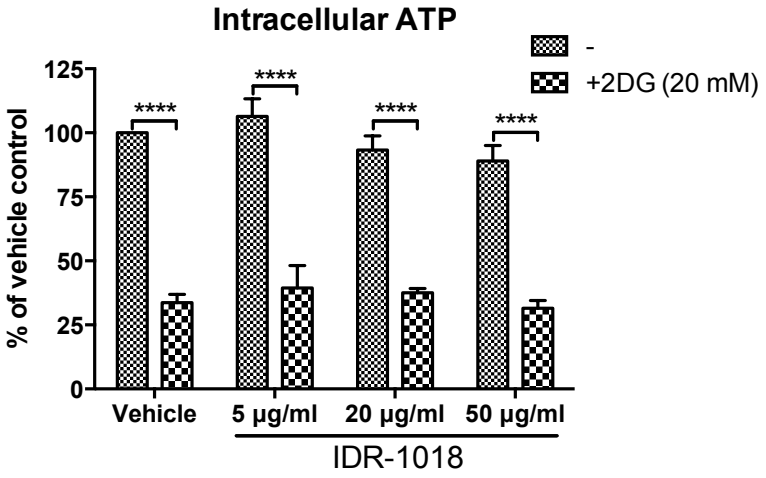

Figure 2.4: Inhibition of glycolysis depleted intracellular ATP levels. Macrophages were stimulated with varying concentrations of IDR-1018 with or without 20 mM of 2DG for 24 hours then intracellular ATP levels were measured. Results are from 5 independent experiments, with error bars representing SEM. Data was compared using two-way ANOVA, **** $p < 0.0001$.

2.3.3 Activation by IDR-1018 of the ERK MAPK pathway, a major regulator of metabolism and the immune response, was suppressed by 2DG

HDP and IDR peptides depend on a variety of signalling pathways to induce chemokine production by cells, including the MAPK pathways (190, 193). Activation of the extracellular signal-regulated kinase (ERK) pathway has also been shown to enhance glucose metabolism in a variety of cell types, including cancer cells, T lymphocytes and macrophages (221-224). Since IDR-1018 induced increases in both glucose metabolism and chemokine production, we sought to determine whether IDR-1018 was activating the ERK pathway and, if so, whether 2DG was modulating IDR-1018 activity through dysregulation of this pathway. IDR-1018 induced significant phosphorylation of ERK at 5 minutes post-stimulation. This was suppressed by the pretreatment of cells with 2DG (**Figure 2.5**), suggesting that impairment of IDR-1018-mediated ERK signaling might, in part, explain 2DG-mediated suppression of chemokine production.

The JNK pathway has also been implicated in the regulation of glycolysis in LPS-stimulated macrophages, though to a significantly lesser degree (222). However, IDR-1018 and 2DG had no effect on JNK phosphorylation, suggesting that unlike the ERK pathway, the JNK pathway was not involved in IDR-1018 induced chemokine production and glucose metabolism or the modulation of IDR-1018 activity by 2DG.

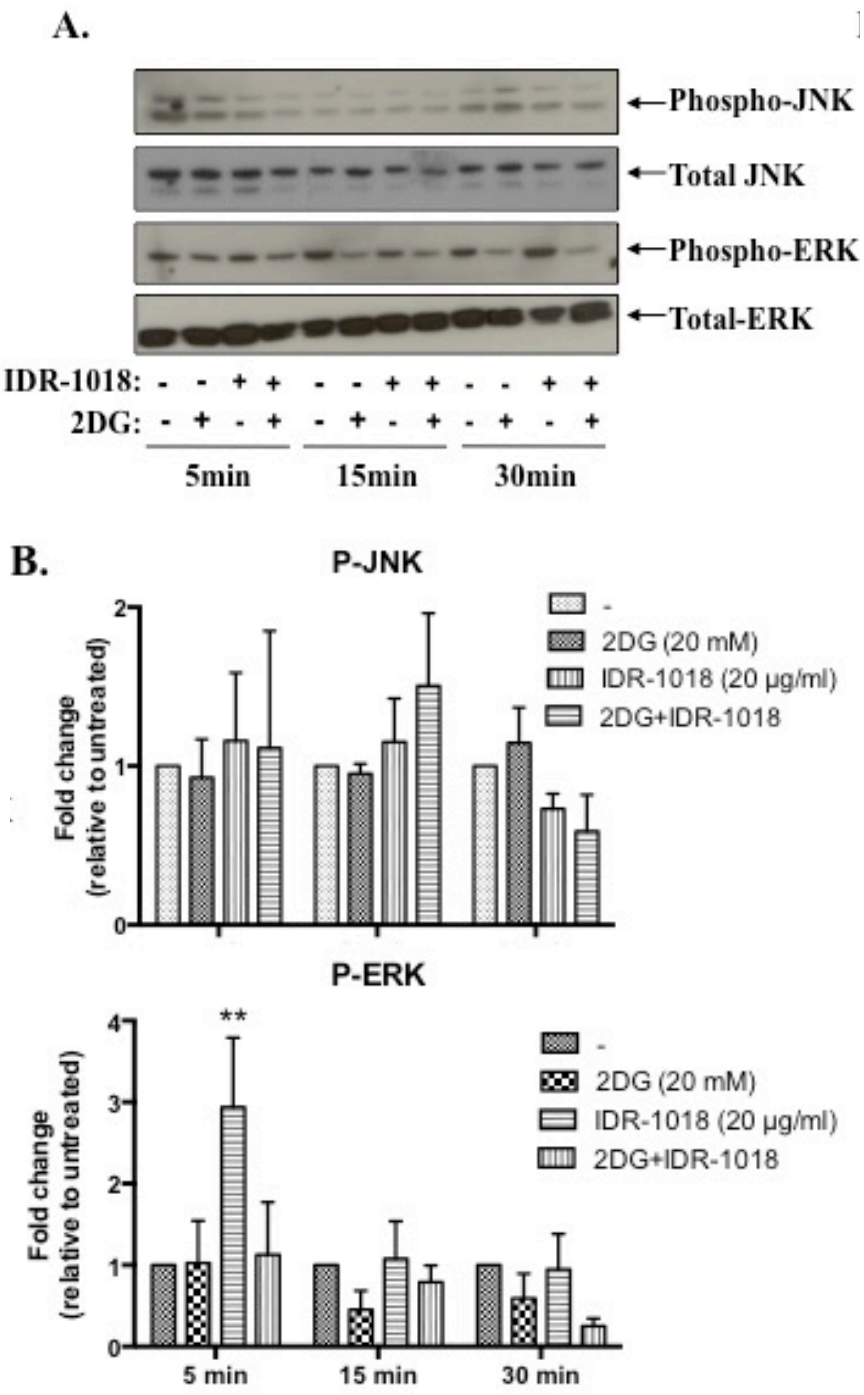

Figure 2.5: IDR-1018 induced ERK phosphorylation was suppressed by pretreatment with 2DG. Macrophages were pretreated with 20 mM of 2DG for 5 min then stimulated with 20 µg/ml IDR-1018 for 5, 15 or 30 min. Cell lysates were used for immunoblot analysis of phosphorylated and total JNK and ERK. Autoradiogram data A) are from one experiment, representative of three independent experiments. Densitometry calculations for phosphorylated JNK and ERK B) were performed using ImageJ software, results are from three independent experiments, with error bars representing SEM. Data was compared using two-way ANOVA **$p < 0.01$.

2.3.4 Early induction of the Unfolded Protein Response significantly modulated IDR-1018 activity

As a result of the complex and significant interconnectivity of innate immunity and cellular metabolism, a systems biology approach was taken to obtain a more in depth understanding of how the glycolysis inhibitor, 2DG was modulating IDR-1018 activity. Transcriptomic analysis by RNA-Seq was carried out on macrophages stimulated with IDR-1018 in the absence or presence of 2DG for 4 hours. The genes with altered expression levels following IDR-1018 stimulation were subjected to a biological pathway overrepresentation analysis using InnateDB (214) to identify the biological processes most affected by IDR-1018. As anticipated, the majority of biological pathways over represented as a result of IDR-1018 treatment were related to the innate immune response (**Table 2.1**).

Table 2.1: Selected overrepresented biological pathways associated with the immune response in IDR-1018 stimulated macrophages compared to untreated controls

Overrepresented biological pathways	**Corrected *p* Value**
NOD-like receptor signalling pathway	7.1E-8
Cytokine - cytokine receptor interaction	1.3E-7
Toll-like receptor signalling pathway	4.6E-5
Apoptosis	1.2E-4
Chemokine signalling pathway	1.4E-4

The same analysis was also carried out on the genes differentially expressed in response to IDR-1018 stimulation in the presence of 2DG compared to IDR-1018 alone. The significantly overrepresented biological pathways for the down regulated genes were predominantly associated with the immune response, which correlated well with the observed suppression of IDR-1018 induced chemokine production by 2DG (**Table 2.2**).

Table 2.2: Selected overrepresented biological pathways associated with the immune response, the unfolded protein response, and amino acid metabolism in macrophages stimulated with IDR-1018 and 2DG compared to macrophages stimulated with IDR-1018

Over represented biological pathways	**Corrected *p* Value**
Down-regulated genes:	
Pertussis toxin-insensitive CCR5 signalling in macrophages	0.003
Toll-like receptor signalling pathway	0.004
GPCR ligand binding	0.007
Chemokine signalling pathway	0.01
Up-regulated genes:	
Unfolded protein response (UPR)	1.3E-12
IRE1α activates chaperones	6.3E-16

Over represented biological pathways	Corrected *p* Value
Up-regulated genes (continued):	
Protein processing in endoplasmic reticulum	9.2E-8
XBP1(S) activates chaperone genes	6.3E-7
PERK regulates gene expression	7.1E-5
ATF6-α activates chaperone genes	1.5E-4
Amino acid synthesis and interconversion (transamination)	8.3E-4

The most interesting results were obtained from the overrepresented biological pathways associated with the genes upregulated in response to 2DG treatment. Several pathways associated with amino acid metabolism were dysregulated in response to 2DG. Pathways like amino acid synthesis and transamination are known to be activated in response to glucose starvation, as a mechanism for generating glucose, alternate amino acids, and TCA cycle intermediates (225). Their presence in the biological pathway overrepresentation analysis supported the hypothesis that 2DG was suppressing glucose metabolism. However, the most significantly dysregulated pathways identified in this analysis of upregulated genes were related to ER stress and the UPR. These results correlated with previous studies, which have shown that, in addition to inhibiting glycolysis, 2DG activates ER stress and the UPR in several different cell lines (226-230).

An analysis of the biological networks to which the differentially expressed genes belong was carried out using the network biology tool NetworkAnalyst (72). The products of the genes upregulated in response to IDR-1018 stimulation with 2DG, compared to IDR-1018 alone, were used to create a first order (upregulated gene plus direct interactors) protein:protein interaction network. Biological function enrichment analysis was then performed and genes belonging to the identified UPR pathway were extracted, demonstrating that the upregulated genes formed a dense network (indicative of mechanistic interconnection). This confirmed the activation of all three stress-sensing arms of the UPR (**Figure 2.6**), namely the ATF-6, PERK and IRE1α pathways (100).

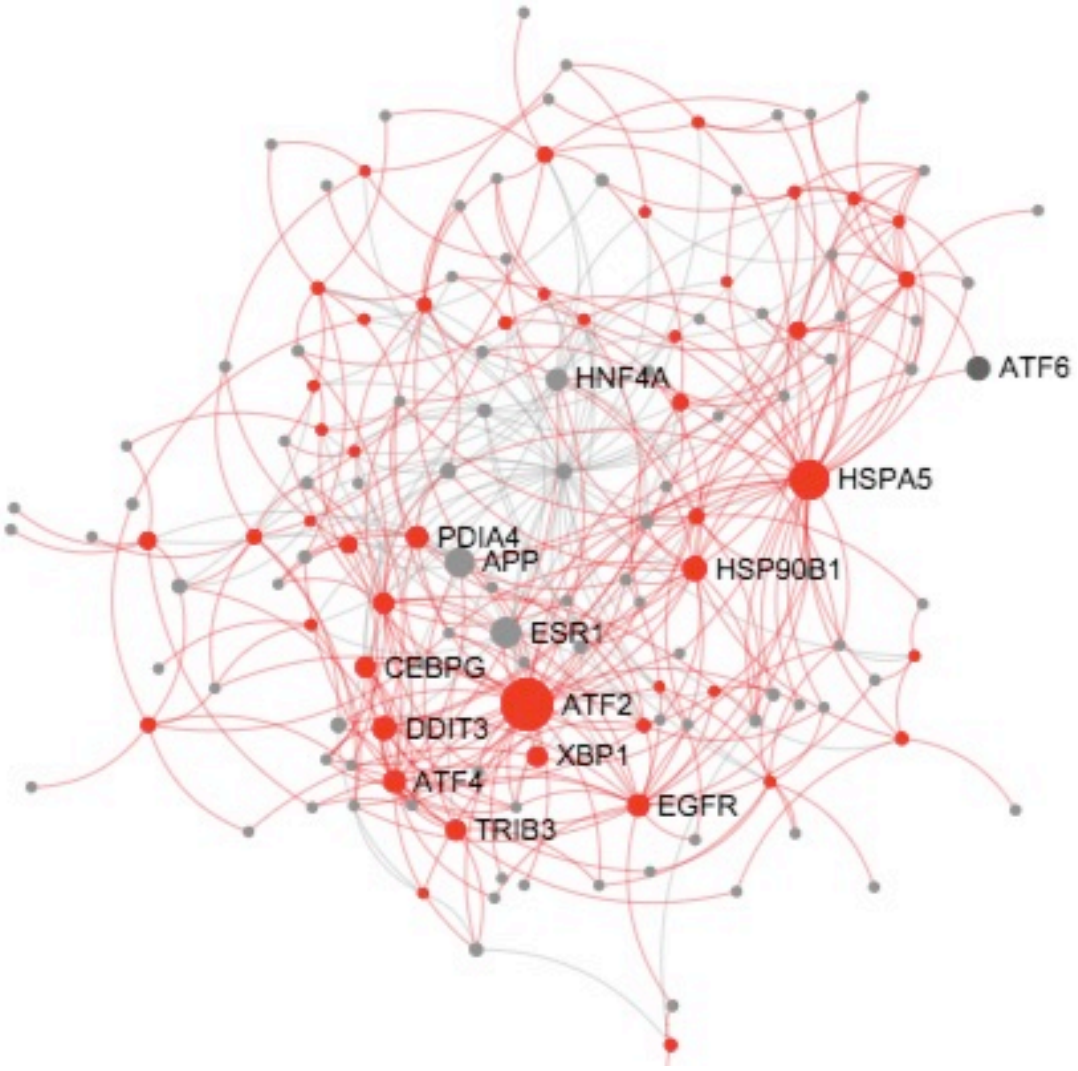

Figure 2.6: 2DG induced expression of genes associated with the UPR in IDR-1018 stimulated macrophages. Macrophages were stimulated with 20 μg/ml IDR-1018 in combination with 20 mM 2DG. A protein:protein interaction network was generated by uploading the genes upregulated in response to IDR-1018 stimulation with 2DG compared to IDR-1018 alone into NetworkAnalyst and extracting the curated UPR pathway from the overall network. Node color denotes gene expression (red, upregulated; grey, interactors that were not differentially expressed).

To confirm the results of the computational analysis, we examined the level of Binding immunoglobulin Protein (BiP; HSPA5), which was significantly transcriptionally upregulated. BiP is not only a major ER protein chaperone, but also a regulator of all three arms of the UPR, and a survival signal (231, 232). It is used as a marker of UPR activation and ER stress since induction of these processes leads to its substantial elevation. BiP was only observed in macrophages after treatment with 2DG (**Figure 2.7**).

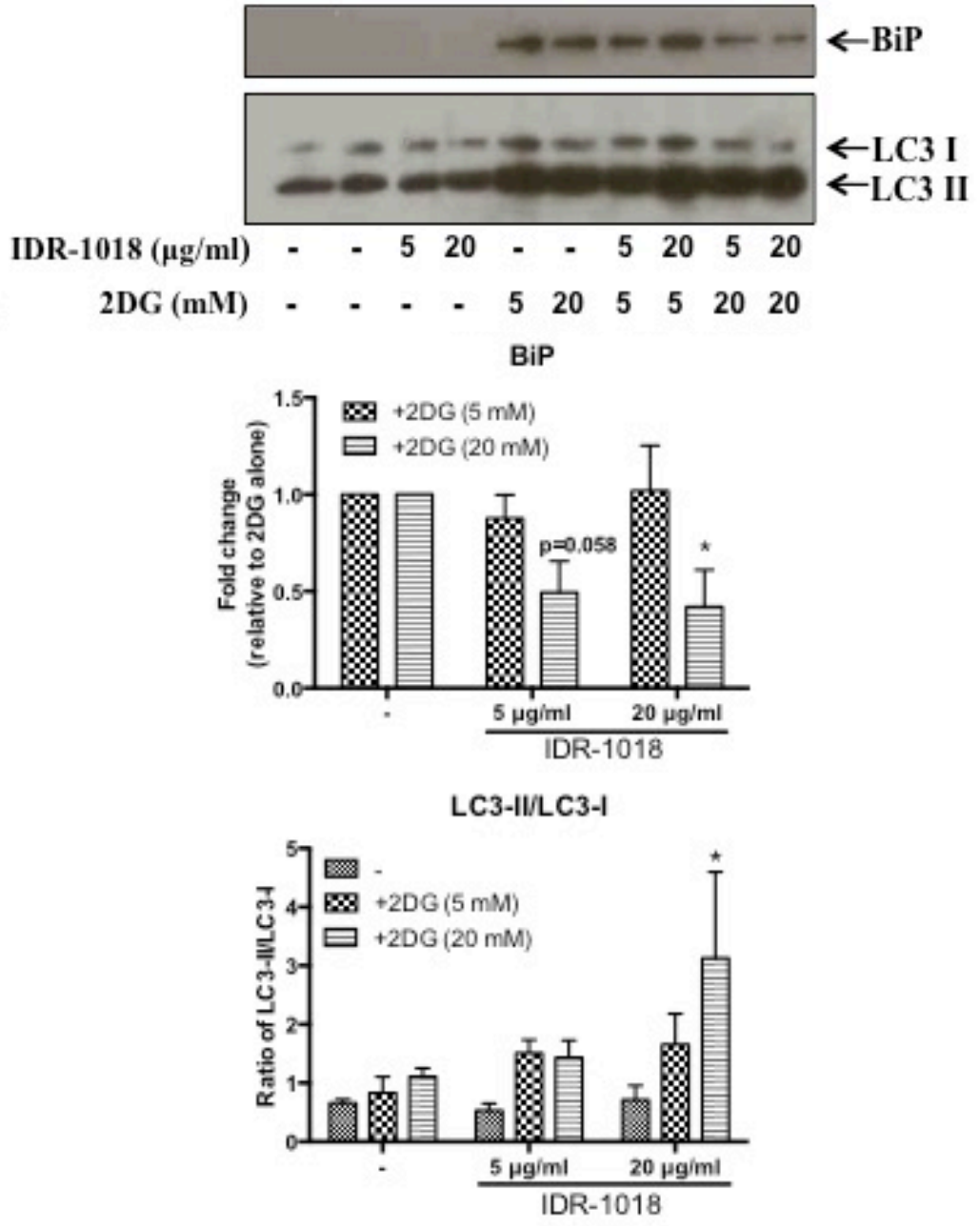

Figure 2.7: 2DG induced the UPR and autophagy in IDR-1018 stimulated macrophages. Macrophages were pretreated with 20 mM 2DG then stimulated with 20 μg/ml IDR-1018 for 16 hours. Cell lysates were used for immunoblot analysis of BiP and LC3-I/II. Data are from one experiment representative of three individual experiments with similar results. Densitometry results collate these three independent experiments, with error bars representing SEM. Data was compared using two-way ANOVA *$p < 0.05$.

The combination of 20 mM 2DG with 5 or 20 μg/ml IDR-1018 resulted in a substantial decrease in BiP (significant in the presence of 20 μg/ml IDR-1018). In cells undergoing an ER stress response, decreases in BiP mRNA and protein have been associated with the induction of apoptosis (233, 234). Therefore we investigated whether the decrease in BiP observed after

treatment with both 20 mM 2DG and IDR-1018 indicated a shift in cellular response from normal growth to apoptosis. However, using a TUNEL assay no DNA fragmentation, a marker of cell death, was observed under these conditions even after 16 hours (**Figure 2.8**), suggesting that the induction of apoptosis was not the cause of reduced IDR-1018 activity. Furthermore, a lactate dehydrogenase-release assay confirmed an absence of cytotoxicity under the experimental conditions.

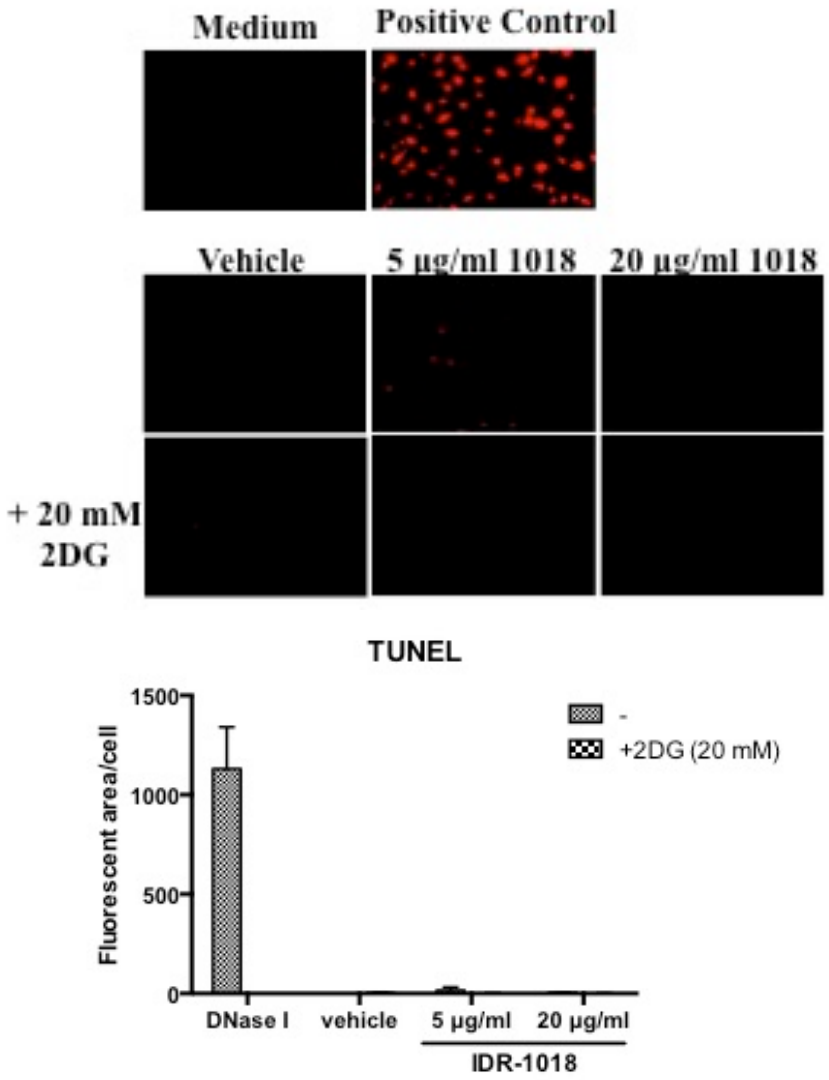

Figure 2.8: 2DG alone or in combination with IDR-1018 did not induce cell death. Macrophages were pretreated with 5 or 20 mM of 2DG for 5 min then stimulated with 5 or 20 µg/ml of IDR-1018. Cells were fixed and stained according to manufacturer's instructions. Data representative of 3 independent experiments was plotted bellow. Quantification of the TUNEL assay was carried out using ImageJ software.

