Characterising the impact of obesity on Dendritic Cell metabolism & function

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List of Abbreviations

1,3-BPG 1,3-bisphosphoglycerate

2-DG 2-deoxy-D-glucose

2-DG6P 2-deoxy-D-glucose-6-phosphate

AA Amino Acid

AAO Amino Acid Oxidation

Ab Antibody

ACC Acetyl-CoA carboxylase

Actb Actin Beta

ADCC Antibody-dependent cell cytotoxicity

ADCP Antibody-dependent cellular phagocytosis

ADP Adenosine Diphosphate

Ag Antigen

ALDO Aldolase

AMP Adenosine monophosphate

AMPK Adenosine monophosphate (AMP)-activated protein kinase

ANOVA Analysis of variance

APC Antigen Presenting Cell

APS Ammonium persulphate

AT Adipose Tissue

ATP Adenosine Triphosphate

AUC Area Under Curve

AZ67 AZ 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3 (PFKFB3) inhibitor

Batf3 Basic Leucine Zipper ATF-Like Transcription Factor 3

BCR B cell receptor

BM-DM Bone marrow-derived Macrophage

BMI Body Mass Index

BSA Bovine Serum Albumin

CCL C-C Motif Chemokine Ligand

CCR C-C Motif Chemokine Receptor

CD cluster of differentiation

cDC conventional dendritic cell

cDNA complementary deoxyribonucleic acid

CDP common dendritic cell progenitor

cGMP Cyclic Guanosine Monophosphate

Clec C-type lectin-like receptor

CLP common lymphoid progenitor

CLR C-type lectin receptor

cMoP common monocyte progenitor

CMP common myeloid progenitor

CoQ Coenzyme Q

COVID-19 Coronavirus disease 2019

COX Cyclooxygenase

CPT Carnitine palmitoyltransferase

Ct Cycle threshold

CTL Cytotoxic T lymphocyte

CVD Cardiovascular Disease

CXCR C-X-C chemokine receptor

Cyt c Cytochrome c

DAMPs Damage-associated molecular patterns

DC Dendritic Cell

Ddx58 DEAD (Asp-Glu-Ala-Asp) Box Polypeptide 58

DEG Differentially Expressed Gene

DH_2O distilled water

DIO Diet-Induced Obesity

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

DPBS Dulbecco's Phosphate Buffered Saline

DPI Dots per Inch

e^- free electron

ECL Enhanced Chemiluminescence

ELISA Enzyme-Linked Immunosorbent Assay

ENO Enolase

ER Endoplasmic Reticulum

ETC Electron Transport Chain

F-2,6-BP Fructose-2,6-bisphosphate

F6P Fructose-6-phosphate

FA Fatty Acid

Fab Fragment antigen binding

FACS Fluorescence-activated cell sorting

FAD oxidized Flavin Adenine Dinucleotide

$FADH_2$ reduced Flavin Adenine Dinucleotide

FAO Fatty Acid Oxidation

FAS Fatty Acid Synthesis

Fasn Fatty acid synthase

FBS Fetal Bovine Serum

Fc Fragment crystallisable

FDR False Discovery Rate

FFA Free Fatty Acid

FL-DC Fms-like tyrosine kinase-3 ligand (Flt3L)-cultured dendritic cell

Flt3L FMS-like tyrosine kinase 3 ligand

FMO Fluorescence Minus One

FOXP3 Forkhead box protein 3

FPKM Fragments Per Kilobase of transcript per Million mapped reads

Fru-2,6-P_2 Fructose-2,6-bisphosphate

FSC-A Forward Scatter-Area

FSC-W Forward Scatter-Width

G6P Glucose-6-phosphate

GA3P Glyceraldehyde 3-phosphate

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

GFI1 Growth Factor Independent Protein 1

GLUT Glucose transporter

GM-CSF Granulocyte-macrophage colony-stimulating factor

GM-DC Granulocyte-macrophage colony-stimulating factor (GM-CSF)-cultured bone marrow-derived dendritic cell