Indeed, based on our RNA-Seq data, IDR-1018 might have actually suppressed apoptosis since it was observed to induce the expression of several anti-apoptotic factors, including BCL-2, BIRC3, TRAF1 and NAMPT (235-238), while suppressing the pro-apoptotic factor

CACNA2D3 (239). Overall, the activation of the UPR in macrophages by 2DG is likely to be the basis for the suppression of chemokine induction, but this did not involve the promotion of cell death.

Recent work has shown that 2DG-induced ER stress activates autophagy as a means to alleviate stress (226). This activation of autophagy was independent of 2DG-mediated ATP depletion. To measure the potential activation of autophagy, we looked at the ratio of Light chain (LC)3-II to LC3-I, a well characterized indicator of autophagy induction. Both 5 and 20 mM 2DG alone caused subtle but insignificant increases in the LC3-II/LC3-I ratio, suggesting that in our experimental system 2DG might have induced autophagy (**Figure 2.7**). Interestingly, the combination of 20 mM 2DG with the highest dose of IDR-1018, 20 μg/ml caused a statistically significant increase in the LC3-II/LC3-I ratio. This occurred under the same conditions for which a decrease in BiP was observed. Autophagy is activated by ER stress, possibly as a mechanism to clear damaged ER and misfolded proteins (240) so it is possible that IDR-1018 was promoting cell survival by activating autophagy to clear the accumulation of misfolded proteins. This was consistent with a previous study demonstrating that IDR-1018 relieved stalled autophagy associated with the CFTR mutation in CF epithelial cells (197).

2.3.5 Suppression of IDR-1018 induced chemokine activity by 2DG might have been due to activation of the PERK arm of the UPR

It has been documented that two of the stress-sensing arms of the UPR, namely IRE1α and PERK, are also major regulators of cellular immune responses (99, 101, 107, 109, 110). Activation of the PERK pathway in particular is associated with anti-inflammatory activity (101, 110). Several components of the PERK pathway, including ATF-4, DDIT3 (also known as C/EBP homologous protein, CHOP), CHAC1 and TRIB3 (100, 241, 242), were significantly upregulated in our RNA-Seq dataset. In fact, CHAC1, DDIT3 and TRIB3 were three of the five most upregulated genes in macrophages treated with IDR-1018 and 2DG compared to those treated with IDR-1018 alone (fold changes of 51.1, 8.0 and 10.7, respectively). The results from my analyses were thus consistent with the explanation that 2DG modulated IDR-1018 activity by activating the anti-inflammatory arm of the UPR. One major outcome of PERK induced DDIT3 and CHAC1 activity is disruption of the cellular redox state via increased ROS production and GSH depletion (240, 243-245). Thus, to determine if 2DG was activating this arm of the UPR, we measured the cellular redox state by assessing intracellular GSH concentrations. GSH levels were significantly depleted in macrophages treated with both 5 and 20 mM 2DG (**Figure 2.9**).

At the higher concentrations, IDR-1018 also caused a modest though insignificant drop in GSH. Addition of IDR-1018 did not alter depletion of GSH compared to 2DG alone, and was unable to restore GSH to the level observed for unstimulated cells. In addition, an increase in H_2DCFDA oxidation was observed in macrophages stimulated with 20 μg/ml IDR-1018, 20 mM 2DG or the combination of the two, suggesting that all three conditions induced ROS production. Thus, the significant decrease in GSH was likely indicative of cellular redox state disruption and activation of the PERK arm by 2DG.

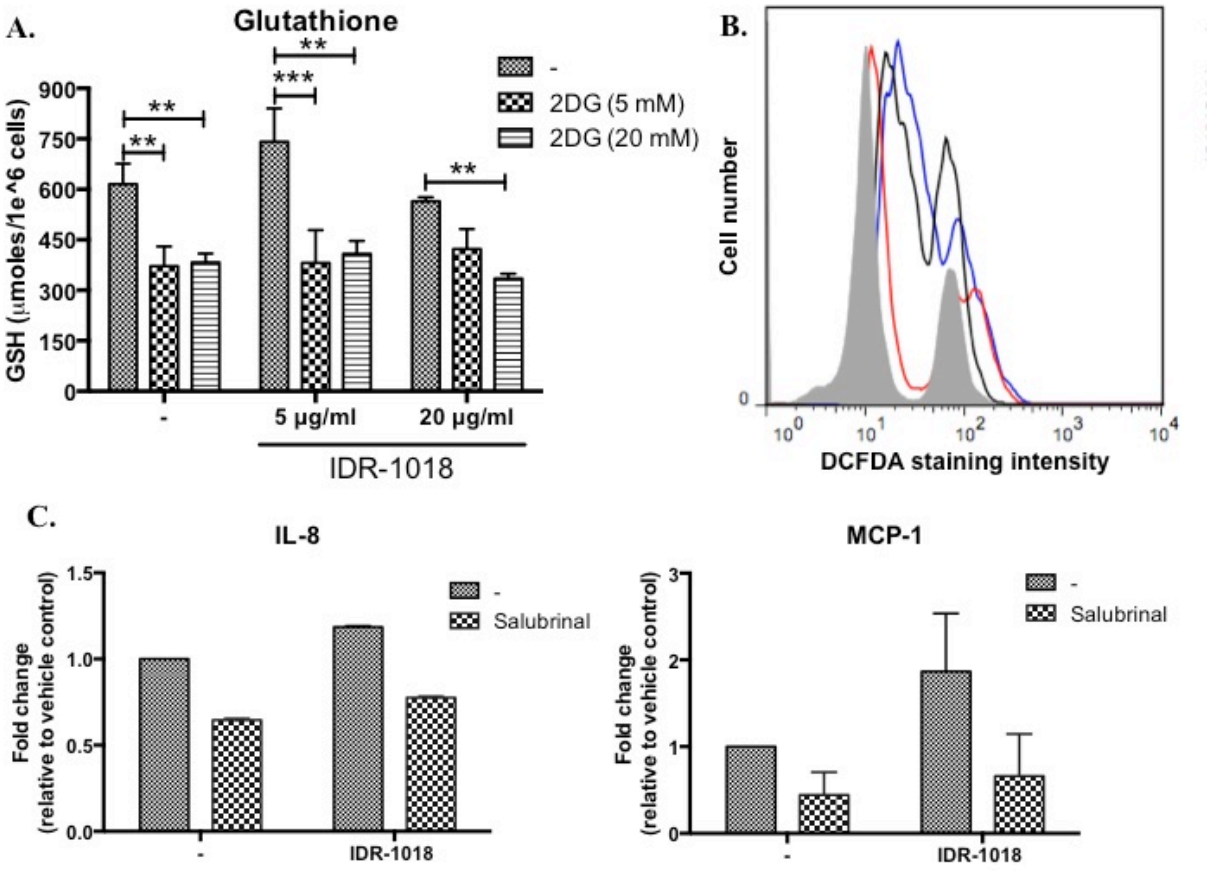

Figure 2.9: 2DG activated the PERK arm of the UPR, which altered cellular redox levels and inhibited IDR-1018 function. A) Macrophages were pretreated with 5 or 20 mM 2DG for 5 min then stimulated with IDR-1018 for 16 hrs after which intracellular GSH was measured. Data was compared using two-way ANOVA *$p < 0.05$. Data are the mean of 3 independent experiments, with error bars representing SEM. B) Macrophages were pretreated with 20 mM 2DG for 5 min then stimulated with 20 μg/ml IDR-1018 for 4 hrs. Cells were washed then incubated with the fluorescent dye, H2DCFDA for 30 min. Fluorescence measured via flow cytometry. Unstimulated cells (grey), IDR-1018 (black), 2DG (red), and IDR-1018+ 2DG (blue). Histogram presented is from one donor, representative of three. C) PBMCs were pre-treated with 5 μM of salubrinal then stimulated with 20 μg/ml IDR-1018. Chemokines in the supernatants were assayed via ELISA; the data are the average of two independent experiments, with error bars representing SEM.

To determine if specific activation of the PERK pathway could mimic the inhibition of IDR-1018 induced chemokine production by 2DG, a pharmacological activator of this pathway, salubrinal, was employed. Salubrinal inhibits GADD34, a phosphatase that negatively regulates the PERK pathway by dephosphorylating eIF2α (101). Activation of the PERK pathway by pre-incubation of cells with salubrinal showed a trend towards suppression of IDR-1018 mediated MCP-1 and IL-8 production by PBMCs (**Figure 2.9 C**) suggesting that activation of the PERK arm might be partially responsible for the modulation of IDR-1018 function by 2DG.

2.3.6 Both induction of the UPR and inhibition of glycolysis mimicked the effects of 2DG on IDR-1018 induced chemokine production

Although these data indicated that the UPR was the major pathway modulating IDR-1018 function, I sought to determine whether the effects of 2DG were due to its interference with glucose metabolism, the UPR or a combination of the two. To investigate whether dysregulation of either process could modulate IDR-1018 activity, I examined IDR-1018 mediated chemokine production using both a specific pharmacological activator of the UPR and an inhibitor of glucose metabolism (**Figure 2.10**). Tunicamycin activates the UPR and ER stress pathways by preventing N-linked glycosylation of proteins (246). Oxamate is an inhibitor of glycolysis dependent ATP production, via inhibition of lactate dehydrogenase alpha, as well as an inhibitor of the TCA cycle and oxidative phosphorylation (247). Both tunicamycin and oxamate significantly suppressed IDR-1018 induced MCP-3 production, suggesting that both pathways might modulate IDR-1018 activity, however, activation of the UPR appeared to have a greater, although not statistically significant, effect on MCP-3 production than inhibition of metabolism. Activation of the UPR also modestly suppressed IL-8 production, while inhibition of metabolism had no appreciable effect. These results thus support the likelihood that the effects of 2DG can be attributed to a combination of UPR activation and inhibition of glucose metabolism, although with activation of the UPR seeming to play a greater role.

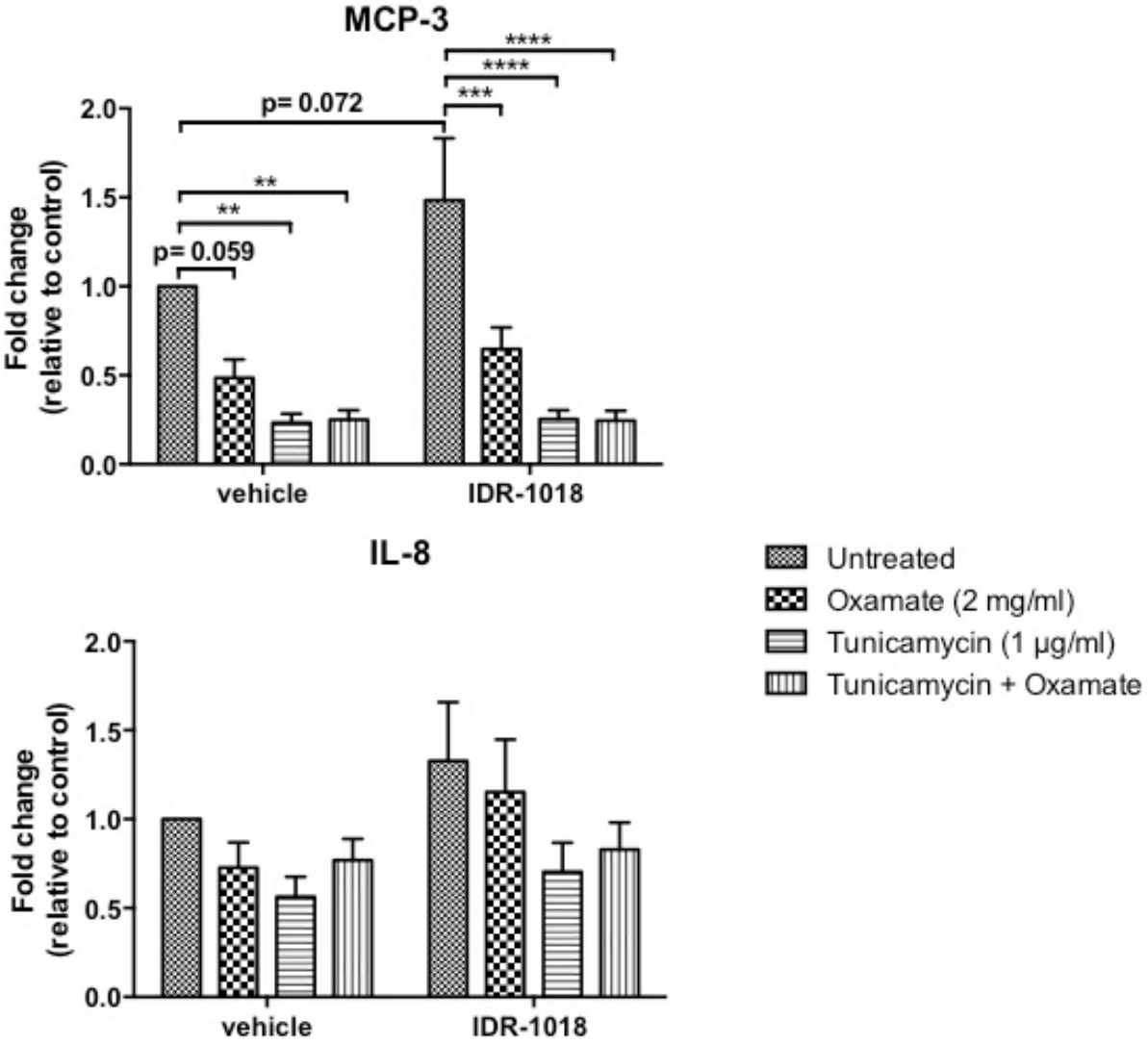

Figure 2.10: Both induction of the UPR and inhibition of glucose metabolism suppressed IDR-1018 mediated chemokine MCP-3 production. Macrophages were pretreated with tunicamycin for 6 hours then stimulated with oxamate and 20 µg/ml IDR-1018 for 16 hours. Supernatants were harvested and chemokines assayed via ELISA. The results represent the mean of seven independent experiments, with error bars representing SEM. Data was compared using one-way ANOVA ****$p < 0.0001$, ***$p < 0.001$ and **$p < 0.01$.

2.4 DISCUSSION

A greater appreciation for the interconnectivity of metabolism and the immune system has developed in recent years with the demonstration that immune cell function is regulated through metabolic reprogramming. Much of this research has utilized immune cells activated by TLR agonists and has applied metabolic inhibitors such as 2DG to demonstrate the inhibition of function. Although there have been some hints that immunomodulatory IDRs were able to influence cell growth (199), no research had been done on the role that nutrients and metabolism play in the immunomodulatory activity of these peptides. Here I showed that one of these

peptides, IDR-1018, not only enhanced metabolism in PBMCs and macrophages, but also that its activity was modulated by 2DG through dysregulation of glucose metabolism and the UPR. A model overviewing the findings is presented in **Figure 2.11**.

Similar to the results observed for cells stimulated with TLR agonists, IDR-1018 significantly enhanced glycolysis by macrophages (36-38, 55) as well as stimulated the production of several chemokines, a hallmark of immunomodulatory peptide activity. In addition, IDR-1018 activated the ERK signalling pathway in macrophages. Activation of the ERK signalling pathway has been linked to IDR peptide-induced chemokine production (172, 175) as well as induction of glycolysis in activated cells (35, 205, 206, 208). Thus, I propose that the activation of ERK is in part responsible for IDR-1018-induced chemokine production and glycolysis in macrophages (**Figure. 2.11**).

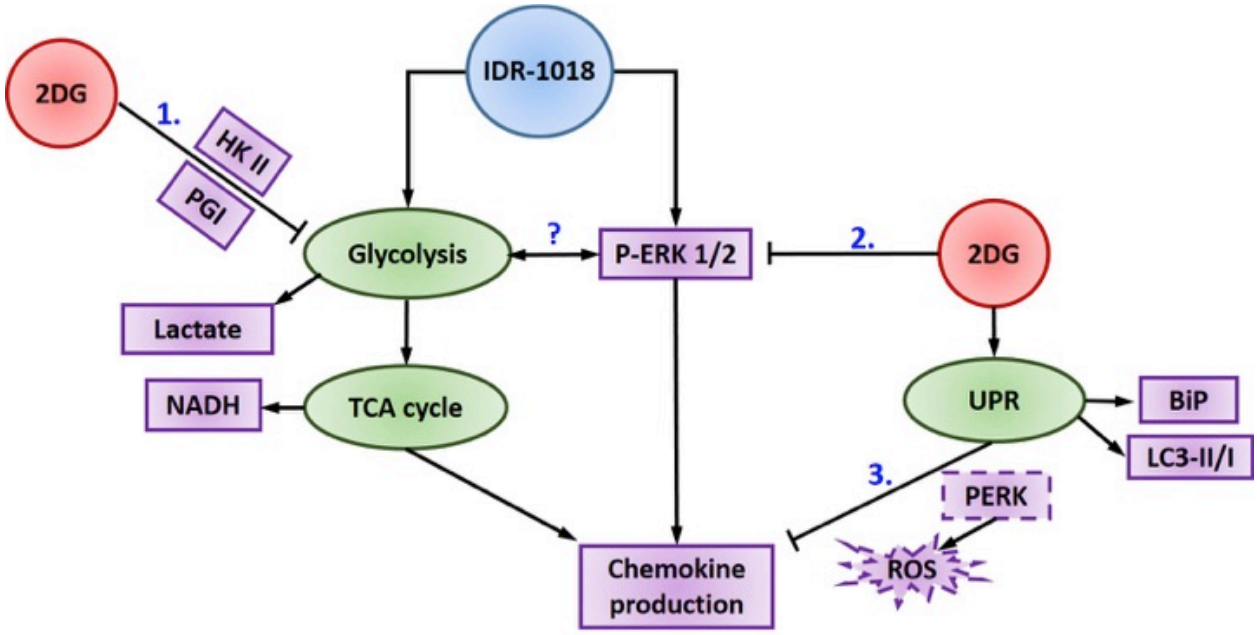

Figure 2.11: Proposed model for the modulation of IDR-1018 activity by 2DG. IDR-1018 induced chemokine production and ERK phosphorylation. ERK activation is required for chemokine induction by several other HDPs and IDRs. ERK activation also enhances glycolysis via c-Myc and HIF-1α activation. Thus, IDR-1018 induced glycolysis and chemokine production may both be via ERK activation. 2DG modulates IDR-1018 activity at three points: 1) direct inhibition of glycolysis via inhibition of HK II and PGI; 2) inhibition of IDR-1018 induced ERK phosphorylation (? symbol indicates a known, but unproven in the context of our study, linkage between pERK phosphorylation and regulation of glycolysis); and 3) activation of the UPR. Inhibition of chemokine synthesis via activation of the UPR maybe a result of one specific arm of this process, the PERK arm, the components of which induce excessive ROS production as well as inhibiting inflammatory signalling and pro-inflammatory cytokine production.

Inhibition of glycolysis using a well-known glycolytic inhibitor, 2DG suppressed IDR-1018-induced chemokine production by macrophages, suggesting that the activation of glycolysis is important to IDR-1018 activity. Although inhibition of glycolysis by 2DG depleted

intracellular ATP, this was unlikely to be the reason for suppressed IDR-1018 activity since Everts et al. (36) showed that inhibition of glycolysis-dependent ATP production had no effect on the inflammatory responses of dendritic cells. It is more likely that inhibition of glycolytic flux suppressed IDR-1018 activity through indirect inhibition of metabolic pathways linked to glycolysis, such as the TCA cycle and the pentose phosphate pathway (220). These pathways produce metabolites required for the synthesis of macromolecules such as nucleic acids and lipids that are essential for an effective immune response (36, 38, 220). In support of this, sodium oxamate, a glycolytic inhibitor that suppresses glucose-dependent macromolecule biosynthesis (247), significantly inhibited IDR-1018 induced MCP-3 production by macrophages. Based on the data presented here, I propose that IDR-1018 induced glycolysis to promote macromolecular biosynthetic processes for its immunomodulatory activities (**Figure 2.11**).

2DG also suppressed IDR-1018 induced ERK phosphorylation by macrophages, suggesting another mechanism by which 2DG suppressed IDR-1018 activity. Traves et al. (222) found that activation of the ERK signalling was key to LPS induced pro-inflammatory responses as well as basal and LPS-enhanced glycolytic flux. Therefore, inhibition of IDR-1018 activity by 2DG might be due to inhibition, downstream of ERK activation, of transcription factors that are responsible for the transcription of chemokines and cytokines such as AP-1 (248). Alternatively, inhibition of ERK activity by 2DG might indirectly suppress IDR-1018 activity by dysregulating ERK-dependent glycolytic flux.

Although 2DG appeared to modulate IDR-1018 function in part by inhibiting glycolysis, analysis of RNA-Seq transcriptomic data revealed that 2DG also significantly upregulated ER stress and UPR pathways. Increased BiP levels as well as the induction of autophagy, a property of 2DG-mediated activation of the UPR (226), confirmed that there was activation of the UPR by 2DG. Activation of the UPR by 2DG has also been reported by several other studies (226-230). Stimulation of macrophages with tunicamycin, also significantly inhibited IDR-1018 mediated MCP-3 production, and reduced IL-8 production, indicating that activation of the UPR was able to modulate IDR-1018 activity. The transcriptomic data also revealed that 2DG was significantly upregulating the anti-inflammatory PERK pathway. This was supported by the fact that 2DG also caused a significant decrease in GSH levels, and the demonstration that DDIT3 and CHAC1, two downstream components of the PERK pathway, were activated. These two proteins disrupt the cellular redox state through ROS production and depletion of GSH (240, 243-245). In addition, activation of the PERK pathway by salubrinal appeared to inhibit IDR-

1018 induced MCP-1 and IL-8 production by PBMCs. Activation of the PERK arm has been shown to suppress chemokine and cytokine production (101, 110, 249, 250). These data collectively suggest that, activation of the UPR, and the PERK arm in particular, by 2DG, also inhibited IDR-1018 activity (**Figure 2.11**).

The results presented in this chapter indicate that, much like TLR-agonists, IDR-1018 enhanced glycolysis, which was important for its ability to induce chemokine production. In addition, this study provided evidence to suggest that the induction of glycolysis and chemokine production might be mediated by activation of the ERK pathway. Finally, the results suggest that 2DG regulated IDR-1018 activity by modulating three distinct biological processes: inhibition of glycolysis, suppression of ERK signalling and activation of the UPR. It is possible that the immunosuppressive effects traditionally attributed to the inhibition of glycolysis by 2DG might actually be due to the combined effect of inhibition of glycolysis and activation of the UPR.

Chapter 3: INHIBITION OF GLYCOLYSIS AND INDUCTION OF THE UNFOLDED PROTEIN RESPONSE BY 2DG MODULATES THE ANTI-ENDOTOXIN ACTIVITY OF IDR-1018

3.1 INTRODUCTION

The ability of host defence peptides and IDRs to suppress the production of pathogen-induced pro-inflammatory mediators while inducing chemokine production and recruitment of immune cells to the site of infection is of particular relevance to their development as anti-infectives (141, 189, 190, 194). Suppression of pro-inflammatory responses by natural and synthetic immunomodulatory peptides is mediated, in part, by their ability to modulate signalling pathways induced by infectious agents and endogenous immune mediators (L. Madera, PhD thesis, UBC, 2012) (141, 251). However, the mechanism(s) underlying the anti-inflammatory activity of immunomodulatory peptides have not been well studied to date. In Chapter 2 it was revealed that IDR-1018 (192) enhanced metabolism in macrophages, and that this was important to its activity, since inhibition of glycolysis using 2DG and sodium oxamate suppressed IDR-1018 induced chemokine production. In addition, 2DG inhibited the activation by IDR-1018 of the ERK signalling pathway, a pathway key to the activation of metabolism and the production of chemotactic agents (35, 222), further supporting the role of glycolysis in IDR-1018 activity. It was hypothesized here that anti-endotoxin activity of IDR-1018 (193, 195) might also be due, in part, to its ability to regulate glycolysis.

Activation of an effective pro-inflammatory response by immune cells requires a rapid shift in cellular metabolism to aerobic glycolysis, a phenomenon known as the Warburg effect (23, 40). Metabolomic studies of macrophages and dendritic cells stimulated with LPS show an upregulation of glycolysis, while perturbation of the glycolytic pathway suppressed initial activation of the cells, expression of cell surface receptors, production of chemokines and cytokines, and phagocytosis (36-38, 55-58). This metabolic shift to aerobic glycolysis involves several processes including mTOR-dependent activation of the transcription factor, HIF-1α, which induces the transcription of several glycolytic enzymes, such as the enzymatic regulator, PFKFB3, an isoform that significantly enhances glycolysis (53). Additionally, Everts et al. (36) found that the initial metabolic reprogramming of dendritic cells required the activation of TBK1 and IKKε, important regulators of the type I interferon response (252, 253) that also activate AKT. Enhanced glycolysis, and glutaminolysis, is essential for the generation of metabolites from associated metabolic pathways such as the pentose phosphate pathway and TCA cycle,

which in turn are used for regulation of transcription factors and *de novo* synthesis of macromolecules (36, 38). Thus, modulation of the metabolic reprogramming induced by LPS might be a mechanism by which IDR peptides exert their anti-endotoxin activity.

Although 2DG was utilized in the previous chapter as an inhibitor of glycolysis, it was also shown to strongly activate all three arms of the UPR. Activation of the UPR, and the anti-inflammatory PERK arm (101, 109, 110) specifically, also suppressed IDR-1018 induced chemokine production. These results suggested that 2DG regulated the immunomodulatory activity of IDR-1018 through dysregulation of both glycolysis and the UPR. Thus, the focuses of the studies reported here was to determine whether the anti-endotoxin activity of IDR-1018 is a result of its ability to regulate metabolism, as well as to determine how 2DG modulates this anti-endotoxin activity. Here it was shown that IDR-1018-mediated suppression of LPS-induced chemokine and cytokine production might be due, in part, to its suppression of LPS-induced glycolytic flux. In addition, although 2DG dysregulated both metabolism and the UPR in peptide and LPS stimulated cells, its ability to enhance IDR-1018 anti-endotoxin activity was largely due to inhibition of glycolysis. Activation of the UPR exhibited a more complex regulation of the anti-endotoxin activity.

3.2 MATERIALS AND METHODS

3.2.1 Ethics statement and cells

Venus blood was collected from healthy volunteers, in accordance with the guidelines of the UBC Clinical Research Ethics Board (UBC-CREB# H04-70232), into vacutainer tubes containing sodium heparin (BD Bioscience, San Jose CA). PBMCs were isolated as described previously (190) and cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (Invitrogen, Carlsbad CA.) in a humidified incubator at 37°C and 5% CO_2.

3.2.2 Reagents

Peptides IDR-1018 (VRLIVAVRIWRR-NH) was synthesized using solid phase Fmoc chemistry and purified by reversed phase HPLC (CPC Scientific, Sunnvale, CA). The LPS of *Pseudomonas aeruginosa* PAO1 strain H103 was isolated from an overnight culture grown in Luria-Bertani broth at 37°C using the Darveau-Hancock method (254). The 2-keto-3-deoxyoctulosonate assay was used to quantify the LPS. 2-Deoxy-d-glucose (2DG) and sodium oxamate were purchased from Sigma Aldrich (St. Louis MO). Tunicamycin was purchased from EMD Millipore (ON, Canada). The potential cytotoxicity of the inhibitors used was measured using the colorimetric lactate dehydrogenase release cytotoxicity detection kit (Roche, Basel

Switzerland) or by assessing trypan blue exclusion. For trypan blue exclusion, the supernatant was removed and cells were washed with PBS before being incubated with a 0.2% trypan blue (Sigma Aldrich) solution for 1 min. Cells were then fixed in 4% methanol-free formaldehyde (Thermo Fisher Scientific, Waltham MA) for 10 mins, washed with PBS and imaged.

3.2.3 Generation of human monocyte-derived macrophages

Human monocyte-derived macrophages were generated as described previously (198). Briefly, PBMCs were seeded at $2x10^6$ cells/ml in serum free RPMI 1640 media for 45 minutes. Media was then gently removed and fresh RPMI 1640 media (HyClone Laboratories-GE healthcare Life Sciences, Mississauga, ON), supplemented with 10% FBS (PAA Laboratories-GE healthcare Life Sciences), was added. Cells were incubated at 37°C and 5% CO_2 for 24 hours after which, the media was removed then replaced with RPMI 1640 containing 10% FBS and supplemented with 10 ng/ml of M-CSF (Research Diagnostic Inc, Concord, MA). The cells were incubated for 5 days. On the sixth day, the cells were washed to remove non-adherent cells and medium replaced with fresh RPMI 1640 containing 10% FBS and 10 ng/ml of M-CSF. On day seven, cells were washed to remove any remaining non-adherent cells and were ready for use.

3.2.4 Metabolism assays

A modified form of the WST-1 cell proliferation assay (Roche) was used to assess the ability of LPS to alter cellular metabolism. PBMCs were seeded at $1x10^5$ cells/ well in a 96 well plate and 10 µl of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) was added. Cells were incubated for 2 hours at 37°C in 5% CO_2, after which the differential absorbance at 450nm (A_{450}) and 900nm (A_{900}) was measured (0-hr measurement) to assess metabolic activity through reduction of the Wst-1 reagent into formazan. The cells were then stimulated with 10 ng/ml LPS and incubated at 37°C and 5% CO_2 for a further 4 hours with metabolic activity measurements made every 30 minutes. Stimulations were completed in triplicate. The results were plotted as the average metabolic activity (A_{450}-A_{900}) as a function of time for four independent experiments.

To measure glycolysis, macrophages were stimulated with 10 ng/ml LPS alone or in combination with 20 µg/ml of IDR-1018 for 4 hours or 20 mM of 2DG for 24 hours. Supernatants were harvested and the end product of glycolysis, lactate, measured using a colorimetric lactate assay kit (Trinity Biotech, Bray Ireland) as per the manufacturer's instructions. Results were the average of three independent experiments.

Intracellular ATP was measured using the Celltiter-Glo 2.0 Luminescent Cell Viability

assay (Promega, Madison WI) according to the manufacturer's instructions with minor modifications. Briefly, macrophages, cultured in 48 well plates, were pretreated with 20 mM 2DG for five min then stimulated with 10ng/ml LPS for 24 hours. Media was removed and used for the above-described colorimetric lactate assay. The cells were lysed in the same plate using the kit reagent and lysates were transferred to a black walled clear bottom 96 well plate and luminescence was measured. Data for both lactate and ATP were reported as percentages of the untreated control. Results were the average of five independent experiments.

3.2.5 RNA-Seq sample preparation and analysis

Genome Analyzer IIx platform. Macrophages were pretreated with 20 mM 2DG then stimulated with 10 ng/ml LPS alone or in combination with 5 μg/ml IDR-1018 for 24 hours. RNA was isolated from cell lysates using the Qiagen RNeasy Isolation Kit (Qiagen, Valencia CA). The RNA concentration, integrity and purity were assessed using the RNA Nano Kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). The Illumina Truseq RNA sample preparation guide was used for library preparation (Illumina catalogue number FC-122-1002). Briefly, mRNA, purified from 500 ng of total RNA using poly-dt beads, was used to synthesize cDNA. This was followed by end repair, 3' end adenylation and ligation of adaptors containing unique barcodes. Finally, DNA containing the adaptor molecules was enriched and amplified using PCR then quantified. Cluster generation was carried out on a CBOT instrument using 8 pM of dsDNA followed by RNA sequencing on a GAIIx instrument (Illumina, San Diego CA), performed as a single read run. Raw data was demultiplexed and converted to FASTQ files using CASAVA. Bowtie 2 (210) and TopHat2 (211) were then used to align the reads to the Ensembl GRCh37.74 reference genome after which SAMtools (212) was used to sort and index the bam and sam files. Read count tables were generated using htseq-count. Differential gene expression analysis was performed using edgeR (213), using a design formula that corrected for donor variability. Bioinformatic analysis of the data sets was carried out using two systems biology tools developed in our laboratory, Innate Db database (214) (www.innatedb.ca) and NetworkAnalyst (72) (www.networkanalyst.ca/). The cut-off for differential expression was defined as a fold change of +/- 1.5 or greater and a p-value <0.05. Samples from two independent experiments were run.

3.2.6 Enzyme-linked Immunosorbent Assay (ELISA)

Tissue culture supernatants were centrifuged at 2000 xg for 5 minutes to remove any cells then frozen at -20°C. Cytokine levels were measured by ELISA, using antibody pairs for

MIP-1α, IL-8 (Biosource), MCP-1, MCP-3 (Research Diagnostic Systems, Minneapolis MN), TNF-α and IL-6 (eBioscience, San Diego CA), according to the respective manufacturers' instructions.

3.2.7 Statistical analysis

Data were normalized to the absolute values obtained for the LPS-treated condition in order to reduce the significant donor-to-donor variability with respect to absolute values. The resulting values were presented as fold-change relative to LPS. Data was analyzed using Graphpad Prism software, version 6. Repeated measures two-way ANOVA was used to analyze the results of the WST-1 assay. Two-way ANOVA was used for all other experiments. In all cases, $p<0.05$ was considered statistically significant.

3.3 RESULTS

3.3.1 The glycolytic inhibitor, 2DG enhanced the anti-endotoxin activity of IDR-1018

In Chapter 2, I showed that 2DG, an inhibitor of glycolysis (218), suppressed the ability of the peptide, IDR-1018 to induce chemokine production, a hallmark response to immunomodulatory peptides. We sought to determine if 2DG was also capable of modulating the anti-endotoxin activity of IDR-1018, another major hallmark function of immunomodulatory peptides. To examine this, macrophages were stimulated with 10 ng/ml LPS alone or in combination with 5 μg/ml IDR-1018 in the presence or absence of 2DG. IDR-1018 significantly suppressed LPS-induced TNF-α, IL-6, IL-8 and MIP-1α to varying degrees (**Figure 3.1**). IDR-1018 also suppressed MCP-1, although not to a statistically significant degree.

2DG appeared to act as an anti-inflammatory agent, significantly inhibiting LPS induced IL-6, TNF-α, MCP-1, MIP-1α and IL-8 production. 2DG also enhanced IDR-1018 anti-endotoxin activity. The combination of 2DG and IDR-1018 suppressed LPS induced cytokine and chemokine production to a greater extent than 2DG or IDR-1018 alone. This was most evident for MIP-1α, IL-8, TNF-α, and IL-6. The inhibition of TNF-α and IL-6 production by 2DG demonstrated here was in agreement with the studies of Everts et al. (36) who found that 2DG suppresses LPS-induced TNF-α, and IL-6 by dendritic cells through inhibition of the rapid glycolytic burst initiated upon activation of the cells. Furthermore, Dieti et al. (255) showed that 2DG dose-dependently suppressed TNF-α production by monocytes. In contrast, Tanahill et al. (38) showed that TNF-α and IL-6 were unaffected by 2DG and therefore not dependent on aerobic glycolysis. It is possible that these discrepancies are due to differences in LPS and 2DG

doses used or the length of exposure.

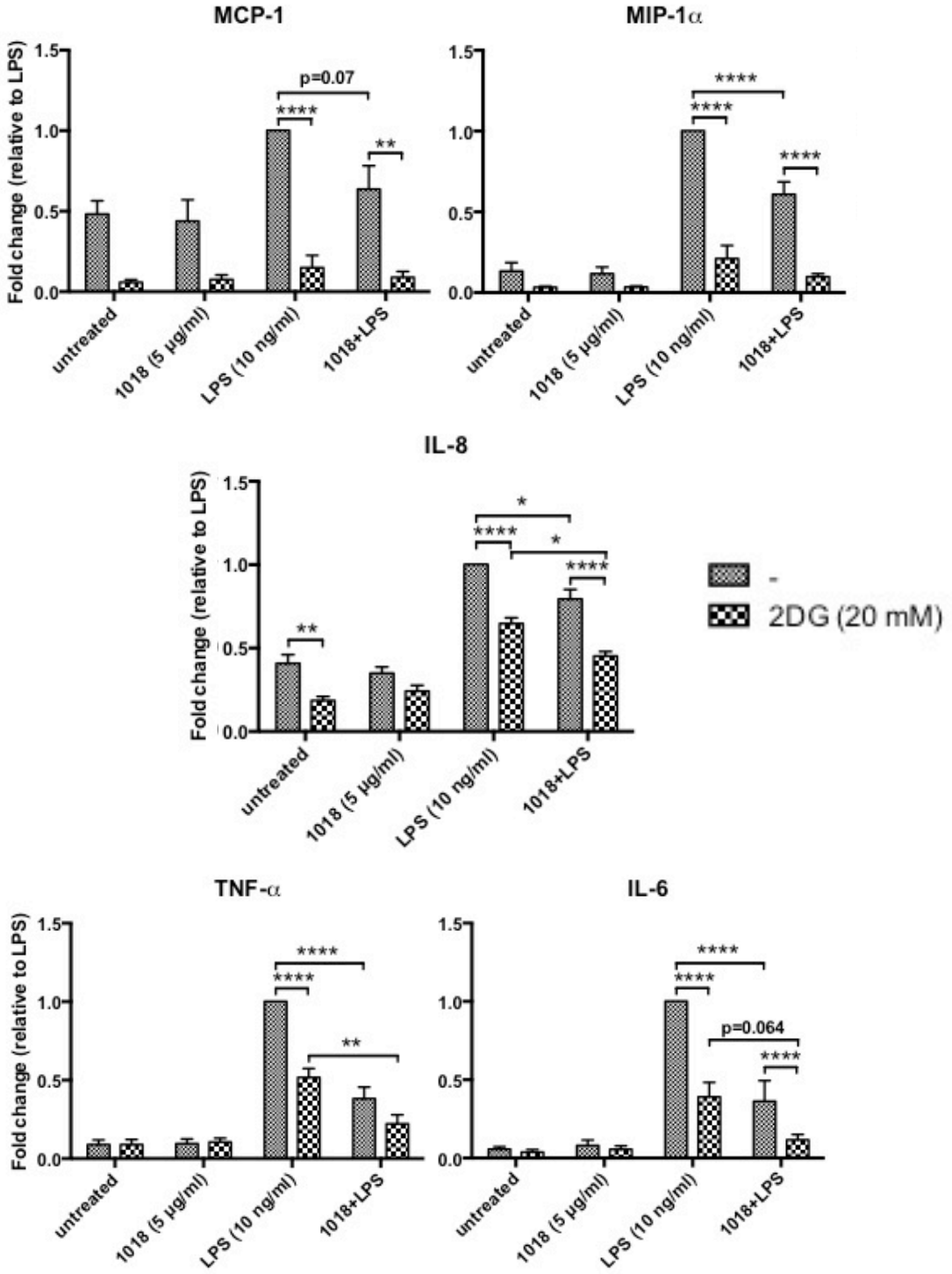

Figure 3.1: 2DG selectively acted as an anti-inflammatory agent and selectively enhanced the anti-inflammatory activity of IDR-1018. Macrophages were pretreated with 20 mM of 2-deoxy-D-glucose (2DG) for 5 min then stimulated with 10 ng/ml LPS, 5 μg/ml IDR-1018 or the combination of the two for 24 hrs. Supernatants were harvested and chemokines assayed via ELISA. Minimum of 4 independent experiments were completed, with error bars representing SEM. Data was compared using two way ANOVA ****P<0.0001, ***P<0.005, **P<0.01 and *P<0.05.

3.3.2 IDR-1018 and 2DG suppressed LPS-induced glycolysis

Previous studies have shown that stimulation of macrophages and dendritic cells with LPS results in the upregulation of glycolysis and that this upregulation was essential to mounting a pro-inflammatory response (36-38, 57, 58). Thus we assessed the ability of LPS to alter cellular metabolism using a tetrazolium salt-based, WST-1 cell viability assay that indirectly measured glycolysis and TCA cycle-dependent NADH and NADPH production by assessing the reduction of the salt into coloured formazan (216, 217). Stimulation of peripheral blood mononuclear cells caused a slight though statistically significant decrease in the reduction of the tetrazolium salt starting at 2.5 hours post-stimulation (**Figure 3.2**).

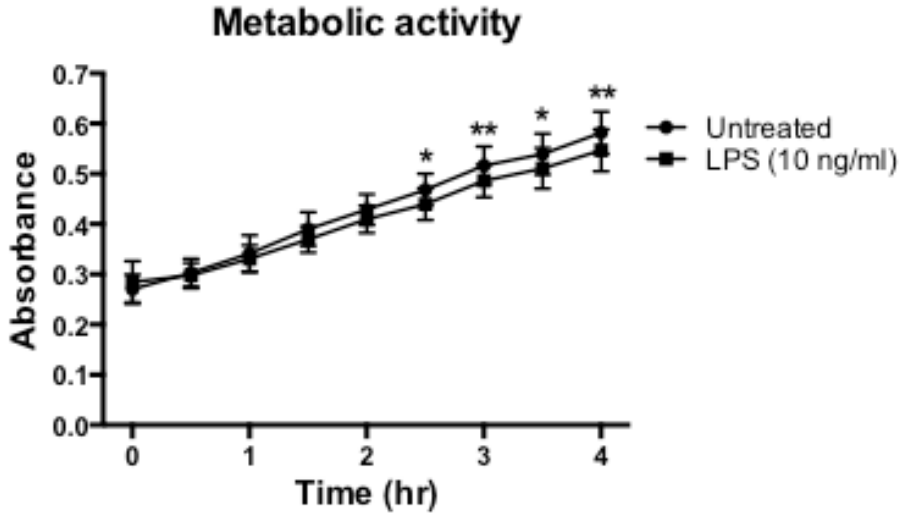

Figure 3.2: LPS appeared to inhibit NADH and NADPH production. PBMCs were loaded with the tetrazolium salt, WST-1, for 2 hours then stimulated with 10 ng/ml of LPS for 4 hours. Absorbance was read every 30 min. Four independent experiments were completed, with error bars representing SEM. Data was compared using repeated measures two-way ANOVA **$p<0.01$ and *$p<0.05$.

A previous study found that inhibition of glycolysis suppressed reduction of tetrazolium salt, which would suggest that LPS was inhibiting glycolysis (216, 217). However, this contradicts the results of several studies, which showed that LPS induces a metabolic shift to aerobic glycolysis (46). LPS-induced metabolic reprogramming results in the suppression of the catabolic activity of the TCA cycle, which would prevent TCA cycle-dependent NADH production (46). Because the TCA cycle produces four times as much NADH as glycolysis, suppression of the catabolic activity of the TCA cycle upon LPS stimulation might explain the significant decrease in tetrazolium salt reduction. To gain a better understanding of LPS-induced changes in glycolysis, I chose, as a better indicator of glycolytic activity, to measure lactate production, the end product of glycolysis. LPS induced a significant increase in lactate

production by macrophages indicating that it was enhancing glycolysis. Stimulation of macrophages with LPS in combination with IDR-1018 or 2DG (**Figure 3.3 A,B**) resulted in a substantial decrease in lactate levels. Interestingly, in Chapter 2 I showed that macrophages stimulated with IDR-1018 by itself demonstrated significantly increased lactate production indicating that IDR-1018 also induced glycolysis and that the combination is antagonistic rather than synergistic. Suppressing glycolysis would prevent the induction of pro-inflammatory responses to TLR agonists like LPS (36-38). This indicates that the anti-endotoxin activity of both IDR-1018 and 2DG could be linked to their ability to suppress LPS-induced increases in glycolysis.

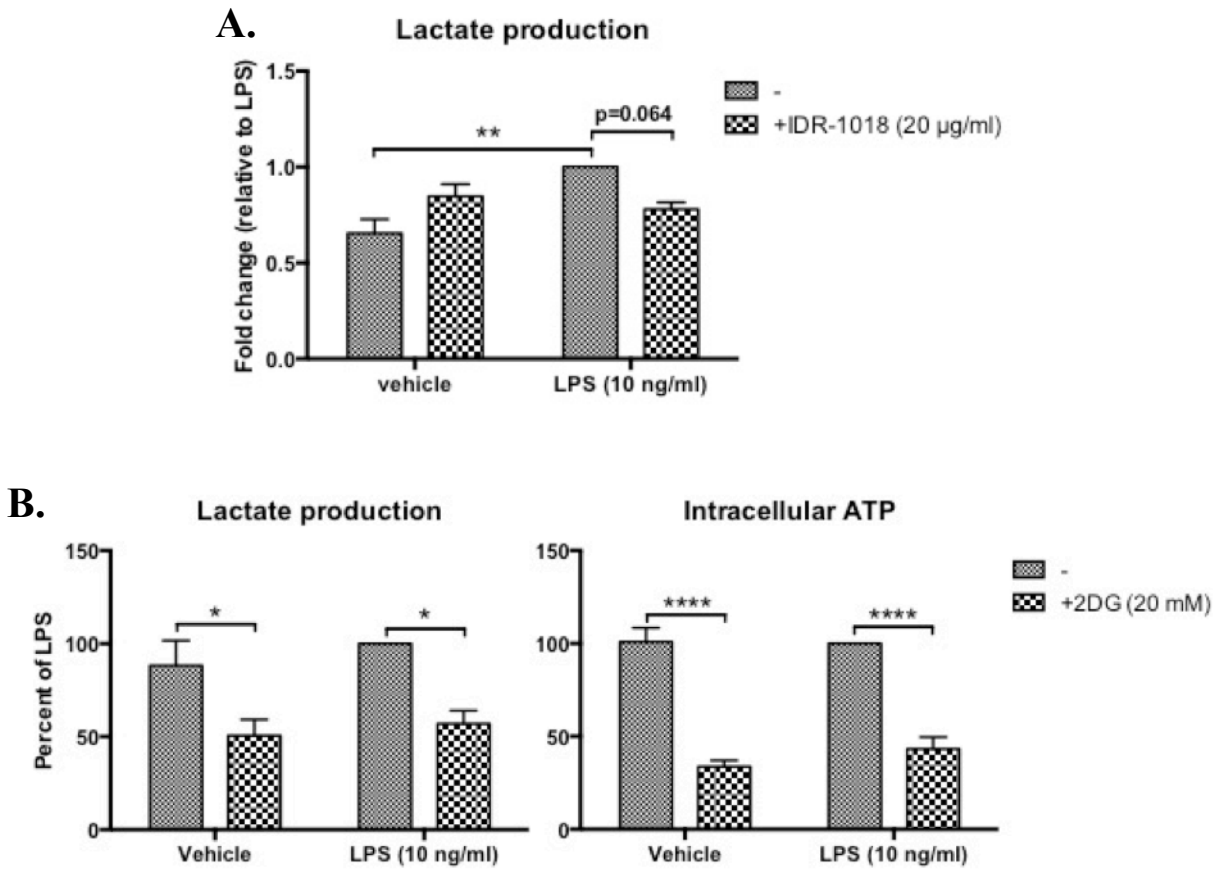

Figure 3.3: IDR-1018 and 2DG suppressed LPS induced increases in glucose metabolism. (A) Macrophages were stimulated with 10ng/ml of LPS alone or in the presence of 20 µg/ml 1018 for 4 hours. Following treatment, supernatants were collected and lactate levels were measured. Data are presented as the mean fold change relative to LPS± SEM. Data were compared using 2-way ANOVA, **p<0.01. (B) Macrophages were stimulated with 10 ng/ml of LPS alone or in the presence of 20 mM 2DG for 24 hours. Supernatants were then collected and assayed for lactate (left). Intracellular ATP (right) was measured after lysing the cells. Data are presented as mean percentage of LPS stimulated cells ± SEM. Data were compared using 2-way ANOVA, *p<0.05, ****p<0.0001.

Mounting a pro-inflammatory response is an energy-intensive process (219) and

inhibition of glycolysis in cells that utilize aerobic glycolysis can result in a substantial decrease of energy stores. Therefore, the ATP level of macrophages stimulated with LPS in the absence or presence of 2DG was also assessed. LPS had no effect on ATP levels. This correlates with the results of Everts et al. (36), who found that although LPS causes an initial decline in ATP levels, these return to pre-stimulation levels approximately 4 hours later. Pretreatment with 2DG significantly decreased ATP levels in the control and LPS stimulated cells (**Figure 3.3 B**). Mounting an effective pro-inflammatory response involves substantial biosynthetic activity, which is highly energy dependent (27, 219). Thus, inhibition of glycolysis by 2DG may suppress pro-inflammatory responses to LPS in part by depleting energy stores. However pro-inflammatory responses to LPS have been shown to be independent of glycolytic or mitochondrial ATP production (36). Inhibition of glycolysis obstructs several downstream metabolic pathways such as the TCA cycle and the pentose phosphate pathway, which produce energy and biosynthetic precursors (27, 36, 38). It is possible that 2DG was suppressing macrophage pro-inflammatory responses to LPS through inhibition of downstream pathways. Regardless, the data indicate that 2DG could suppress LPS-induced pro-inflammatory responses through the inhibition of glycolysis. However in Chapter 2 I showed that 2DG regulated IDR-1018 activity not only through inhibition of glycolysis but through activation of the UPR as well. Several groups have also reported activation of the UPR by 2DG and the resultant inhibition of innate immune cell responses (229, 256, 257). Thus, it remained unclear whether inhibition of glycolysis was the sole reason for 2DG-mediated inhibition of LPS induced pro-inflammatory responses and by extension the enhancement of IDR-1018-mediated anti-endotoxin activity.

3.3.3 LPS stimulation upregulated pathways associated with the induction of aerobic glycolysis as well as the type 1 interferon response, which was downregulated by IDR-1018

To further characterize the anti-endotoxin activity of IDR-1018 and to provide evidence as to whether 2DG caused an enhancement of the anti-endotoxin activity of IDR-1018 through its ability to suppress glycolysis and/or activate the UPR, a transcriptomic analysis using RNA-Seq was performed. Macrophages were stimulated with LPS, IDR-1018 or a combination of the two in the presence or absence of 2DG. The sequencing was carried out using cells stimulated for 24 hours in order to characterize the effects of prolonged exposure to the various treatments. Biological pathway overrepresentation analysis was carried out using InnateDB (214) on the genes differentially expressed as a result of these treatments. In addition, NetworkAnalyst (72), a systems biology tool developed in our lab, was used to generate networks, based on curated protein-protein interactions, of biological pathways containing the differentially expressed genes.

Treatment of macrophages with LPS alone resulted in the altered expression of genes associated with the inflammatory response and TLR signalling, as expected (**Table 3.1**).

Table 3.1: Selected overrepresented biological pathways associated with the immune response, as determined by assessing differentially expressed genes in LPS stimulated macrophages compared to untreated controls and IDR-1018 and LPS stimulated cells compared to LPS stimulated cells.

Condition	Over represented Biological Pathway	*p* Value
LPS vs. no LPS	Cytokine signalling in immune system	3.1E-22
	Immune System	4.5E-19
	Interferon signalling	6.2E-16
	Interferon alpha/beta signalling	1.4E-13
	Interferon gamma signalling	5.5E-9
	Toll-like receptor signalling pathway	7.0E-5
IDR-1018+LPS vs. LPS:	Interferon alpha/beta signalling	1.6E-6
	Immune System	5.7E-5
	Interferon signalling	7.2E-5
	Cytokine signalling in immune system	8.1E-5
	Antiviral mechanism by IFN-stimulated genes	1.1E-3

Biological pathway overrepresentation analysis revealed that both the MyD88 dependent and independent arms of TLR signalling were activated in response to LPS stimulation with the Type I interferon response being one of the most overrepresented pathways.

LPS stimulation also upregulated pathways associated with the induction and maintenance of aerobic metabolism. Stimulation of macrophages with LPS resulted in the dysregulation of the mTOR pathway, a major regulator of metabolism and macrophage polarization (46, 258) (**Figure 3.4**).

Of particular interest was the increased expression of four components of this pathway, RICTOR, the catalytic gamma subunit of PI3K, HIF-1α and metabolic enzyme PFKFB3, which have been identified as critical regulators of LPS-induced activation of glycolysis (37, 46). Two intermediates of the Type I interferon response, TBK-1 and IKKε (252, 253), are also important for the early induction of glycolysis by LPS through activation of AKT and regulation of hexokinase-II activity (36). TBK-1, but not IKKε, was upregulated in response to LPS stimulation. These results indicate that LPS was inducing a shift to aerobic glycolysis by macrophages at the transcriptional level, in order to activate pro-inflammatory responses.

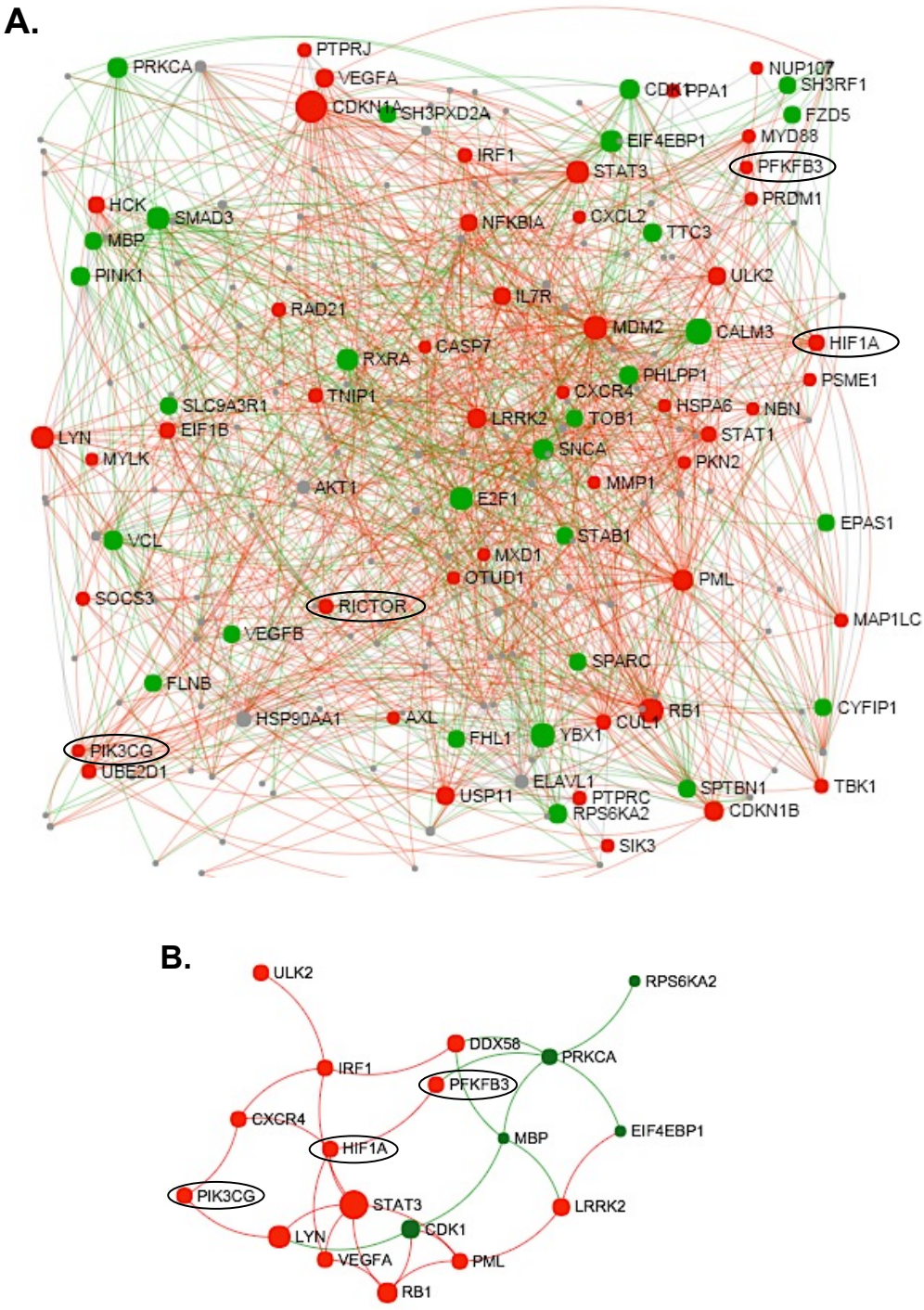

Figure 3.4: Stimulation of macrophages with LPS dysregulated the mTOR signalling pathway. Protein:protein interaction networks for macrophages stimulated with LPS were generated using NetworkAnalyst to identify the biological network represented by the genes dysregulated in response to LPS compared to control cells. The mTOR signalling pathway was extracted from the larger network (Panels A and B). Panel (A) contains dysregulated genes as well as their non-dysregulated interacting partners (first-order) while panel (B) was generated from dysregulated genes with curated direct (zero order) interactions (green, downregulated; red, upregulated; grey, not differentially expressed). Genes of interest are highlighted in black circles.

The concentration of IDR-1018 used to study its anti-endotoxin activity was sub-optimal for stimulating an immune response. IDR-1018 did demonstrate a mild anti-endotoxin effect at the transcriptional level, causing the differential expression of 23 genes (21 downregulated, 2 upregulated) compared to LPS alone. Biological pathway overrepresentation analysis and construction of a biological network of the differentially expressed genes revealed that IDR-1018 downregulated the TLR mediated type I interferon response (**Table 3.1 and Figure 3.5**).

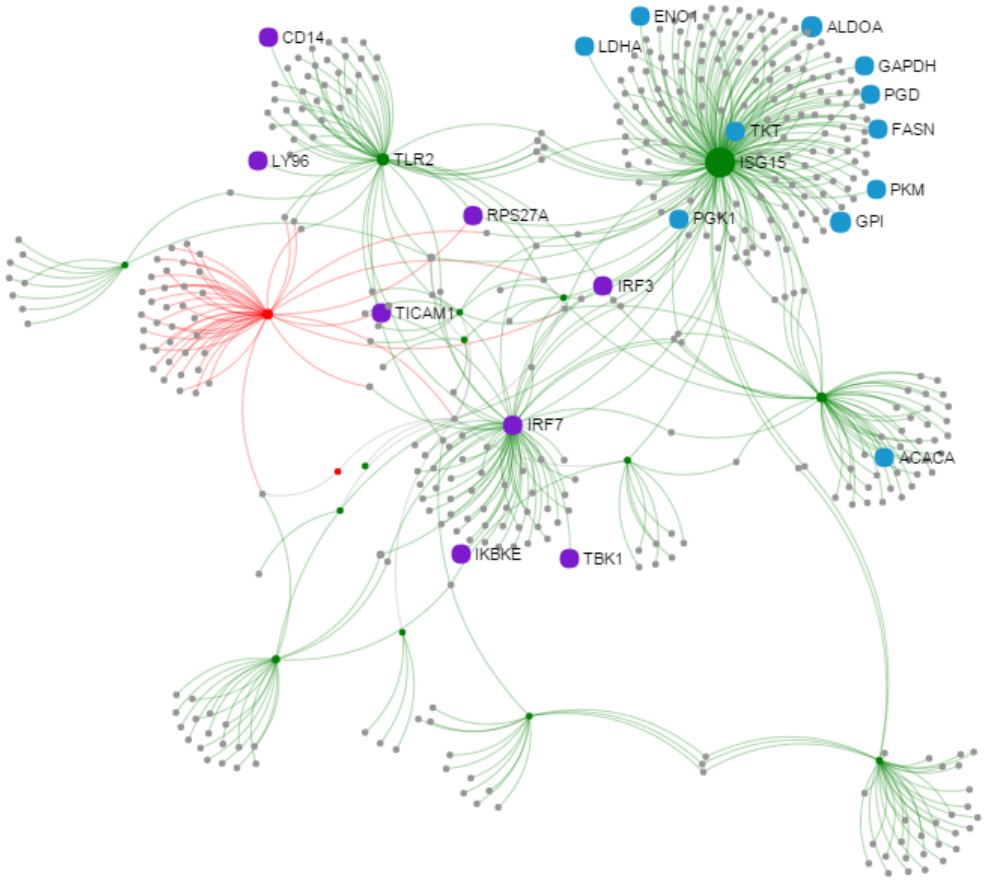

Figure 3.5: IDR-1018 suppressed LPS-induced interferon signalling. Protein:protein interaction networks for macrophages stimulated with LPS alone or in combination with IDR-1018 were generated using NetworkAnalyst by assessing genes differentially expressed in response to LPS combined with IDR-1018 compared to cells stimulated with LPS alone. In network A) only curated genes related to the interferon signaling pathways are presented. Node and edge color denotes gene expression (green, downregulated; red, upregulated; grey, not differentially expressed). The TBK1/IKKε pathway is highlighted in purple; ISG15 interactors are highlighted in blue.

Interestingly, one of the pathways within this network that appeared to be down-regulated by IDR-1018 was the TBK1/IKKε signalling pathway, the regulator of early metabolic reprogramming in dendritic cells (36), suggesting that IDR-1018 might have mediated early suppression of glycolysis via interference with this pathway. Another component of the type I

interferon response down-regulated by IDR-1018, interferon stimulated gene (ISG)15 (259), has also been linked to the regulation of glycolysis possibly by covalent conjugation to glycolytic enzymes, although the effect that this has on glycolytic flux remains unexplored (260). In some cases, ISGylation of a target protein appears to inhibit polyubiquitination and proteosomal degradation, consequently preventing protein turnover and potentially leading to prolonged activation (261, 262). If ISGylation of glycolytic proteins prevents their turn over and extends their activity, then it is possible that IDR-1018 might have inhibited LPS-induced glycolytic flux by suppressing ISG15 expression. However since so little is known about the function of ISGylation apart from its role in the anti-viral response, more research into this area is required. Regardless, these results suggest that IDR-1018 might regulate LPS induced glycolysis at the transcriptional level, possibly through modulation of the type I interferon response.

3.3.4 Exposure to 2DG enhanced IDR-1018 anti-endotoxin activity through inhibition of the TCA cycle and oxidative phosphorylation combined with induction of the UPR

To understand how 2DG was enhancing the anti-endotoxin activity of IDR-1018, I compared the transcriptional responses of macrophages stimulated with LPS, IDR-1018 and the combination of the two, with those also treated with 2DG. Treatment with 2DG resulted in altered expression of nearly 5000 genes. To carry out a biological pathway overrepresentation analysis, the differentially expressed genes from each comparison were split into lists containing either upregulated or downregulated genes. The biological pathways overrepresented as a result of 2DG treatment were nearly identical for all three comparisons (**Table 3.2**).

Table 3.2: Selected biological pathways overrepresented in response to 2DG stimulation in all comparison analyzed as determined by examining genes that were differentially expressed cf. non-2DG stimulated cells.

Overrepresented Biological Pathway	
Upregulated genes	**Downregulated genes**
Translation	The citric acid (TCA) cycle and respiratory electron transport
Peptide chain elongation	Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.
Protein processing in the endoplasmic reticulum	Respiratory electron transport
Influenza life cycle	Cytokine signalling in the immune system
Unfolded protein response	Interferon signalling
Golgi to ER transport	Endogenous TLR signalling
mTOR signalling	Chagas disease

Biological pathway overrepresentation analysis of the downregulated genes from each comparison revealed that a longer exposure to 2DG resulted in significant dysregulation of the citric acid (TCA) cycle and oxidative phosphorylation (**Figure 3.6**).

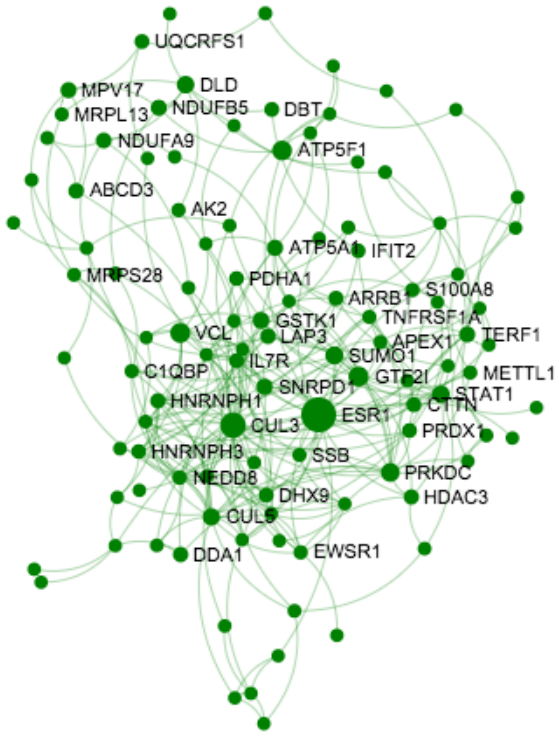

Figure 3.6: 2DG downregulated the expression of genes involved in the TCA cycle and oxidative phosphorylation. Protein:protein interaction network for macrophages stimulated with LPS and IDR-1018 in combination with 2DG. The networks was generated using NetworkAnalyst by assessing genes downregulated in response to LPS and IDR-1018 combined with 2DG compared to cells stimulated with only LPS and IDR-1018.

Previous studies have shown that induction of glycolysis in activated macrophages and dendritic cells is not only essential for ATP generation but also for fueling the TCA cycle (27, 36, 38). Two TCA cycle metabolites, succinate and citrate, were identified as being essential to pro-inflammatory responses. They are required for activation of pro-inflammatory transcription factors like HIF-1α, which enhances glycolysis and inflammation through increased transcription of glycolytic enzymes and pro-inflammatory mediators as well as fatty acid synthesis, which is required for increased biogenesis of the ER and Golgi membranes (36, 38, 55). These results indicate that 2DG was, in fact, enhancing IDR-1018 anti-endotoxin activity through inhibition of metabolism. However, the genes upregulated as a result of 2DG treatment were substantially associated with ER stress and the UPR (**Table 3.2 and Figure 3.7**). Pathways associated with protein translation, processing, folding and transport were also significantly overrepresented indicating that 2DG also modulated the anti-endotoxin activity of IDR-1018 activities through

UPR activation.

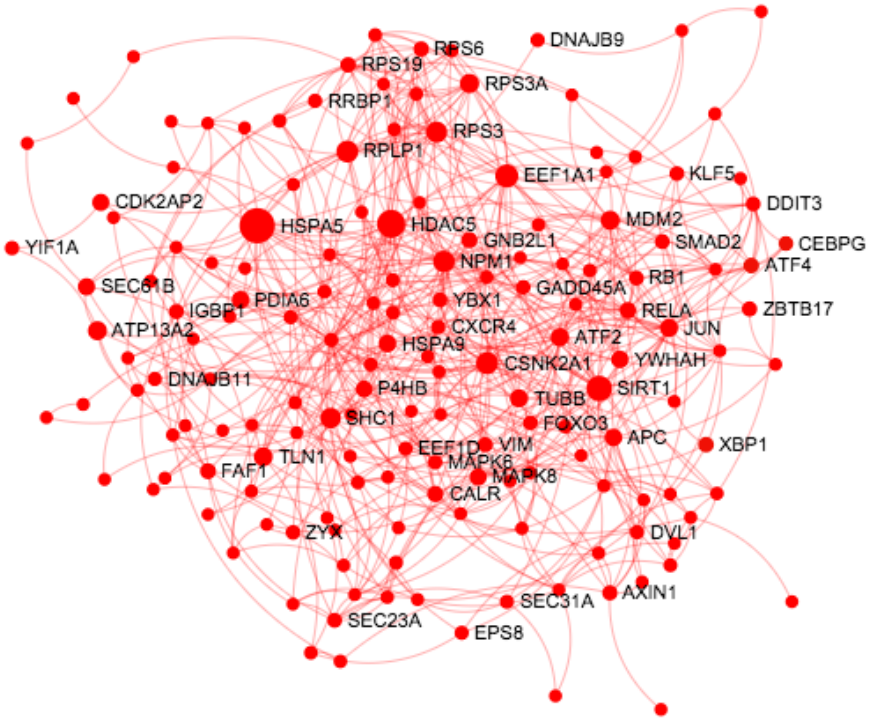

Figure 3.7: 2DG upregulated genes involved in ER stress and the unfolded protein response. Protein interaction network for macrophages stimulated with LPS and IDR-1018 in combination with 2DG. The networks was generated using NetworkAnalyst by assessing genes upregulated in response to LPS and IDR-1018 combined with 2DG compared to cells stimulated with only LPS and IDR-1018.

Interestingly, when 2DG was used in combination with LPS, only genes downstream of IRE1α activation and a few genes downstream of PERK were upregulated. However, when in the presence of IDR-1018 or the combination of IDR-1018 and LPS, 2DG upregulated the expression of genes associated with all three arms of the unfolded protein response, IRE1α, PERK and ATF6, Thus, 2DG enhanced IDR-1018 anti-endotoxin activity while suppressing its ability to induce chemokines, likely through the activation of the UPR coupled with suppression of the TCA cycle.

3.3.5 Inhibition of glucose metabolism and activation of the unfolded protein response mimicked the anti-endotoxin effect of 2DG

To determine if inhibition of metabolism, activation of UPR, or a combination of the two, could modulate IDR-1018 anti-endotoxin activity I assessed LPS-induced chemokine and cytokine production in the presence of IDR-1018 with or without the addition of sodium oxamate and tunicamycin. Sodium oxamate is a metabolic inhibitor (247) while tunicamycin is an activator of the UPR (246) but has no known effect on glycolysis. Sodium oxamate alone and

in combination with IDR-1018 significantly suppressed LPS-induced MCP-1, MCP-3, MIP-1α and IL-6 as well as inducing a modest reduction in IL-8 and TNF-α (**Figure 3.8**).

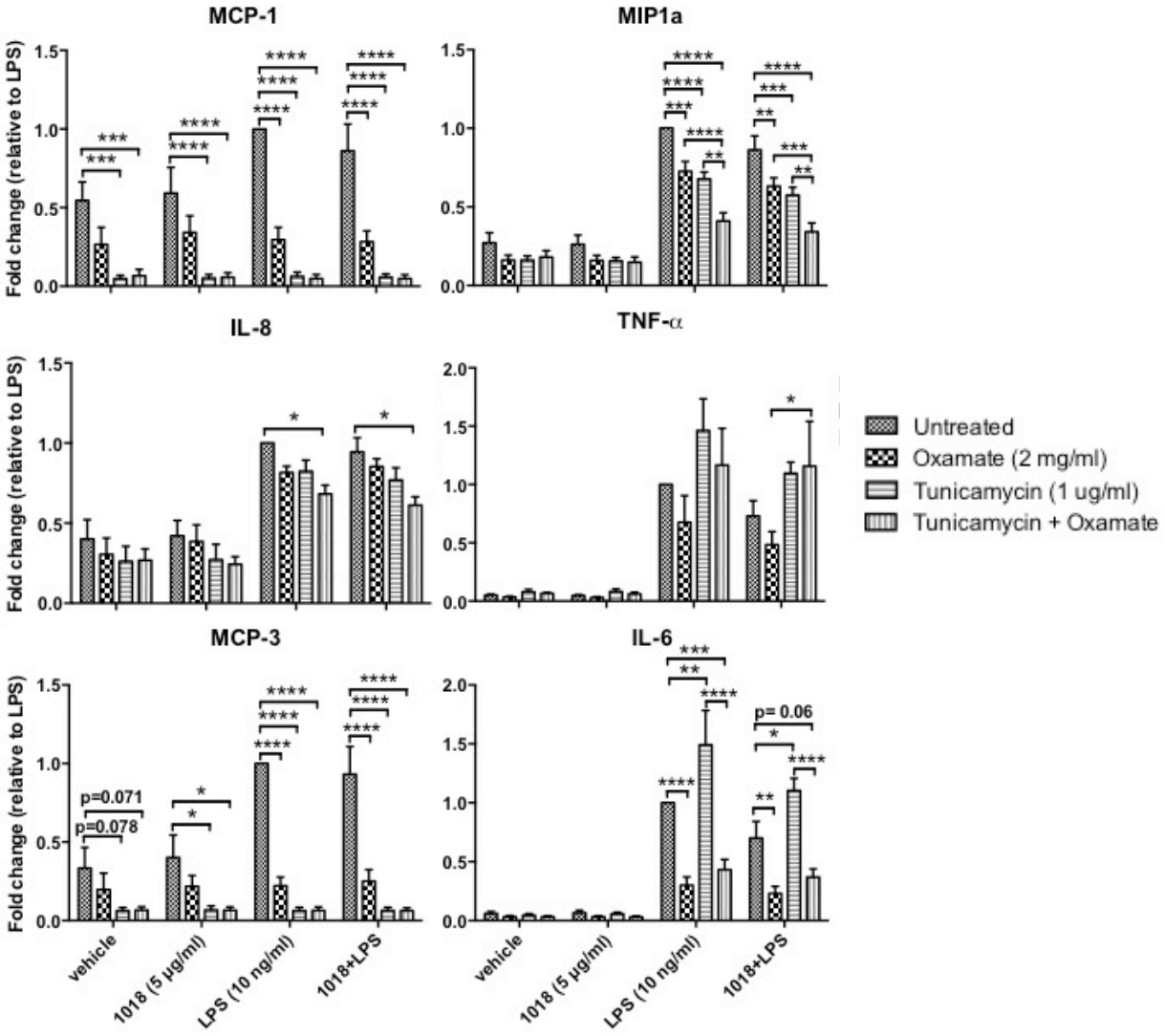

Figure 3.8: Inhibition of glucose metabolism and activation of ER stress mimicked the anti-inflammatory effect of 2DG. Macrophages were pretreated with tunicamycin for 6 hours then stimulated with sodium oxamate and 5 µg/ml IDR-1018, 10 ng/ml LPS or the combination of the two for 16 hours. Supernatants were harvested and cytokines assayed via ELISA. Average of seven independent experiments were completed, with error bars representing SEM. Data was compared using one way ANOVA ****P<0.0001 and *P<0.05.

The results suggested that inhibition of metabolism was sufficient to suppress pro-inflammatory responses to LPS. Tunicamycin also significantly inhibited MCP-1, MCP-3 and MIP-1α, and modestly inhibited IL-8 production induced by LPS when used alone or in combination with IDR-1018 (**Figure 3.8**). This is similar to the suppression of IDR-1018 induced chemokine production that was reported in Chapter 2.

Unlike sodium oxamate, tunicamycin caused a significant increase in IL-6 and a modest increase in TNF-α. Previous studies found that LPS reduces the activation of the PERK pathway and suppresses ATF6 while activating IRE1α and its downstream transcription factor, XBP-1 (108, 109). XBP1 is recruited to the promoters of pro-inflammatory mediators such as IL-6, inducing their expression. Because both LPS and tunicamycin induce activation of the IRE1α pathway, a synergistic increase in IL-6 is observed (108). This might explain the increased TNF-α and IL-6 production by cells stimulated with tunicamycin and LPS compared to those stimulated with LPS alone.

Combining sodium oxamate and tunicamycin was significantly more effective in suppressing LPS-induced MIP-1α and IL-8 production than the use of the inhibitors individually, but had no significant effect on MCP-1, MCP-3, IL-6 or TNF-α production. Overall, these results suggest that 2DG modulated chemokine production through inhibition of glucose metabolism combined with UPR activation, while inhibition of cytokine production occurred predominantly through the inhibition of metabolism.

3.4 DISCUSSION

In Chapter 2 I showed that one major facet of the immunomodulatory activity of the IDR-1018, namely induction of chemokine production, was modulated by 2DG through a combined dysregulation of glycolysis and activation of the UPR. In this Chapter, I examined the role of these two pathways in another major facet of IDR-1018, its anti-endotoxin activity. Based on the results of this study, I propose a model in which the anti-endotoxin activity of IDR-1018 stems in part from its ability to suppress LPS-induced glycolysis, possibly through inhibition of Type I interferon response pathways that also regulate glycolysis. 2DG enhanced the anti-endotoxin activity of IDR-1018 largely by inhibiting glycolysis. Activation the UPR by 2DG also contributed to selective enhancement of anti-endotoxin activity, by suppressing LPS-induced chemokine production while inducing production of some cytokines (**Figure 3.9**).

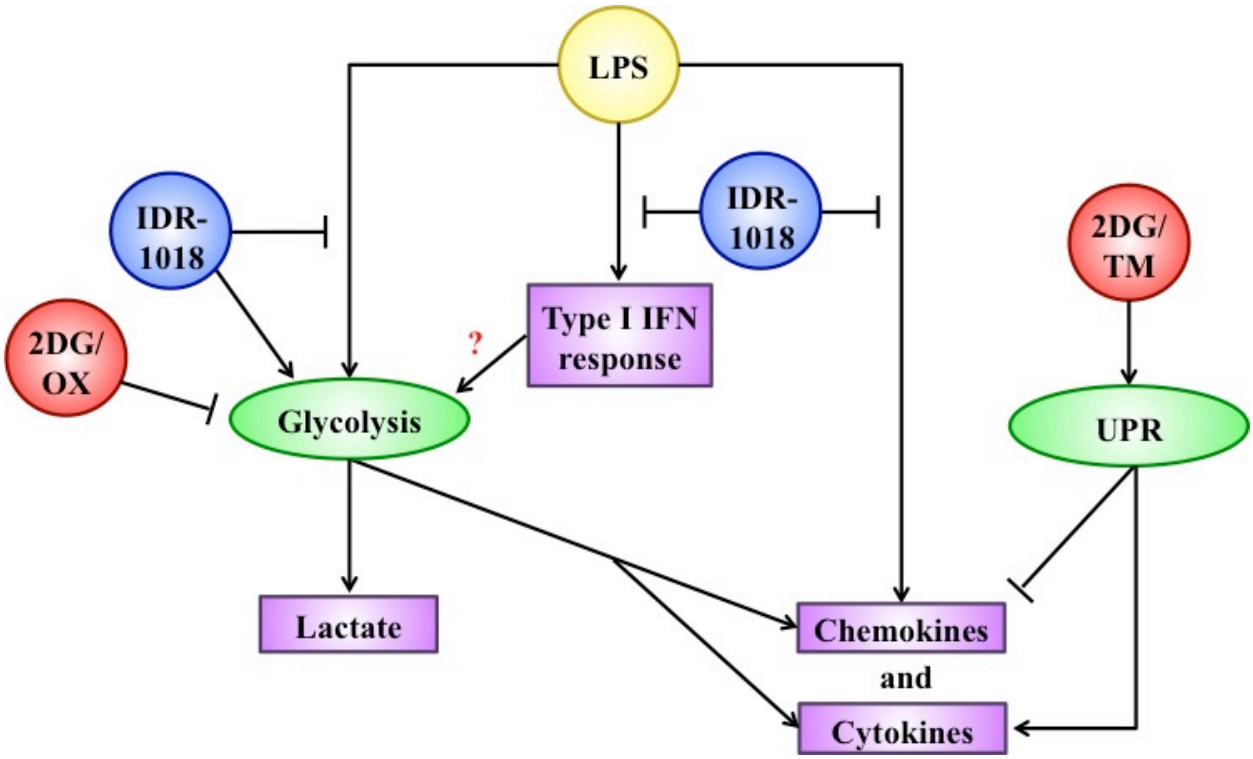

Figure 3.9: Inhibition of glycolysis and activation of the UPR selectively enhanced the anti-endotoxin activity of IDR-1018. The inhibition of cytokine and chemokine production by IDR-1018 may have been mediated by its ability to inhibit LPS-induced glycolysis, possibly through suppression of specific pathways in the type I interferon response. The anti-endotoxin activity of IDR-1018 was selectively enhanced by pharmaceutical inhibition of glycolysis and activation of the UPR. 2-deoxy-d-glucose (2DG) appeared to enhance the anti-endotoxin activity through two distinct points 1) inhibition of glycolysis and 2) activation of the unfolded protein response (UPR). The glycolytic inhibitor sodium oxamate (OX) also significantly inhibited LPS induced chemokine and cytokine production while activation of the UPR by tunicamycin (TM) suppressed LPS induced chemokines but induced pro-inflammatory cytokine production. Taken together the results suggest that 2DG enhances IDR-1018 anti-endotoxin activity largely through inhibition of glycolysis and, to a lesser extent, via activation of the UPR.

Stimulation of macrophages with LPS resulted in the increased expression genes essential to the maintenance of aerobic glycolysis, including several components of the mTOR signalling pathway, namely RICTOR, PI3K, HIF-1α and PFKFB3 (37, 46, 51-53, 263). LPS stimulation also upregulated TBK1, which along with IKKε and AKT, initiates rapid metabolic reprogramming in LPS stimulated dendritic cells (36). In addition to the apparent upregulation of glycolysis at the transcriptional level, LPS also induced a significant increase in extracellular lactate, an indicator of glycolytic activity. Taken together the results suggest that, as seen in previous studies (30, 37, 38, 57, 58), LPS induced a metabolic shift to aerobic glycolysis.

IDR-1018 significantly suppressed LPS-induced chemokine and cytokine production. This was suspected to be due, in part, to suppression of LPS-induced glycolysis by IDR-1018 since IDR-1018 induced an apparent decrease ($p=0.064$) in LPS-induced lactate production compared to that of non-LPS-treated cells, which is consistent with a decrease in glycolytic flux. It is unclear how IDR-1018 was able to suppress LPS-induced glycolysis, although this might have stemmed from IDR-1018-mediated suppression certain Type 1 interferon response pathway components that also regulate glycolysis such as the TBK1/IKKε signalling pathway (36, 252, 253, 264). We propose that this occurred as an early event preventing the TBK1/IKKε induced rapid shift towards aerobic glycolysis, which is required for the production of inflammatory mediators such as IL-6 and TNF-α (36) (**Figure 3.9**).

Consistent with previous studies (36-38), inhibition of glycolysis, in this case using 2DG or sodium oxamate, significantly suppressed LPS-induced cytokine and chemokine production. In addition, 2DG significantly improved IDR-1018-mediated suppression of LPS induced MCP-1, MIP-1α, IL-8, TNF-α, and IL-6. Biological pathway overrepresentation analysis of the down-regulated genes from the RNA-Seq study (**Table 3.2**) revealed that 2DG significantly dysregulated pathways associated the TCA cycle. Glycolysis feeds into several pathways such as the pentose phosphate pathway and the TCA cycle, which produce metabolites that can serve as activators of pro-inflammatory transcription factors and substrates for macromolecule synthesis (29, 36, 38). Inhibition of glycolysis by 2DG might therefore have enhanced the anti-endotoxin activity of IDR-1018 by preventing the accumulation of essential TCA cycle intermediates.

However, in Chapter 2 I showed that in addition to inhibiting glycolysis, 2DG also upregulated the UPR in macrophages stimulated with IDR-1018 and that activation of this pathway suppressed IDR-1018 induced chemokine production. Based on biological pathway over-representation analysis of the upregulated genes from the RNA-Seq study (**Table 3.2**), a similar result was observed here, with 2DG dysregulating pathways associated with protein synthesis, processing and transport, and with the UPR. In fact, activation of the UPR using tunicamycin suppressed LPS-induced chemokine production. Furthermore, inhibition of glycolysis using sodium oxamate coupled with activation of the UPR using tunicamycin had a significantly greater effect on the suppression of LPS induced MIP-1α and IL-8 production compared to individual modulation of the pathways. These results appeared to suggest that 2DG-mediated modulation of IDR-1018 anti-endotoxin activity might also be due to the combined effect of dysregulating glycolysis and the UPR.

Activation of the UPR actually exhibited a more complex regulation of LPS-induced pro-inflammatory responses than expected, enhancing LPS-induced TNF-α, and IL-6 production. Even in the presence of pharmacological UPR activators, LPS has been shown to upregulate the IRE1α pathway while suppressing the PERK pathway in order to sustain cytokine production (108, 109). Interestingly, in my study stimulation of macrophages with 2DG and LPS upregulated numerous genes associated with IRE1α activation but only a few associated with the PERK arm compared to LPS alone. This suggests that the combination of 2DG and LPS results in selective upregulation of the pro-inflammatory arm of the UPR, making it unlikely that activation of the UPR is the mechanism behind 2DG-mediated suppression of cytokine production. Rather, it appears that with respect to cytokine production, 2DG-mediated inhibition of glycolysis enhanced the anti-endotoxin activity of IDR-1018 (**Figure 3.9**).

In summary, this study has provided evidence that suppression of LPS-induced glycolysis might be a mechanism behind IDR-1018 anti-endotoxin activity. In addition, I have shown that 2DG enhances the anti-endotoxin activity of IDR-1018 through inhibition of glycolysis and, with respect to chemokine production, activation of the UPR.

Chapter 4: IDR-1018 ENHANCED HDL-MEDIATED CHOLESTEROL EFFLUX FROM MACROPHAGES

4.1 INTRODUCTION

In the previous chapters, IDR-1018 was shown to modulate glycolysis in order to exert its immunomodulatory activities. The general aim of the present chapter was to assess whether IDR-1018 was also capable of modulating metabolic pathways dysregulated in response to nutrient excess specifically, the intracellular cholesterol efflux pathway of lipid-laden macrophages, a major cell type associated with the pathogenesis of atherosclerosis (75, 84, 265).

Atherosclerosis is characterized by chronic inflammation mediated, in part, by the aberrant accumulation of lipids and immune cells in arteries (265). During the development of atherosclerosis, macrophages internalize and store substantial amounts of cholesterol, eventually developing into foam cells. These cells exhibit elevated pro-inflammatory responses due to their production of inflammatory mediators and the release of inflammatory intracellular contents during death (75, 84). Reverse cholesterol transport combats this process though the removal of excess cholesterol from peripheral tissues. The initial stages of reverse cholesterol transport involve efflux of cholesterol from cells via the cholesterol transporters ABCA1 and ABCG1 to preβ-HDL and mature HDL particles, respectively (75, 86, 266, 267). While HDL and its major protein component and cholesterol acceptor, ApoA1, are essential for cellular cholesterol efflux and reverse cholesterol transport, they also regulate the immune response; modulating chemokine and cytokine production, promoting the differentiation of alternatively activated macrophages and dampening pro-inflammatory responses to lipopolysaccharide (95, 268-272).

In the development of new therapeutic agents for the treatment of atherosclerosis, substantial efforts have focused on enhancing the cholesterol efflux activity of HDL [reviewed in Kingwell et al. (88)]. This includes ApoA1 peptide mimetics (88). These peptides were designed based on the structural and physical properties thought to be responsible for the ability of ApoA1 to induce cholesterol efflux, rather than specific amino acid sequences [reviewed in Navab et al. (90) and, Getz et al. (89)]. This resulted in a series of peptides with one or two short amphipathic α-helixes and an ability to induce cellular cholesterol efflux and modulate immune responses under a variety of conditions, both *in vivo* and *in vitro* (92, 94-97, 273-279).

IDR-1018 shares several physical and immunomodulatory characteristics with ApoA1 and ApoA1 peptide mimetics, suggesting that, IDR-1018 might also be capable of promoting cellular cholesterol efflux. It is a 12-amino acid peptide that adopts an amphipathic α-helical

conformation in the presence of zwitterionic micelles (280) and exhibits potent and pleiotropic immunomodulatory activities including promoting chemokine production, modulating pro-inflammatory responses, enhancing wound healing, and modulating the differentiation state of macrophages (193, 197-199, 280). IDR-1018 has also proven an effective treatment in animal models of *Mycobacterium tuberculosis* and *Staphylococcus aureus* infections and in preventing inflammation-mediated death due to severe (cerebral) malaria (281, 282). Based on the similarities between ApoA1 peptide mimetics and IDR-1018, the aim of this study was to determine if IDR-1018 was also capable of promoting cholesterol efflux from cells. Here I showed that IDR-1018 bound serum ApoA1 and facilitated its interaction with macrophages and, at low concentrations, enhanced HDL-mediated cholesterol efflux from macrophages and smooth muscle cells. Based on these results, I propose that IDR-1018 enhanced cholesterol efflux from cells by binding to HDL, and improved its interaction with cells, thus facilitating the transfer of cholesterol to HDL particles.

4.2 MATERIALS AND METHODS

4.2.1 Blood collection and ethics statement

Blood was collected from healthy volunteers using vacutainer tubes with ethical approval and according to the guidelines of the University of British Columbia clinical research ethics board (UBC-CREB# H04-70232).

4.2.2 Reagents

Solid phase F-moc chemistry followed by reverse phase HPLC was used to synthesize and purify the peptide, IDR-1018 (VRLIVAVRIWRR-NH_2) (CPC Scientific, Sunnyvale CA), to greater than 95% purity. Cholesterol and fatty acid free bovine serum albumin (FAFA) were purchased from Sigma Alrich (St. Louis MO) and ApoA1 was obtained from Academy Biomedical (Huston TX). [1,2-^{3}H]-cholesterol and [1-^{14}C]-oleate were purchased from PerkinElmer (Waltham, MA). Serum was collected from healthy donors using BD vacutainer serum collection tubes with clot activator (BD bioscience, San Jose CA).

Delipidated human serum was generated by first collecting blood into vacutainer tubes containing EDTA and centrifuging (2000 rpm at 4°C for 15 minutes) to isolate plasma. The density of the plasma was adjusted to 1.21 using KBr, then plasma was spun at 400,000 xg for 4 hours at 8°C using a TLA 100.3 Beckman rotor (Pasadena, CA), after which lipoproteins were removed. Following lipoprotein removal, the plasma was dialyzed overnight (at 4°C) against a buffer containing 150 mM NaCl, 50 mM Tris-HCl and 0.3 mM EDTA, pH 7.4.

HDL_3 was isolated as described previously (283). Briefly blood was collected from healthy individuals to obtain plasma. The plasma was then subjected to a series of ultracentrifugation steps (284) to isolate serum lipoproteins.

4.2.3 Cell lines and generation of human monocyte-derived macrophages

All cells used in this study were cultured in a humidified incubator at 37°C and 5% CO_2. Macrophages were generated as described previously (198) with some modifications. Briefly, blood was collected from healthy volunteers using BD vacutainer tubes containing sodium heparin (BD bioscience). Peripheral blood mononuclear cells (PBMCs) were isolated according to the methods described by Mookherjee et al. (141) and Nijnik et al. (251), and resuspended in serum-free RPMI 1640 media (HyClone Laboratories-GE healthcare Life Sciences, Mississauga, ON). Cells were seeded at a concentration of $2x10^6$ cells/ml for 45 minutes, then media was gently removed and replaced with RPMI 1640 supplemented with 10% FBS (PAA Laboratories-GE healthcare Life Sciences). Cells were incubated for 24 hours. On day one of the differentiation process, the media was removed and replaced with RPMI 1640 media supplemented with 10% FBS and 10 ng/ml M-CSF (Research Diagnostic Inc, Concord MA). Cells were then incubated for 5 days to allow for macrophage differentiation. On day 6, the cells were washed and cultured for a further 24 hours in RPMI 1640 media containing 10% FBS and 10 ng/ml M-CSF. Finally, the cells were washed to remove any remaining lymphocytes prior to use. For certain experiments, the differentiation process was carried out in media containing penicillin (100 units/ml) and streptomycin (100 μg/ml) (Life technologies, Burlington ON) to prevent bacterial growth.

The human skin fibroblast (HSF) cell line was obtained from ATCC (Manassas, VA). The rat smooth muscle (SMC) cell line WKY3M-22 (285) was a generous gift from Dr. Joan Lemire (University of Washington, Seattle). Both cell lines were cultured in DMEM supplemented with 10% FBS (both from GE Healthcare Life Technologies), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Life Technologies).

4.2.4 Immunoblotting analysis

Following stimulation, cells were washed with PBS and lysed in NP-40 (150 mM NaCl, 1% nonidet-40, and 50 mM pH 8.0 Tris-HCl) or maltoside (0.5% maltoside, 20 mM Tris, 5 mM EDTA, 5 mM EGTA) buffer containing complete protease inhibitor (Sigma Aldrich). Total protein concentration was determined using a colorimetric assay. Normalization of the data was carried out by loading an equal amount of protein from each sample. Samples were run on SDS-

polyacrylamide (SDS-PAGE) gels after which proteins were transferred to polyvinylidene difluoride Immobilon-P (EMD Millipore, Billerica CA) or nitrocellulose membranes (VWR International, Mississauga ON). Membranes were probed for ApoA1 (EMD Millipore), ApoE (Meridian Life Science, Memphis TN), ABCA1 (Santa Cruz Biotechnology Inc., Dallas TX), low density lipoprotein receptor (LDLR) (Research Diagnostic Inc), and β-actin (Cell signaling Technology Inc., Danvers MA). ImageJ software was used for densitometric calculations of amounts of these proteins, and values were normalized to the β-actin values.

4.2.5 RNA isolation and quantitative real-time PCR (qRT-PCR)

RNA was isolated using the Qiagen RNeasy Isolation Kit (Qiagen, Toronto ON) according to the manufacturer's instructions. RNA concentration and high quality was confirmed using a Nanodrop spectrophotometer. cDNA was generated using the qScriptTM cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg MD) according to manufacturer's instructions. qRT-PCR was carried out using SuperScript III Platinum Two-Step qRT-PCR kit with SYBR Green (Life Technologies) in conjunction with the ABI Prism 7000 sequence detection system (Applied Biosystems-Life Technologies). The comparative Ct method (286) was used to calculate fold changes for the genes of interest. Beta-2-microglobulin was used as the housekeeping gene. Primer sequences are listed in **Table 4.1**.

Table 4.1: Sequences of primers used in quantitative real-time PCR experiments.

Gene	Forward primer	Reverse primer
ApoA1	CCCTGGGATCGAGTGACGGA	TGAGCACATCCACGTACACAG
ApoE	GTTGCTGGTCACATTCCTGG	GCAGGTAATCCCAAAAGCGAC
ABCA1	GCACTGAGGAAGATGCTGAAA	AGTTCCTGGAAGGTCTTGTTCAC
SRB1	AGGAGATCCCTATCCCCTC	GGATGTTGGGCATGACGATGT
NCP1	CTCCTGTGTGGGAAGGACG	GGAGTGATGGTAAAAGGTGC
NCP2	CAAAGGACAGTCTTACAGCGT	GGATAGGGCAGTTAATTCCACTC
CETP	CATGGCCGATTTTGTCCAGAC	CTGTCAGGGAAATGTCCACCC
LCAT	CCCCGCACCTACATCTACGA	CCACCGTGTCATCACCATCC
B2M	CTCGCGCTACTCTCTCTCTTTCT	TGCTCCACTTTTTCAATTCTCT

4.2.6 Cholesterol efflux

Cholesterol efflux experiments were carried out as described previously (287) with minor modifications described below. Macrophages, SMCs and HSF cells were cultured in medium containing 100 units/ml penicillin and streptomycin during all stages.

4.2.6.1 Labeling cells with tritiated cholesterol

To enable cholesterol loading of macrophages, on day one of the differentiation phase,

media was removed from PBMCs (containing monocytes that uniquely adhered) and RPMI 1640 media supplemented with 10% FBS, 10 ng/ml M-CSF, and 0.2 μCi [1,2-^{3}H]cholesterol was added. Cells were incubated in this medium for 5 days. On the sixth day, the medium was removed and cells were washed with PBS containing fatty acid-free albumin (FAFA) (1 mg/ml). The cells were then cholesterol loaded in RPMI 1640 media containing 2 mg/ml FAFA, 10 ng/ml M-CSF and 30 μg/ml free cholesterol for 24 hours. Subsequently, the medium was removed and cells were washed with PBS+FAFA (1 mg/ml) then equilibrated for 24 hours in RPMI 1640 medium supplemented with 1 mg/ml FAFA. Finally, macrophages were rinsed with PBS+FAFA (1 mg/ml) then stimulated to induce cholesterol efflux.

SMC cells and fibroblasts were grown to approximately 60% confluence before being incubated with DMEM containing 10% FBS and 0.2 μCi [1,2-^{3}H]cholesterol. Labeling continued until cells reached 100% confluence, at which point they were washed with PBS+FAFA (1 mg/ml) then cholesterol loaded.

To study cholesterol efflux, all cell types were stimulated for 16 hours with HDL alone or in combination with IDR-1018 in RPMI medium containing 1 mg/ml FAFA. For the combination treatments, the HDL_3 concentration was kept constant at 0.12 μM while IDR-1018 concentration was varied, generating a range of peptide:HDL molar ratios.

4.2.6.2 Cholesterol extraction and counting

Cells were put on ice and medium was removed, centrifuged, and transferred to scintillation vials along with scintillation fluid and [^{3} H] was counted. Cells were washed with ice cold PBS + bovine serum albumin (BSA), then PBS alone. To extract intracellular cholesterol, cells were incubated at room temperature in a 3:2 (v:v) hexane:isopropanol solution for 30 minutes. After the incubation, the extraction solution was transferred to glass tubes. The wells were rinsed with the same solution; this was also transferred to the glass tubes. The samples were dried under air with low heat and reconstituted in ice-cold chloroform. Free and esterified cholesterol were isolated using thin layer chromatography. The amounts of extracellular as well as intracellular free cholesterol and cholesterol esters in the respective spots were calculated as the percent of total radioactivity recovered.

4.2.7 ACAT activity- cholesterol esterification

On day 6 of the differentiation procedure, macrophages were cholesterol loaded in RMPI 1640 media containing 2 mg/ml FAFA, 10 ng/ml M-CSF and 30 μg/ml free cholesterol for 24 hours. Cells were washed and treated as described for the cholesterol efflux experiments. After

treatment, cells were washed and incubated RMPI 1640 containing [^{14}C]-oleate-albumin (100:1 v/v) for 1 hour. Cells were then placed on ice and media was discarded. Cells were rinsed first with ice-cold PBS+BSA then PBS. Lipids were extracted as described above however, only cholesterol esters were collected (287). Counts were normalized to cellular protein content. Data are presented as a percentage of the BSA control.

4.2.8 Statistical analysis

Data are expressed as mean ± SEM. Statistical differences were calculated with Graphpad Prism 6.0 (GraphPad Software, La Jolla CA) using a paired two-tailed Student's t-test. P values <0.05 were considered statistically significant.

4.3 RESULTS

4.3.1 Stimulation with IDR-1018 increased the expression of proteins associated with the cholesterol efflux pathway in macrophages

Since peptide IDR-1018 shares several physical and immunomodulatory properties with ApoA1 and its peptide mimetics (89, 92, 95, 193, 197, 198, 268, 280), we determined if it could also modulate cellular cholesterol efflux. In medium containing human serum, IDR-1018 dose dependently increased the levels of ApoA1 and ABCA1, two major components of the cholesterol efflux pathway (86) (**Figure 4.1**).

Figure 4.1: IDR-1018 dose dependently increased ApoA1 and ABCA1. Macrophages were stimulated with increasing concentrations of IDR-1018 for 24 hr in RPMI media containing 2% human serum. Cell lysates were run on a 12% gel and probed for ApoA1, ABCA1and β-actin. A minimum of three independent experiments were completed.

Stimulation of macrophages with 13 μM of IDR-1018 significantly upregulated the levels of ApoA1 and ABCA1 as well as another important apolipoprotein associated with cholesterol efflux, ApoE. IDR-1018 also induced a slight, statistically insignificant increase in the low-density lipoprotein receptor, a transporter involved in the uptake of lipids by cells (288) (**Figure 4.2**). These results suggest that IDR-1018 might modulate cholesterol efflux by increasing the levels of key proteins involved in the process.

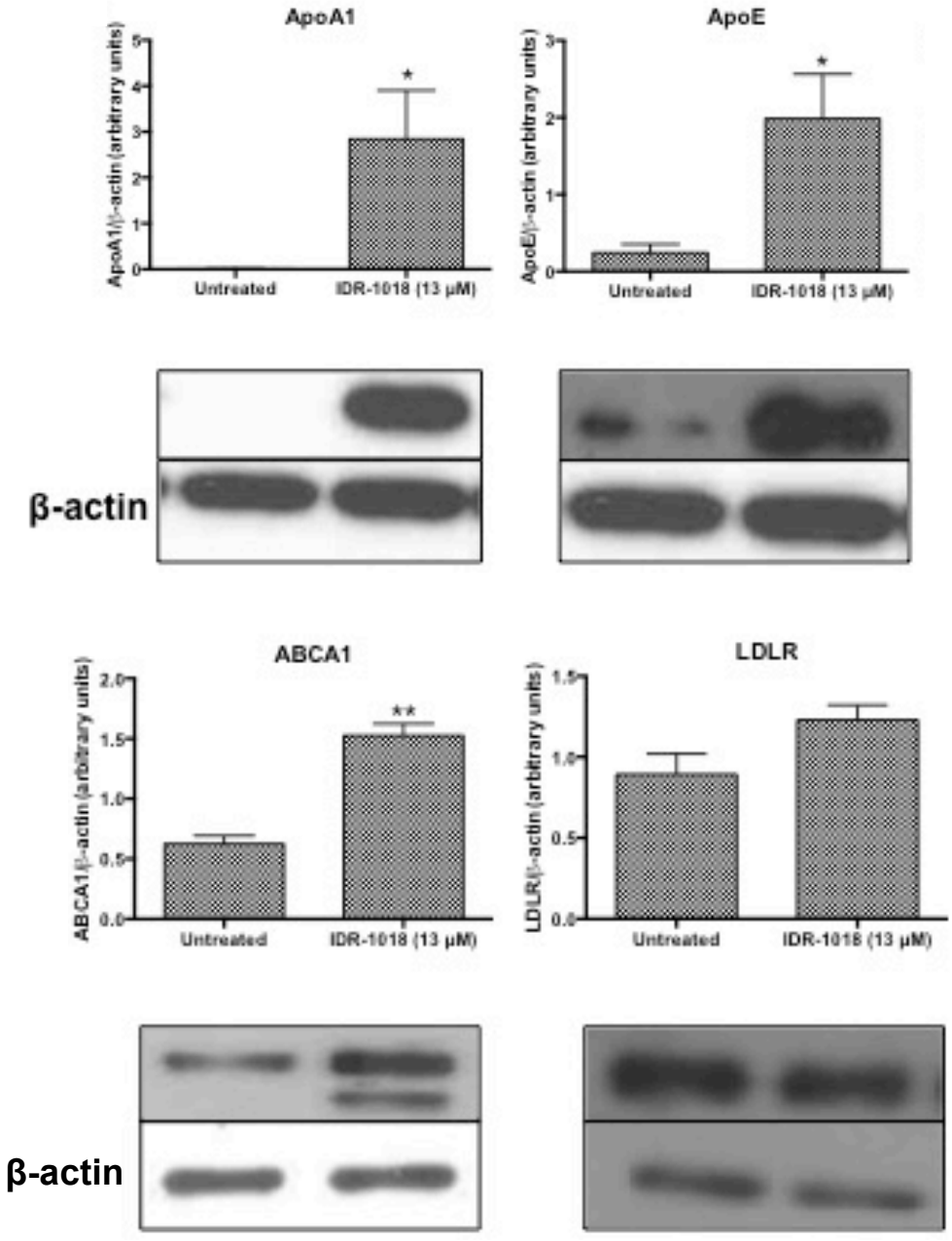

Figure 4.2: IDR-1018 increased the level of proteins associated with cellular cholesterol trafficking in macrophages. Macrophages were stimulated with 13 μM IDR-1018 for 24 hr in RPMI media containing 2% human serum. Cell lysates were run on a 12% gel and probed for ApoA1, ApoE, ABCA1, LDLR and β-actin (blots shown below). Densitometry was completed using Image J software (shown above). Data is presented as the mean ± SEM. A minimum of three independent experiments were completed, data analyzed using student's t-test. *$p<0.05$, **$p<0.01$.

Gene expression analysis revealed that the IDR-1018 induced upregulation of ApoA1, ApoE and ABCA1 proteins did not occur at the transcriptional level (**Figure 4.3**). Furthermore,

IDR-1018 did not alter the expression of any other genes associated with the cellular cholesterol efflux pathway, indicating post-transcriptional regulation by IDR-1018 was likely involved.

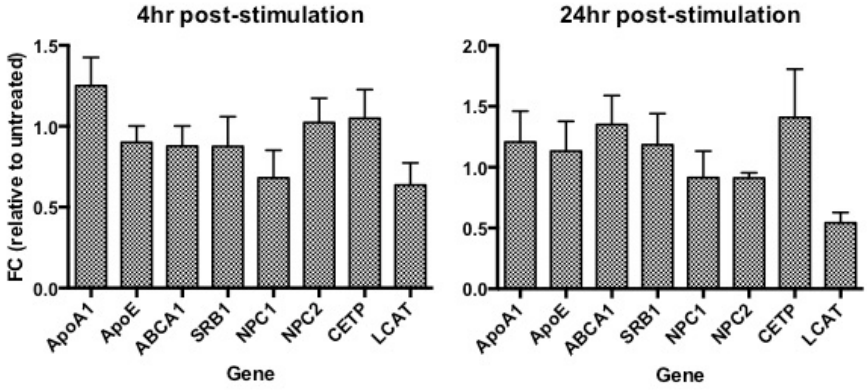

Figure 4.3: IDR-1018 induced increases in protein levels of cellular cholesterol trafficking components but these were not due to increased gene expression. Macrophages were stimulated with 13 μM IDR-1018 for 4 or 24 hr in RPMI media containing 2% human serum. RNA was extracted and ApoA1, ApoE, ABCA1, SRB1, NPC1/2, CETP and LCAT expression were assayed using qRT-PCR. Data is presented as the mean of at least three independent experiments, with error bars representing SEM.

4.3.2 IDR-1018 bound to HDL in serum as well as exogenous lipid-free ApoA1 and promoted their interaction with macrophages

Stimulation of macrophages with IDR-1018 for 15-60 minutes in medium containing human serum resulted in markedly elevated levels of ApoA1 protein, with increased ApoA1 observed as early as 15 minutes post-stimulation (**Figure 4.4**).

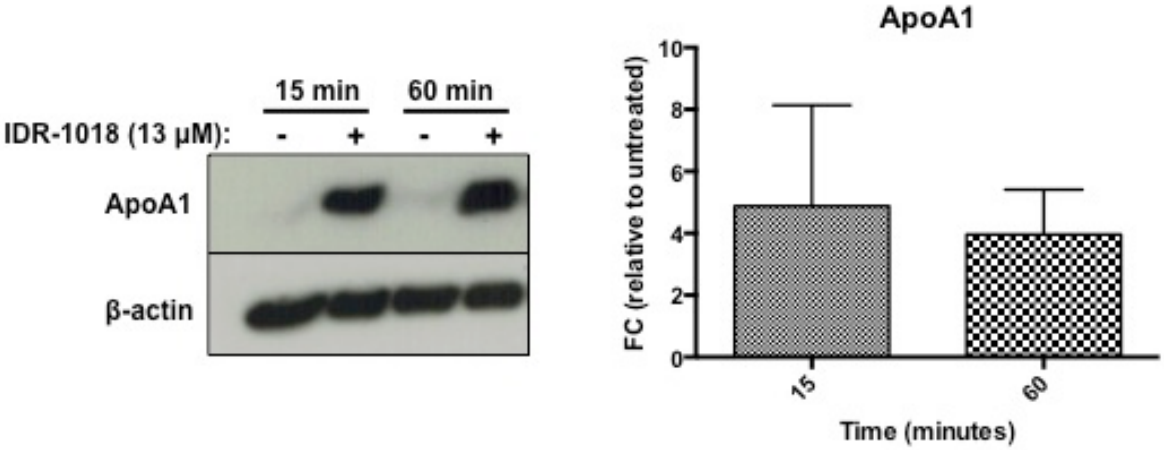

Figure 4.4: Stimulation of macrophages with IDR-1018 induced a rapid increase in ApoA1 protein levels. Macrophages were stimulated with 13 μM IDR-1018 for 15 or 60 min in RPMI media containing 2% human serum. Cell lysates were run on a 12% gel and probed for ApoA1 and β-actin (left). Densitometry (right) was calculated using Image J software. Three independent experiments were completed. Data are presented as the mean ± SEM.

This rapid increase in ApoA1 in response to IDR-1018 was further consistent with the

observation that IDR-1018 was not regulating ApoA1 transcriptionally. Several natural immunomodulatory peptides have previously been shown to bind lipoproteins and, in some cases, facilitate the interaction of lipoproteins with cells (128, 289, 290). Thus, the rapid increase in ApoA1 induced by IDR-1018 might have been a result of IDR-1018 binding to HDL in the serum, which then associated with cells. To assess whether IDR-1018 bound ApoA1 in serum, macrophages were stimulated with IDR-1018 under a variety of serum conditions. Macrophages were also loaded with free cholesterol to mimic conditions under which macrophages would upregulate their cholesterol efflux pathway. As for previous experiments, a substantial increase in ApoA1 was observed for cholesterol-loaded macrophages stimulated with IDR-1018 in the presence of human serum (**Figure 4.5**). This interaction was nearly abolished when delipidated serum was used.

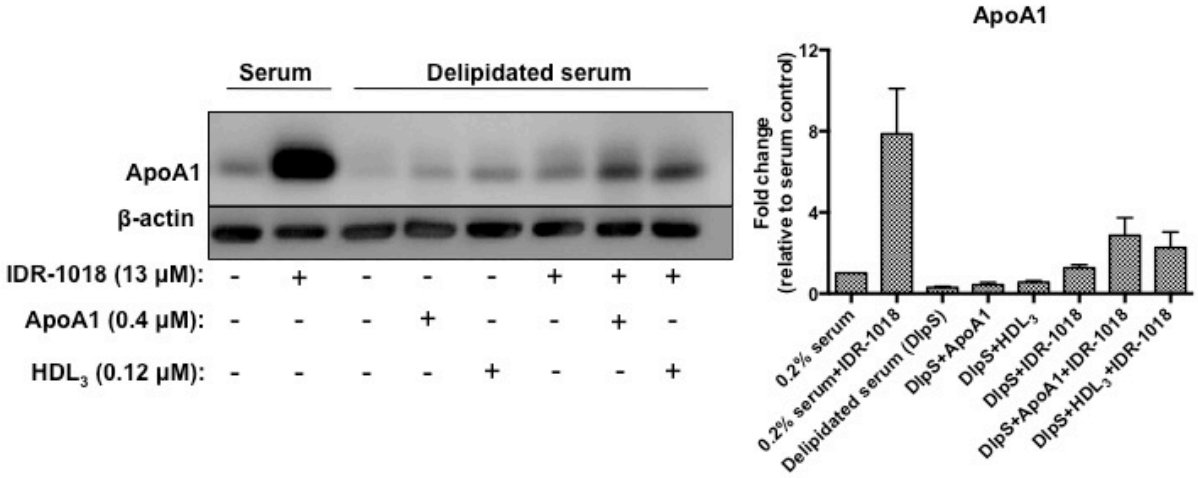

Figure 4.5: IDR-1018 interacted with HDL and exogenous lipid free ApoA1. Macrophages were stimulated with 13 μM IDR-1018 in the presence of media containing 0.2% human serum or de-lipidated human serum (DLPS) with or without lipid-poor ApoA1 or HDL for 16 hrs. Cell lysates were run on a 12% gel and probed for ApoA1 and β-actin (left). Densitometric values (right) were calculated using Image J software. Three independent experiments were completed. Data are presented as the mean ± SEM.

These data are consistent with those of Sorenesen *et al. (128)*, who showed that binding of LL-37 to ApoA1 and B does not occur in dilipidated serum. Addition of exogenous lipid-free ApoA1 and HDL both partially restored IDR-1018 mediated association of ApoA1 with macrophages. However, very little lipid-free ApoA1 is found *in vivo (291)* therefore, the substantial increase in ApoA1 observed when macrophages were stimulated with IDR-1018 in the presence of serum were likely the result of IDR-1018 binding to serum HDL, thus facilitating its interaction with cells.

4.3.3 Low molar concentrations of IDR-1018 enhanced HDL-mediated suppression of cholesterol esterification by ACAT and cellular cholesterol efflux

To determine if there were any functional consequences of the binding of IDR-1018 to HDL, and the subsequent interaction of this complex with macrophages, we examined whether IDR-1018 influenced HDL-induced cholesterol efflux from macrophages. In these studies the concentration of HDL was kept constant at 0.12 μM while the concentration of IDR-1018 was varied, resulting in a range of peptide:HDL molar ratios. Based on the dose-dependent increase in ApoA1 observed when cells were stimulated with 3.3 to 33 μM of IDR-1018, high peptide:HDL ratios were initially utilized for the cholesterol efflux studies. Although, IDR-1018 alone had no effect on cholesterol efflux (data not shown), high concentrations inhibited HDL-mediated cholesterol efflux from cells (**Figure 4.6**). Therefore, the concentration of IDR-1018 was reduced substantially to generate peptide:HDL molar ratios ranging from 1:5 to 5:1.

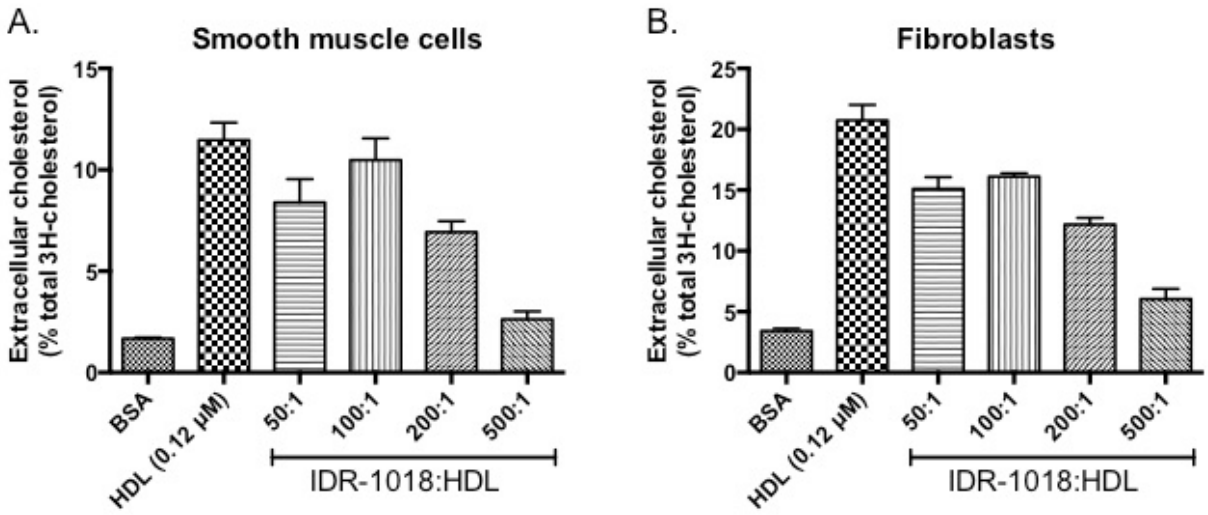

Figure 4.6: At high concentrations, IDR-1018 inhibited HDL–mediated cholesterol efflux from free cholesterol loaded cells. SMCs (A) and fibroblasts (B) were loaded with ^{3}H-cholesterol. Cells were stimulated with HDL (0.12 μM) alone or in combination with IDR-1018 for 16 hrs. For the combination treatments (peptide:HDL), the concentration of IDR-1018 was varied while the HDL concentration was kept constant. Following treatment, media was removed and used to measure extracellular cholesterol content. The level of extracellular cholesterol, presented as the percentage of total radiolabelled cholesterol recovered, was used as a measure of cholesterol efflux. The results presented are of one experiment, with each condition completed in triplicate.

To study the effect of these lower peptide:HDL ratios on cellular cholesterol levels, we initially utilized a cholesterol esterification assay. This assay accurately measures the degree to which cells are storing residual cholesterol, and thus also acts as an indicator of cholesterol efflux. To store cholesterol, cells must convert the free cholesterol that is taken up into

cholesterol esters, a reaction carried out by the endoplasmic reticulum enzyme Acyl-CoA cholesterol acyltransferase (ACAT) (292). To carry out this assay, cells were stimulated with HDL alone, or in combination with IDR-1018, and then incubated with radiolabelled oleic acid. The oleic acid is used by ACAT as a substrate for the esterification of cholesterol (287). The amount of radioisotope incorporated into cholesterol esters estimates the level of ACAT activity and therefore the degree to which cells are storing cholesterol. A decrease in cholesterol esterification is consistent with an increase in cholesterol efflux, which would reduce the amount of intracellular free cholesterol available for esterification. HDL significantly reduced cholesterol esterification in macrophages by 55% (**Figure 4.7**). Of the peptide:HDL ratios tested, the 1:1 ratio (0.12 μM IDR-1018), significantly reduced cholesterol ester formation compared to HDL alone by an additional 25%, suggesting that at low concentrations, IDR-1018 enhanced HDL-mediated suppression of cholesterol esterification.

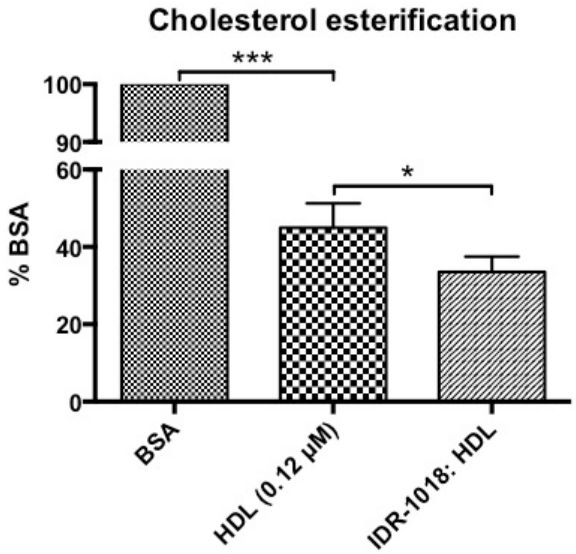

Figure 4.7: At low concentrations, IDR-1018 enhanced HDL-mediated inhibition of cholesterol esterification. Macrophages were loaded with free cholesterol for 24 hrs then stimulated with HDL (0.12 μM) alone or in combination with IDR-1018 for 16 hrs. For the combination treatments (IDR1018:HDL), 0.12 μM of IDR-1018 and HDL were added to the cells for a 1:1 ratio. Following the treatments, cells were incubated with [14C]oleate-albumin for 1 hr. Media was removed and cholesterol esters isolated from cell lysates. Data is presented as the mean percentage of BSA control. Five independent experiments were completed, data analyzed using the paired Student's t-test ***$p<0.001$, *$p<0.05$.

In contrast to the cholesterol efflux studies presented in **Figure 4.6**, higher concentrations of IDR-1018 appeared to have little effect on HDL-mediated suppression of cholesterol esterification. Thus these results are consistent with the suggestion that, at low concentrations IDR-1018, promoted the efflux of free cholesterol from macrophages, thus enhancing HDL-

mediated reduction of cholesterol esterification.

To further characterize the effect of lower concentrations of IDR-1018 on cholesterol efflux from HDL stimulated macrophages and SMCs, a cholesterol efflux assay was again employed, at the lower peptide:HDL molar ratios that proved effective in the ACAT assay. HDL induced a significant ~4-fold increase in extracellular cholesterol compared to the BSA control indicating that HDL was potently inducing cholesterol efflux (**Figure 4.8**). The 1:1 and 5:1 HDL:IDR-1018 ratios induced approximately 15% more cholesterol efflux from macrophages and SMCs, respectively compared to HDL alone suggesting that IDR-1018 improved HDL-mediated cholesterol efflux from SMCs ($p<0.05$) and possibly macrophages.

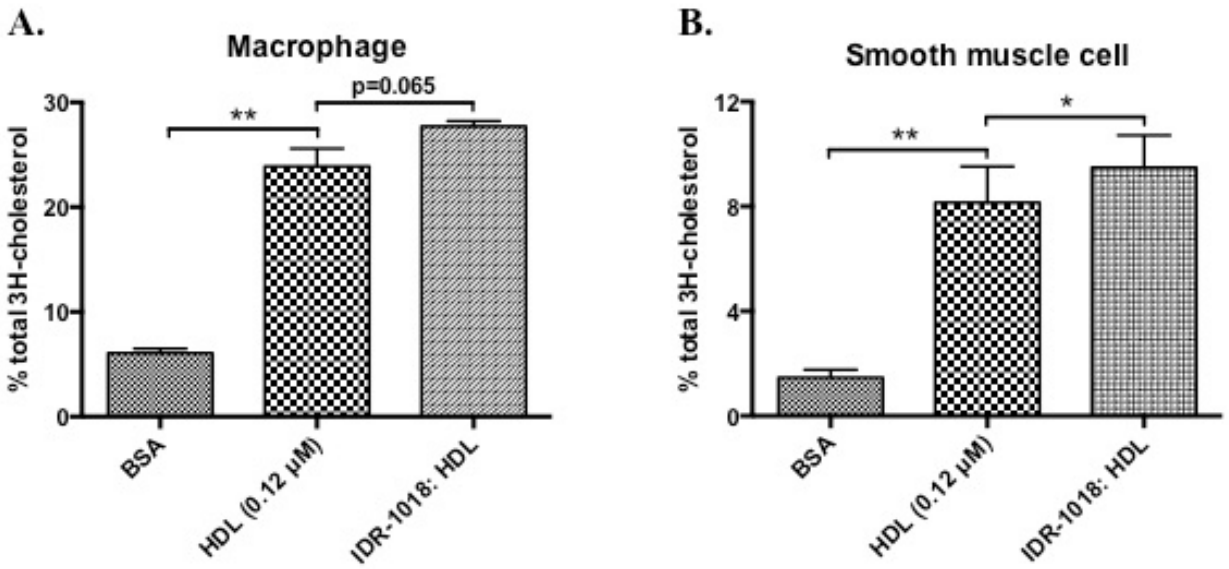

Figure 4.8: At low concentrations, IDR-1018 enhanced HDL mediated cholesterol efflux from free cholesterol loaded cells. Macrophages (A), smooth muscle cells (B) were loaded with 3H-cholesterol. Cells were stimulated with HDL (0.12 μM) alone or in combination with IDR-1018 for 16 hrs. For the combination treatment (peptide:HDL), 0.12 μM of HDL was combined with 0.12 μM IDR-1018 for macrophages (1:1 ratio) or 0.6 μM for smooth muscle cells (5:1 ratio). Following treatment, media was removed and used to measure extracellular cholesterol content. Five independent experiments were completed for both macrophages and smooth muscle cells with one experiment on macrophages excluded as an outlier. Data is presented as the mean percentage of total cholesterol, data analyzed using the paired Student T-test **$p<0.01$, *$p<0.05$.

Although the difference for macrophages approached statistical significance, the poor sensitivity of the assay limited accuracy of these analyses. Consistent with the ACAT assay, HDL also induced a significant decrease in intracellular free cholesterol and cholesterol esters compared to BSA controls (**Figure 4.9**). Addition of IDR-1018 significantly decreased the free cholesterol content compared to HDL alone, but had no significant effect on cholesterol esters. This indicates that the increase in extracellular cholesterol observed with the addition of IDR-

1018 likely resulted from IDR-1018 promoting the release of cholesterol that had yet to be esterified. Alternatively, IDR1018 might have enhanced the transfer of radiolabelled cholesterol from the plasma membrane to HDL via the aqueous diffusion pathway (267).

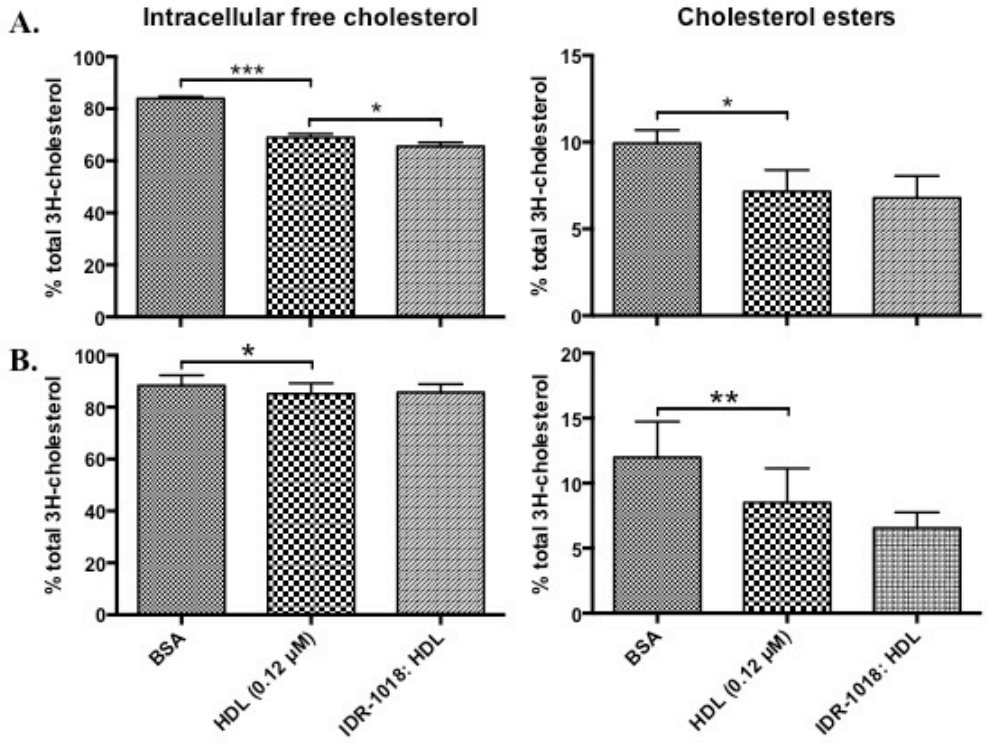

Figure 4.9: IDR-1018 further reduced the intracellular cholesterol content of HDL stimulated cells. Macrophages (A), smooth muscle cells (B) were loaded with 3H-cholesterol. Cells were stimulated with HDL (0.12 μM) alone or in combination with IDR-1018 for 16 hrs. For the combination treatment (peptide:HDL), 0.12 μM of HDL was combined with 0.12 μM IDR-1018 for macrophages (1:1 ratio) or 0.6 μM for smooth muscle cells (5:1 ratio). Following treatment, cell lysates were used to measure intracellular free cholesterol (right graph) and cholesterol esters (left graph). Five independent experiments were completed for both macrophages and smooth muscle cells; one experiment on macrophages was excluded as an outlier. Data is presented as the mean percentage of total cholesterol, data analyzed using the paired Student t-test ***$p<0.001$, **$p<0.01$, *$p<0.05$.

As mentioned above, IDR-1018 also enhanced HDL-dependent cholesterol efflux from SMCs (**Figure 4.8**). This increase correlated with a moderate but non-significant reduction in intracellular cholesterol esters (**Figure 4.9**). However in contrast to the results with macrophages, IDR-1018 had no effect on the intracellular free cholesterol content. These results indicate that, with respect to SMCs, the enhanced HDL-mediated cholesterol efflux induced by IDR-1018 might be a result of increased mobilization of intracellular cholesterol stores. The results for the SMCs are of particular importance as these cells are now thought to represent more than 50% of the foam cells present in arteries (207).

4.3.4 At low molar concentrations, IDR-1018 did not alter ApoA1 or ABCA1 protein expression

Immunoblot analyses of ApoA1 and ABCA1 were completed for macrophages stimulated with the peptide:HDL ratios that enhanced HDL–mediated cholesterol efflux as well as the 114:1 ratio which is equivalent to 13μM IDR-1018, a concentration that increased ApoA1 and ABCA1 in macrophages stimulated in serum. HDL alone induced a modest increase in ApoA1. The 1:5 to 5:1 ratios (0.023 μM, 0.12 μM and 0.58 μM, IDR-1018 respectively) induced a modest decrease in ApoA1 compared to HDL alone (**Figure 4.10**).

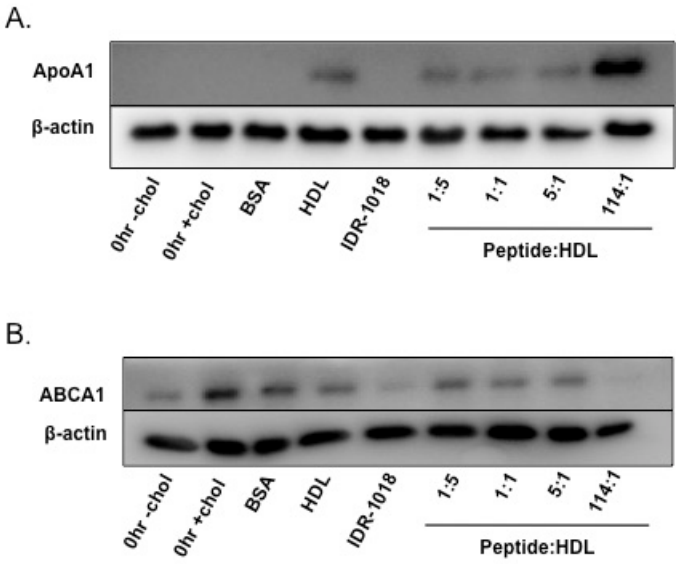

Figure 4.10: At low concentrations, IDR-1018 did not alter HDL-mediated changes in ApoA1 or ABCA1 levels. Macrophages were loaded with free cholesterol then stimulated with HDL (0.12 μM) or IDR-1018 (13 μM) alone or a combination of the two for 16 hrs. For the combination treatments (peptide:HDL), the concentration of IDR-1018 was varied while the HDL concentration was kept constant at 0.12 μM. Cell lysates were run on a 12% gel and probed for ApoA1 (A), ABCA1 (B) and β-actin. Images are one experiment representative of three.

Although 13 μM of IDR-1018 had no effect on ApoA1 when used alone, combined with HDL (114:1 ratio) it increased ApoA1 substantially, further supporting the hypothesis that IDR-1018 could interact with HDL in serum and promote its interaction with cells. Interestingly, similarly high peptide:HDL ratios caused a strong suppression of HDL-mediated cholesterol efflux from cells (**Figure 4.6**).

With respect to ABCA1, a different trend was observed compared to the results from macrophages stimulated with IDR-1018 in media containing human serum. At time zero, the

cholesterol-loaded macrophages exhibited elevated ABCA1 levels (**Figure 4.10**), which was expected given that ABCA1 expression is regulated, in part, by intracellular cholesterol levels with cholesterol loading leading to its upregulation (293-295). After 16 hours the ABCA1 level declined slightly in the BSA control. This was attributed to passive aqueous diffusion of cholesterol from the cells (267). Treatment with HDL further decreased ABCA1, presumably as a result of HDL-mediated induction of cholesterol efflux from the cells, reducing intracellular cholesterol content (294). Interestingly, ABCA1 was nearly undetectable in cells stimulated with 13μM of IDR-1018 alone or in combination with HDL (114:1 peptide:HDL ratio). This contrasted with the results obtained when macrophages were stimulated with 13μM of IDR-1018 in the presence of human serum, but without additional HDL (**Figures 4.1 and 4.2**). Much like the results for ApoA1, the low peptide:HDL ratios had little effect on ABCA1 levels compared to HDL alone.

4.4 DISCUSSION

This study shows that low concentrations of IDR-1018 elevated the level of several proteins involved in cellular cholesterol efflux, aided HDL in the suppression of cholesterol esterification by ACAT and enhanced HDL-mediated cholesterol efflux from SMCs and macrophages. Based on these data, I propose a model in which IDR-1018 enhanced HDL-mediated cholesterol efflux by binding to HDL in serum and promoting its association with cells. It is possible that this improved the transfer of cholesterol to HDL particles while still allowing HDL to dissociate from the cell after efflux. In addition, this interaction significantly improved HDL-mediated suppression of cholesterol esterification. At higher concentrations, IDR-1018 strongly enhanced association of HDL with cells but efflux was inhibited, possibly because the dissociation of HDL from the cell was reduced (**Figure 4.11**).

Stimulation with IDR-1018 of macrophages not loaded with cholesterol induced a significant increase in several components of the cholesterol efflux pathway including a dose-dependent increase in two of the most important components, ApoA1, and ABCA1. The increase in ApoA1 observed in response to IDR-1018 stimulation, was likely the result of IDR-1018 interacting with HDL in serum, thus facilitating its interaction with the plasma membranes of macrophages, since delipidation of serum abolished the IDR-1018-mediated increases in ApoA1. Several immunomodulatory peptides interact with both mammalian membranes and biomolecules such as lipoproteins and DNA (203, 290, 296-298). This allows the peptides to

facilitate interactions between these biomolecules and cells (290, 296, 299). I propose that IDR-1018 interacted with HDL in a similar manner.

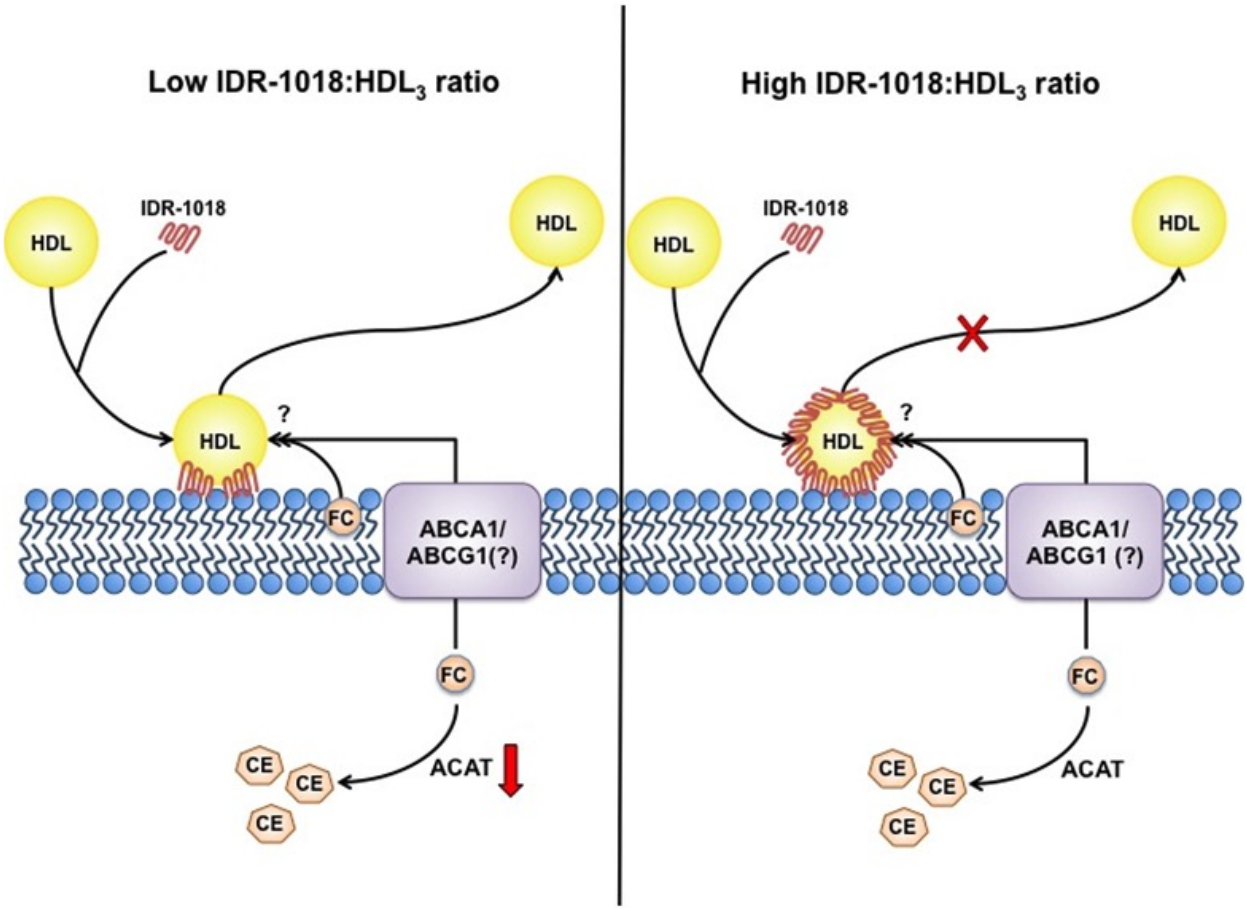

Figure 4.11: Proposed model for the enhancement of HDL-mediated cellular cholesterol efflux by IDR-1018. At low concentrations, IDR-1018 enhanced HDL-mediated cholesterol efflux by binding to HDL particles in serum and facilitating their association with cells. This improved the transfer of cholesterol to HDL particles via ABCA1, ABCG1 or passive aqueous diffusion, while still allowing its dissociation from cells. The addition of IDR-1018 also enhanced HDL-mediated suppression of cholesterol esterification in cells. At high concentrations, IDR-1018 became inhibitory possibly because the interaction between IDR-1018, HDL and the plasma membrane was too strong, preventing the dissociation of HDL from the cell. However, suppression of cholesterol esterification appeared unaffected by IDR-1018.

Although IDR-1018 was unable to promote cholesterol efflux from macrophages directly, IDR-1018 did enhance HDL-mediated cholesterol efflux from macrophages and SMCs. However, this was dependent on the concentration of IDR-1018 used. Concentrations of IDR-1018, similar to those that substantially increased ApoA1 association with macrophages, actually inhibited HDL-mediated cholesterol efflux from cells. Conversely, the low IDR-1018 concentration used to generate the 1:1 peptide:HDL molar ratio (0.12 μM IDR-1018), enhanced cholesterol efflux from macrophages, as well as significantly reduced intracellular free

cholesterol and the esterification of cholesterol for intracellular storage. IDR-1018 also significantly enhanced HDL-mediated cholesterol efflux from SMCs and reduced cholesterol ester levels compared to HDL alone, at low concentrations. Macrophages and SMCs play a significant role in the pathogenesis of atherosclerosis, in part by harbouring excessive levels of cholesterol in arteries (207, 265). Interestingly, the concentrations of IDR-1018 that enhanced HDL-mediated cholesterol efflux from macrophages and SMCs did not visibly alter ApoA1 protein levels. Based on these results I hypothesize that the ability of IDR-1018 to enhance HDL-mediated cholesterol efflux was partly dependent on the number of IDR-1018 molecules available to interact with each HDL particle. Low concentrations of IDR-1018 enhanced cholesterol efflux since only a few positively charged IDR-1018 molecules bound to each HDL particle in serum and were able to improve their interaction with the negatively charged cell surface. However, the interaction was still weak enough to allow the HDL particles to dissociate from the cells after taking up the effluxed cholesterol. Significantly increasing the concentration of IDR-1018 inhibited cholesterol efflux because the substantially higher number of IDR-1018 molecules present might have bound to and coated the HDL particles present and facilitated stronger association with the cell surface. This was likely also the cause for the visible increase in ApoA1 association with macrophages observed. However, this interaction might have been too strong to allow HDL dissociation from the cell and normal cholesterol efflux (**Figure 4.11**).

The results presented in this chapter showed that IDR-1018 enhanced HDL-mediated cholesterol efflux from multiple cell types, by facilitating the interaction of HDL with cells. In addition, IDR-1018 improved the ability of HDL to suppress cholesterol esterification in macrophages. Animal models of reverse cholesterol transport would be needed to determine if the enhanced cellular cholesterol efflux observed is physiologically relevant however, I predict that there is strong potential for success based on the results of others with ApoA1 peptide mimetics (92, 94-97, 273-279). Regardless, the results presented here suggest that immunomodulatory peptides like IDR-1018 represent a large untapped, group of molecules that could be developed for the treatment of atherosclerosis.

Chapter 5: DISCUSSION: MODULATION OF MACROPHAGE METABOLISM AND ACTIVITY BY IDR-1018

An extremely close association between the metabolism and the immune response has been observed. Clinically, malnourished patients exhibit frequent chronic infections as well as increased rates of hospitalization and morbidity; a syndrome that has become known as nutritionally acquired immunodeficiency (16, 20). These clinical findings have resulted in the development of a new research field, nutritional immunology. Cells of the innate immune system, the first line of defense against insults, rely on glucose, glutamine and fatty acid metabolism to generate energy and the metabolites required for macromolecule biosynthesis as well as to regulate their response to infectious agents (25-27). Interestingly their metabolism, and therefore function, is regulated by pathogen and host-derived external signals such as cytokines and TLR agonists (23, 300). This has been best characterized in two myeloid cell types, macrophages and dendritic cells. Withdrawal of specific macronutrients such as glucose and glutamine or inhibition of their metabolic pathways negatively impacts macrophage and dendritic cell survival, activation, differentiation and function (23, 31, 33, 36-38, 44, 55, 57, 59). Nutrient excess also has deleterious effects, causing chronic inflammation and cell death. Atherosclerosis is a particularly interesting case linking nutrient excess, in this case cholesterol, with dysregulation of the immune response, specifically macrophage activity. Hyperlipidemia leads to the development of lipid-laden macrophages known as foam cells, a major pro-inflammatory component of arterial plaques (75). IDRs, synthetic derivatives of HDPs, were developed as new anti-infective therapeutics, which act by supporting the immune response rather than directly targeting pathogens. Although a lot is known about the immunomodulatory activity of both IDRs and HDPs, the mechanisms underlying these activities remain to be fully elucidated. The results of this thesis identified a novel activity of IDR peptides, the ability to modulate immune cell metabolism under normal conditions as well as conditions of nutrient excess. This regulation of cellular metabolism was also found to be a potential mechanism by which IDR peptides exert their immunomodulatory activity. In addition, the research in this thesis also demonstrated that 2DG, a well-known glycolytic inhibitor, actually exhibited a more complex regulation of inflammatory responses. Specifically, 2DG modulated inflammatory responses through inhibition of metabolism coupled with activation of ER stress and the UPR.

5.1 IDR-1018 MODULATES GLUCOSE METABOLISM IN ORDER TO EXERT ITS IMMUNOMODULATORY ACTIVITY

The metabolic reprogramming of immune cells induced by external signals, such as TLR agonists and cytokines, defines how these cells will function (23). Macrophages and dendritic cells activated by pro-inflammatory mediators rely on aerobic glycolysis and glutamine metabolism to generate energy and metabolites that can act as transcriptional regulators or building blocks for macromolecules, both of which are necessary for an effective inflammatory response (36-38). There was some evidence to suggest that IDRs and HDPs modulate metabolism at the whole organism and cellular level, and that this might be important to their immunomodulatory activity (199-201, 203). However, little research had been performed in this area prior to my thesis research. Thus, the first aim of this thesis was to determine whether IDR peptides modulate macrophage metabolism and if so, what role this plays in their immunomodulatory activity. The results presented in Chapter 2 showed that IDR-1018 modulated glucose metabolism and that this was important to its ability to induce chemokine production, a hallmark of IDR peptide immunomodulatory activity.

In Chapter 2, IDR-1018 was shown to enhance glycolysis and TCA cycle activity by PBMCs. It also stimulated the production of lactate by macrophages, suggesting that, like TLR agonists, IDR-1018 stimulates glycolysis in immune cells (36-38, 55). This study also demonstrated that IDR-1018 activated the ERK signalling pathway in macrophages and stimulated the production of several chemokines. IDR peptides rely on several signalling pathways, including the ERK pathway, to induce chemokine production by cells (190, 193). The ERK pathway is also a major regulator of glucose metabolism in a variety of cell types, including macrophages (221-224). Activation of ERK leads to phosphorylation of pyruvate kinase M2, which stimulates the activity of transcription factors c-myc and HIF-1α. Both transcription factors increase the expression of several pro-glycolytic enzymes, leading to an increase in aerobic glycolysis (55, 221). Thus, I propose that the activation of ERK is, in part, responsible for both IDR-1018 induced chemokine production and enhanced glycolysis in macrophages.

IDR peptides are known to trigger a mixed response, with both anti- and pro- inflammatory elements, including the pro-inflammatory promotion of chemokine production and chemotaxis by cells (202). In Chapter 2 it was demonstrated that the IDR-1018 mediated production of several chemokines was suppressed when macrophages were pretreated with the glycolytic inhibitor 2DG. This also occurs in the case of TLR agonists and is consistent with the suggestion

that the activation of glycolysis is important to the pro-inflammatory aspects of IDR-1018 immunomodulatory activity. One possibility was that this was mediated by reduction in ATP levels since inhibition of glycolysis decreased ATP levels by 45-65%. This substantial drop in energy stores did not affect cell viability but might have potentially prevented energy-intensive biosynthetic pathways induced by IDR-1018 such as chemokine production and secretion (219). However this seems unlikely given that Everts *et al*. (36) previously found that ATP generated through glycolysis was not required for dendritic cell activation by TLR agonists and production of pro-inflammatory cytokines, and that inhibiting the generation of ATP through oxidative phosphorylation also had no effect. Indeed this result contradicts one model of pro-inflammatory immune cell activation that suggests the involvement of a metabolic switch from oxidative phosphorylation to glycolysis (55, 301), resulting in a dependence on glycolysis for ATP generation. Instead, it appears that immune cells are flexible with respect to the pathway used for energy generation (36, 302). Therefore, it is unlikely that a decrease, by 2DG, in glycolysis-dependent ATP stores was solely responsible for the suppression of IDR-1018 activity.

On the other hand 2DG significantly reduces glycolytic flux resulting in dysregulation of downstream metabolic pathways like the TCA cycle (220). These pathways are essential for the generation of the metabolites needed for gene expression and macromolecular biosynthesis, rather than energy generation (36, 38, 220). Consistent with the involvement of glycolytic flux, the pre-treatment of macrophages with sodium oxamate, another inhibitor of glycolytic flux (247), significantly inhibited IDR-1018 induced MCP-3 production. Lu et al. (247) previously found that the treatment of cells with oxamate led to reduced glucose-dependent macromolecule biosynthesis. Based on the collective data presented here, I propose that, for its immunomodulatory activities, IDR-1018 induces glycolysis to promote glucose-dependent macromolecular biosynthetic processes, such as those involved in chemokine production.

Pretreatment of macrophages with 2DG also suppressed IDR-1018 induced ERK phosphorylation. The ERK signalling pathway is partly responsible for IDR peptide-induced chemokine production (190, 193) as well as the upregulation of glycolysis in several cell types (221-224). Inhibition of the ERK signalling pathway in macrophages suppresses LPS induced cytokine and ROS production as well as basal and LPS-enhanced glycolytic flux (222). Although it is unclear how 2DG suppressed IDR-1018 induced ERK phosphorylation and therefore activation, this suppression could affect IDR-1018 induced chemokine production through one of two different mechanisms. Inhibition of ERK activation by 2DG might prevent the activation of downstream transcription factors such as AP-1, which is responsible for the

transcription of chemokines and cytokines (248). Conversely, inhibition of ERK activity might represent an alternative mechanism by which 2DG inhibits glycolysis, preventing IDR-1018-mediated stimulation of glycolysis, and thus limit the promotion of macromolecular biosynthesis.

Overall, the results from Chapter 2 showed that IDR-1018 induced glycolysis and that its activation was important for IDR-1018 induced chemokine production. It also provided evidence to suggest that the induction of glycolysis and chemokine production might be mediated by activation of the ERK pathway. Further research is required to determine how IDR-1018 affects the flux of glucose-derived carbon into metabolic pathways such as the TCA cycle and pentose phosphate pathway, as well as how 2DG acts through the ERK signaling pathway to perturb metabolism. HDP and IDR peptides act on a variety of different immune cell types, all of which rely on metabolic reprogramming to ensure an appropriate response to a given signal (23, 202). The data presented in Chapter 2 hinted that IDRs are also capable of regulating metabolism by multiple immune cell types since IDR-1018 was shown to enhance glycolysis and TCA cycle activity by PBMCs, which is a mixed population of immune cells. Finally, HDPs and IDR peptides have been shown to modulate the differentiation of macrophages and T lymphocytes (202). Macrophages and T lymphocytes have multiple differentiation states, each with different phenotypes that are triggered by external signals modulating metabolic pathways. Thus, future research should also focus on how IDR peptides modulate metabolism in other cell types as well as whether peptide-induced differentiation of cells is associated with metabolic reprogramming.

5.2 REGULATION OF ENDOTOXIN-INDUCED INFLAMMATION BY IDR-1018, AND 2DG INVOLVES SUPPRESSION OF GLYCOLYSIS

Another hallmark of HDP and IDR peptide immunomodulatory activity is their ability to suppress TLR-agonist induced pro-inflammatory responses. In fact, this is one of the markers used to identify IDR peptides with enhanced activity compared to their parent peptides. Although some research into the mechanism(s) behind this action has been performed, examining how IDR peptides modulate signalling pathways activated by TLR agonists, much still remains to be learned. In Chapter 2 IDR-1018 was shown to induce chemokine production, in part, through induction of glycolysis. However we are starting to appreciate that the chemokine inducing and anti-inflammatory properties of IDRs are separately determined having different structural determinants (188). The aim of Chapter 3 was to determine if the anti-

inflammatory activities of IDR peptides, specifically their anti-endotoxin activity, also resulted from modulation of cellular metabolism. The results of Chapter 3 indeed showed that IDR-1018 was able, in part, to suppress LPS-induced pro-inflammatory responses by inhibiting LPS-induced glycolysis. Inhibition of glucose metabolism using 2DG and sodium oxamate enhanced this activity.

Macrophages stimulated with LPS for 24 hours upregulated the expression of PI3K, HIF-1α and PFKFB3, all three of which are components of the mTOR signaling pathway and major regulators of sustained aerobic glycolysis (37, 46, 51-53, 263). TBK1, which along with IKKε and AKT, initiates the rapid metabolic reprogramming in LPS stimulated dendritic cells (36) was also upregulated. However, given the 24 hour length of stimulation, it is possible that the upregulation of TBK1 was associated more with induction of the Type I interferon response (264) than early metabolic reprogramming, which occurs within the first hour of stimulation (36). Regardless, these results suggested that stimulation of macrophages with LPS resulted in induction of aerobic glycolysis at the transcriptional level. Furthermore, stimulation of macrophages with LPS induced a significant increase in extracellular lactate, an indicator of increased glycolytic activity. Taken together the results suggest that, as seen in previous studies (30, 37, 38, 57, 58), LPS-stimulated macrophages undergo a metabolic shift to aerobic glycolysis.

The shift towards aerobic glycolysis induced by TLR agonists, such as LPS, is necessary for the generation of a pro-inflammatory response (27, 36-38, 55-58). IDR-1018 significantly suppressed LPS-induced chemokine and cytokine production and induced an apparent decrease ($p=0.064$) in early lactate production to a level equal to that of non-LPS-treated cells, which was consistent with a suppression of glycolysis. Based on this data, I hypothesized that the anti-endotoxin activity of IDR-1018 might involve suppression of LPS-induced glycolysis. Suppression of LPS-induced glycolysis by IDR-1018 may have stemmed from IDR-1018-mediated suppression of the components of the Type 1 interferon response. IDR-1018 downregulated the TBK1/IKKε signalling pathway, which is a key mediator of metabolic reprogramming in dendritic cells and is required for the production of inflammatory mediators such as IL-6 and TNF-α (36). IDR-1018 also suppressed the expression of ISG15, which is an important component of the anti-viral and anti-mycobacterial responses of macrophages (259, 303). ISG15 becomes covalently linked to numerous proteins including several glycolytic enzymes through a process known as ISGylation (260, 304). Although it is unknown how ISGylation affects the activity of glycolytic enzymes, inhibition of ISG15 expression might be

another mechanism by which IDR-1018 suppresses LPS induced glycolysis.

Both 2DG and sodium oxamate suppressed LPS-induced chemokine and cytokine production, suggesting that suppression of glycolysis was sufficient to inhibit pro-inflammatory responses induced by TLR agonists. 2DG also significantly improved IDR-1018 mediated suppression of LPS-induced MCP-1, MIP-1α, IL-8, TNF-α, and IL-6. In addition, biological pathway over-representation analysis and network construction, using the down-regulated genes from an RNA-Seq study (Table 3.2), revealed that 2DG significantly dysregulated pathways associated with inflammation as well as the TCA cycle. Several studies have shown that the activation of glycolysis in response to LPS stimulation is required for the generation of metabolites through connected pathways, such as the pentose phosphate pathway and the TCA cycle, that can serve as activators of pro-inflammatory transcription factors and substrates for macromolecule synthesis (29, 36, 38). Thus, the suppression of glycolysis by 2DG and sodium oxamate might have enhanced the anti-endotoxin activity of IDR-1018 by preventing the accumulation of TCA cycle intermediates.

The results of Chapter 3 indicated that the anti-endotoxin activity of IDR-1018 might be due in part to the ability of IDR-1018 to suppress LPS-induced upregulation of glycolysis, and that pharmacological inhibition of glucose metabolism enhanced the anti-endotoxin activity of IDR-1018. Further research is required to pinpoint the mechanism by which IDR-1018 induces glycolysis while being able to inhibit LPS-mediated activation of the same pathway. Macrophages and dendritic cells exposed to other TLR agonists and certain pro-inflammatory cytokines such as IFN-γ undergo the same metabolic reprogramming as those exposed to LPS (23, 46). It would be worthwhile to examine whether IDR peptides like IDR-1018 also suppress the inflammatory responses induced by these mediators through modulation of metabolism. Finally, the data gathered in Chapters 2 and 3 are all a result of *in vitro* experiments; it would be of interest to determine whether modulation of metabolism by IDR-1018 occurs at the whole animal level and whether it plays a role in the protective effects of IDRs in murine infection models.

5.3 MODULATION OF IDR-1018 ACTIVITY BY 2DG THROUGH ACTIVATION OF ER STRESS AND THE UPR

2DG has been predominantly used as a glycolytic inhibitor since it was found to inhibit hexokinase II and phosphoglucose isomerase, the first two enzymes in glycolysis (218). Numerous immunological studies utilize 2DG to assess the role of glycolysis in pro-

inflammatory responses. Indeed, in Chapters 2 and 3, I showed that 2DG modulated the immunomodulatory activity of IDR-1018 in part though inhibition of glycolysis. However, biological pathway overrepresentation analysis of upregulated genes from the RNA-Seq transcriptomic studies presented in Chapters 2 and 3 revealed that, in addition to inhibiting glycolysis, 2DG was also upregulating pathways associated with ER stress and the UPR. Although this activity of 2DG is fairly well established, various mechanisms have been proposed to explain it (226-230). Van der Harg et al. (228) suggested that 2DG-mediated inhibition of glycolysis and consequent depletion of ATP led to activation of the UPR in *in vitro* models of neurodegeneration. Conversely several other groups studying the anti-tumor activity of 2DG concluded that activation by 2DG of the UPR, as well as autophagy, was due to inhibition of N-linked glycosylation of proteins (226, 227, 230). This stemmed from the structural similarity of 2DG to mannose, which allows it to compete with mannose in the initial stages of N-linked glycosylation, resulting in the accumulation of misfolded proteins in the ER and consequent activation of the UPR (229). Therefore, in Chapters 2 and 3 I sought to determine to what extent 2DG-mediated activation of the UPR played a role in the regulation of IDR-1018 immunomodulatory activity.

2DG activated the UPR alone and in combination with IDR-1018, as demonstrated by increased BiP levels as well as the induction of autophagy, a property of 2DG-mediated activation of the UPR that was recently described by Xi et al. (226). A well-known inducer of the UPR, tunicamycin, like 2DG, also suppressed IDR-1018-mediated chemokine production, demonstrating that activation of the UPR was capable of modulating IDR-1018 activity. The RNA-Seq transcriptomic data also revealed that the PERK arm of the UPR was highly affected by 2DG, with several of its major components significantly upregulated. Activation of the PERK arm was of particular interest since it has been identified as a negative regulator of TLR-mediated chemokine and cytokine production (101, 110, 249, 250). A hallmark of prolonged activation of the PERK pathway is the disruption of the cellular redox state through excessive ROS production and depletion of GSH (240, 243-245). Treatment with 2DG significantly decreased GSH levels indicating that 2DG was activating the PERK pathway. Similarly, specific activation of the PERK pathway by salubrinal appeared to inhibit IDR-1018 induced MCP-1 and IL-8 production by PBMCs. Taken together, these data suggest that, in addition to inhibition of glycolysis, activation by 2DG of the UPR, and specifically the PERK arm, modulates IDR-1018 pro-inflammatory activities.

2DG also upregulated pathways associated with ER stress and the UPR when used alone or

in combination with LPS, or LPS together with IDR-1018. Furthermore, activation of the UPR by tunicamycin also suppressed LPS-induced chemokine production. These results suggest that activation of the UPR by 2DG also played a role in enhancing the anti-endotoxin activity of IDR-1018 with respect to chemokine production. In contrast, activation of the UPR by tunicamycin increased LPS-induced TNF-α and IL-6 production. LPS has been shown to suppress the PERK and ATF6 pathways while upregulating the IRE1α pathway (108, 109). Induction of the IRE1α pathway leads to activation of XBP1, a transcription factor that is essential to the sustained production of pro-inflammatory cytokines, specifically IL-6 and TNF-α (108). Interestingly, analysis of the genes upregulated in response to the combination of 2DG and LPS revealed that numerous genes associated with IRE1α activation were substantially upregulated, while only a few associated with the PERK arm were. These results suggest that LPS was able to modulate the activation of the UPR by 2DG, enhancing the pro-inflammatory IRE1α arm while suppressing the anti-inflammatory PERK arm. This makes it unlikely that activation of the UPR was responsible for the suppression of IL-6 and TNF-α production caused by 2DG. Rather, it appears that with respect to cytokine production, 2DG suppressed LPS activity, and enhanced IDR-1018 anti-endotoxin activity, through inhibition of glycolysis.

The data presented in Chapters 2 and 3 demonstrated that the glycolytic inhibitor 2DG actually has pleiotropic effects on immune cells, suppressing pro-inflammatory responses through modulation of both glycolysis and the UPR. Interestingly, most other studies utilizing 2DG as a glycolytic inhibitor to indicate the necessity of glycolysis for the pro-inflammatory responses in immune cells, have failed to identify the UPR as another pathway by which 2DG might be suppressing activity. Nevertheless, several components of the UPR, including CHOP, were upregulated in microarray data for murine macrophages simulated with 2DG and LPS (38), which is consistent with my assertion that the UPR is activated by 2DG. Further research is required to delineate the extent to which the pathways dysregulated by 2DG, glycolysis and the UPR affect the inflammatory responses of immune cells. Finally, the results of this project illustrate the need for a better understanding of how pharmacological compounds like 2DG and immunomodulatory peptides act.

5.4 IDR-1018 REGULATION OF CHOLESTEROL EFFLUX

Based on the results of previous studies as well as those presented in Chapters 2 and 3, it was clear that IDR peptides modulate immune cell metabolism and that this was important for their immunomodulatory activity. Another major goal of this thesis was to determine whether

IDR peptides were capable of modulating dysregulated metabolic pathways associated with chronic inflammatory conditions such as obesity, type-2 diabetes and atherosclerosis. HDPs and IDR peptides possess many of the physical and functional attributes of ApoA1 peptide mimetics, which are currently in clinical trials for the treatment of atherosclerosis, as a result of their ability to enhance reverse cholesterol transport (91). Therefore, I chose to assess the ability of IDRs to regulate intracellular cholesterol efflux, the first step in reverse cholesterol transport, in cholesterol-loaded macrophages, a major cellular component of atherosclerotic plaques and mediators of the chronic inflammation observed in atherosclerosis.

IDR-1018 significantly increased the levels of three major components of the cholesterol efflux pathway, ApoE, ApoA1, and ABCA1. Interestingly, this was not a result of transcriptional regulation by IDR-1018. With respect to ApoA1, this was further supported by the fact that ApoA1 was found associated with macrophages as early as 15 minutes post stimulation with IDR-1018, making transcriptional regulation less likely. Analysis of ApoA1 levels in cholesterol-loaded macrophages stimulated under varying serum conditions revealed that delipidation of serum abolished the IDR-1018-mediated increase in ApoA1, indicating that IDR-1018 was interacting with the lipidated form of ApoA1, HDL (291). Based on the data, we propose that the elevated ApoA1 levels observed in macrophages stimulated with IDR-1018 were due to the peptide binding to serum ApoA1, in the form of HDL, and consequently facilitating the interaction of HDL with cells. Numerous immunomodulatory peptides bind to mammalian membranes (203, 296-298). Some of these peptides also interact with biomolecules such as lipoproteins and DNA, which facilitates interactions between the biomolecules and cells. (296, 299). For example defensins bind lipoproteins and facilitate their interaction with endothelial cells and smooth muscle cells (290). LL-37 is also capable of binding lipoproteins, non-covalently interacting with both lipid-poor ApoA1 and HDL (128, 289). However the ability of LL-37 to subsequently promote the interaction between these biomolecules and cells has not been well studied and in fact serum inhibited LL-37 mediated apoptosis of human airway cells (305). LL-37 has been shown to bind at least two other biomolecules, DNA and heparin sulfate, allowing LL-37 to facilitate their uptake by cells through interactions with lipid rafts (296, 299). It is possible that IDR-1018 is capable of doing the same with lipoproteins such as HDL. Although IDR-1018 is likely binding to HDL, the nature of its interaction is unclear. LL-37 is believed interact with HDL through its hydrophobic C-terminus interacting with the lipids of HDL (128). It is possible that similar interactions might be involved in the binding of IDR-1018 to HDL.

The increase in ABCA1 induced by IDR-1018 in macrophages stimulated in the presence of human serum likely also occurred post-transcriptionally. ApoA1 and several ApoA1 peptide mimetics increase ABCA1 protein levels (306-308) through stabilization of the ABCA1 protein and prevention of its degradation, an important mode of post-translational regulation for this transporter (306, 307, 309, 310). The results suggest that IDR-1018 was able to increase ABCA1 levels by stabilizing it, although it is unclear if it did so directly or through its interaction with HDL. Conversely, in cholesterol-loaded macrophages, IDR-1018 caused a substantial decrease in ABCA1 levels. Because the intracellular cholesterol level is also a major regulator of ABCA1, this result was initially thought to be due to IDR-1018 inducing cholesterol efflux from cells, which would result in a downregulation of ABCA1 (294). However, IDR-1018 alone had no effect on cholesterol efflux and at higher concentrations inhibited HDL-mediated cholesterol efflux, making this explanation unlikely. It is not completely clear why IDR-1018 suppressed ABCA1 in cholesterol-loaded macrophages, while in the non-cholesterol loaded cells it induced an increase, however this might reflect differential regulation in the two backgrounds at either the transcriptional or translational levels.

The inability of IDR-1018 to directly induce cholesterol efflux was unexpected given that it possesses many of the physical attributes of ApoA1 peptide mimetics, chiefly in forming an amphipathic α-helix (278, 279). Several factors associated with this structure are important to ApoA1 peptide mimetic activity including the number of helices per peptide, stability, hydrophobicity, and lipid affinity as well as the location of acidic residues along the polar face of the helices (275, 277-279, 311). There are several possibilities for why IDR-1018 did not induce cholesterol efflux directly. IDR-1018 may not maintain a helical conformation; in fact, it is largely unstructured in buffer and although it forms an α-helix in the neutral membrane mimetic DPC, it forms an array of structures when interacting with anionic lipids (or upon aggregation, Evan Haney, personal communication) and the effect of cholesterol is indeed unknown (280). Stabilizing the helicity of IDR-1018 might allow it to induce cholesterol efflux on its own or improve its ability to enhance HDL-mediated efflux. Alternatively, the inability of IDR-1018 to induce cholesterol efflux might be because it contains basic rather than acidic residues. Natarajan et al. (311) found that the peptides capable of stabilizing ABCA1 and inducing cholesterol efflux were both a lot larger than IDR-1018 (22-33 vs. 12 amino acids) and contained acidic residues (net charge -1) that were aligned linearly along the polar face of the amphipathic helices. However, Sethi et al. (277) generated several inactive ApoA1 mimetic peptides with this acidic motif, indicating that although this motif may be important to peptide activity it is not solely

responsible for it.

IDR-1018 at low concentrations did, however, enhance HDL-mediated cholesterol efflux from macrophages, and SMCs, although high peptide:HDL molar ratios inhibited HDL-mediated cholesterol efflux from cells. The concentrations of IDR-1018 used to generate these inhibitory peptide ratios were similar to the ones that substantially increased ApoA1 association with macrophages. Conversely, low peptide:HDL molar ratios (1:1 and 5:1) enhanced cholesterol efflux from macrophages and smooth muscle cells as well as significantly reducing cholesterol esterification in macrophages compared to HDL alone. Interestingly, the concentrations of IDR-1018 used to generate the low peptide:HDL ratios did not visibly alter ApoA1 protein levels associated with macrophages. I hypothesize that this was because at these the low peptide:HDL ratios, fewer peptide molecules were present to interact with each HDL particle. Thus, although IDR-1018 enhanced HDL association with cellular membranes, this interaction would have been relatively weak, allowing HDL particles to dissociate from the cell after taking up cholesterol. As the peptide:HDL ratio increased, more peptide molecules were present to interact with each HDL particle, eventually coating HDL particles with peptide. While this likely enhanced the interaction of HDL with cellular membranes significantly, it also potentially prevented the HDL particles from dissociating from the cells, thus inhibiting cholesterol efflux. A model of this hypothesis is presented in Chapter 4 (**Figure 4.11**).

The results presented in Chapter 4 suggest that IDR-1018 enhanced HDL-mediated cholesterol efflux from multiple cell types, in part through facilitating the interaction of HDL with cells and also improved HDL-induced suppression of cholesterol esterification in macrophages. These represent new biological properties for IDR-1018. Further research is required to clarify the exact mechanisms behind these activities. Of particular importance is defining which cholesterol transporters were involved in the enhanced efflux observed. ABCA1 predominantly transfers cholesterol to preβ-HDL particles, which consist of fewer lipids, while ABCG1 transfers cholesterol to more mature HDL particles, like HDL_3 [267, 312]. Considering that the experiments in this study were completed using HDL_3, it is more likely that IDR-1018 was enhancing the transfer of cholesterol to HDL via ABCG1. The increase in cholesterol efflux may also have been a result of passive removal of cholesterol from the plasma membrane (267). In addition, animal models of reverse cholesterol transport are needed to determine if the enhanced cellular cholesterol efflux induced by IDR-1018 is physiologically relevant. This is especially important given that there has been little development for many of the ApoA1 peptide mimetics that entered clinical trials for the treatment of atherosclerosis (88) and although ApoA1 mimetic

peptides are able to directly induce cholesterol efflux, high concentrations (10-80 μg/ml) are required to achieve this effect (277, 279, 308). In our system, a minimal amount of IDR-1018 was needed, 0.12 to 0.58 μg/ml, to enhance cholesterol efflux normally induced by HDL. The results presented here thus suggest that the immunomodulatory peptides currently in development as anti-infectives could potentially be used for the treatment of chronic inflammatory conditions associated with excess nutrients such as atherosclerosis.

5.5 CONCLUDING REMARKS

Metabolism at the whole organism and cellular level is intimately linked to the immune system and its activity. Research into the interconnectivity of these two major biological pathways has significantly increased as a result of the substantial malnutrition observed in the world today. Much of the research has focused on how external signals can modulate metabolism in order to modify immune cell activity. In this thesis, the ability of IDR peptides to modulate metabolism by macrophages was examined. As a result of their vast immunomodulatory activity and relatively low toxicity, IDRs are being developed as novel therapeutics for a wide range of applications including as a treatment of bacterial infections, a potential adjunctive therapy for cerebral malaria, and as vaccine adjuvants (202). Although a significant amount of research has been done to characterize the variety of ways in which IDRs modulate the immune response, the mechanisms underlying these immunomodulatory activities remain to be fully elucidated. Clarifying the mechanisms by which IDR peptides exert their anti-infective activities is essential for their clinical development as well as the generation of new, more effective IDRs. Here I identified the modulation of glycolysis as a possible mechanism by which IDRs regulate immune cell responses. In addition, my investigation identified a novel use of IDRs in the modulation of metabolic pathways dysregulated by dietary excess specifically, atherosclerosis. Finally, I also characterized the complex regulation of inflammatory responses by 2DG, which involved dysregulation of both glycolysis and the UPR, a combination not identified in previous immunological studies. Overall, the results presented here expand the understanding of IDR peptide activities to include regulation of cellular metabolism, a system essential to cellular survival and function, and identify this as a mechanism by which IDR peptides modulate immune cell responses. These insights could aid in the development of more effective IDRs as anti-infective therapeutic agents, as well as the development of IDRs as therapeutic agents for the treatment of chronic inflammatory conditions induced by nutrient excess, a major problem in the world today.

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