GMPs Granulocyte-Macrophage Progenitors

GMP Guanosine Monophosphate

GO Gene Ontology

GPI Glucose-6-phosphate isomerase

GSEA Gene Set Enrichment Analysis

GTP Guanosine Triphosphate

GYS Glycogen Synthase

H^+ Hydrogen ion

H_2O water molecule

H_2SO_4 sulphuric acid

HA Haemagglutinin

HBP Hexosamine Biosynthetic Pathway

HBV Hepatitis B Virus

HDM House Dust Mite

HFD High Fat Diet

HIF Hypoxia-Inducible Factor

HISAT2 Hierarchical Indexing for Spliced Alignment of Transcripts 2

HIV Human Immunodeficiency Virus

HK Hexokinase

Hprt Hypoxanthine Phosphoribosyltransferase

HSC Haematopoietic Stem Cell

I Complex I

ICU Intensive Care Unit

ID2 Inhibitor of DNA Binding 2

IDH Isocitrate Dehydrogenase

Ifih1 Interferon Induced With Helicase C Domain 1

IFN interferon

IFNb Interferon Beta

Ig Immunoglobulin

II Complex II

III Complex III

IL Interleukin

ILC innate lymphoid Cell

iNOS Inducible Nitric Oxide Synthase

IRE1a Inositol-Requiring Enzyme 1 alpha

IRF Interferon Regulatory Factor

ISG interferon stimulated gene

ITGAX Integrin Subunit Alpha X

IV Complex IV

JAK Janus Kinase

KCl Potassium chloride

KH_2PO_4 Potassium dihydrogen phosphate

LBP Lipopolysaccharide-Binding Protein

LC Langerhans cell

LDH Lactate Dehydrogenase

LFD Low Fat Diet

LPS lipopolysaccharide

MAIT Mucosal Associated Invariant T cell

MAMs Mitochondria-associated membranes

MAVS Mitochondrial antiviral-signalling protein

MCP1 Monocyte Chemoattractant Protein 1

MD2 Myeloid Differentiation Protein-2

MDA5 Melanoma differentiation-associated protein 5

MDP common macrophage-dendritic cell-restricted precursor

MFI Geometric Mean Fluorescence Intensity

MHC Major Histocompatibility Complex

MIP Macrophage Inflammatory Protein

Mo-DC Monocyte derived-Dendritic Cell

MPC1 Mitochondrial Pyruvate Carrier 1

MSigDB Molecular Signatures Database

mTOR Mammalian Target of Rapamycin

Mx2 MX Dynamin Like GTPase 2

NAD^+ oxidised nicotinamide adenine dinucleotide

NADH reduced nicotinamide adenine dinucleotide

NAFLD Non-Alcoholic Fatty Liver Disease

NaH_2PO_4 Sodium dihydrogen phosphate

NCD Non-Communicable Diseases

NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells

NK Natural killer cell

NLR Nucleotide-binding oligomerization domain (NOD)-like receptor

NO Nitric oxide

Notch2 Neurogenic locus notch homolog protein 2

O_2 Oxygen molecule

Oas 2'-5' oligoadenylate synthetase

Oligo Oligomycin

OXPHOS Oxidative phosphorylation

PAMP Pathogen-associated molecular pattern

PANTHER Protein Analysis Through Evolutionary Relationships

PBMC Peripheral Blood Mononuclear cell

PBS Phosphate Buffered Saline

PCOS Polycystic Ovary Syndrome

PCR Polymerase Chain Reaction

pDC plasmacytoid dendritic cell

PDH Pyruvate dehydrogenase

PFK Phosphofructokinase

PFKFB3 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3

PGAM Phosphoglycerate mutase

Pgc1a Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1alpha

PGE_2 Prostaglandin E2

PGK Phosphoglycerate kinase

pIKBa phosphorylated-Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells (NF-κB) Inhibitor, Alpha

PKM Pyruvate kinase muscle isozyme

PPAR Peroxisome proliferator-activated receptor

PRR Pattern recognition receptor

PTGS Prostaglandin-endoperoxide synthase

Puro Puromycin

PWO People With Obesity

PYG Glycogen Phosphorylase

RANTES Regulated Upon Activation, Normally T-Expressed, And Presumably Secreted

RelB Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells 3

RIG-I Retinoic acid-Inducible Gene I

RLR Retinoic acid-inducible gene I (RIG-I)-like receptor

RNA Ribonucleic acid

RNAse L Ribonuclease L

RNAseq RNA sequencing

ROS Reactive Oxygen Species

ROUT Robust regression and Outlier removal

RPMI Roswell Park Memorial Institute

RT-PCR Real Time Polymerase Chain Reaction

SCENITH Single Cell ENergetIc metabolism by profilIng Translation inhibition

SD Standard Diet

SDS-PAGE Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis

SEM Standard Error Mean

SeV Sendai virus

SIRPa Signal regulatory protein alpha

Slc2a1 Solute carrier family 2 member 1

Slc2a3 Solute carrier family 2 member 3

Slfn Schlafen

SREBP Sterol regulatory element binding protein

SSC-A Side Scatter-Area

STAT Signal Transducer and Activator of Transcription

Strep-HRP Streptavidin-Horse Radish Peroxidase

T2DM Type 2 Diabetes Mellitus

TBS Tris-Buffered Saline

TBST Tris-Buffered Saline with Tween® 20

TC4 Transcription factor 4

TCA Tricarboxylic acid

TCR T cell receptor

TEMED Tetramethylethylenediamine

TGFB Transforming Growth Factor Beta

Th cell T helper cell

TIF Tagged Image Format

TLR Toll Like Receptor

TMB Tetramethyl Benzidine

TME Tumour Microenvironment

TNF Tumour Necrosis Factor

TNT tunnelling nanotubes

UK United Kingdom

V Complex V

Vit. D3 Vitamin D3

WAT White Adipose Tissue

WHO World Health Organisation

WT wild type

XCL1 X-C Motif Chemokine Ligand 1

XCR1 X-C Motif Chemokine Receptor 1

Abstract

Obesity is a worldwide epidemic, as of 2016, over 650 million people were living with obesity. Obesity is strongly associated with over 200 comorbidities. In addition, people with obesity (PWO) are more susceptible to infection, and have increased morbidity and mortality following infection, as highlighted during the H1N1 influenza and COVID-19 pandemics. Obesity has also previously been linked to reduced antibody titres following immunisation, depicting dampened vaccine efficacy. Dendritic cells (DCs) play an essential role in activating the adaptive immune response, particularly in stimulating the differentiation of naïve T cells into memory cells. DCs have been shown to be reduced in the periphery of PWO, and this was paired with dysfunctional cytokine production. However, the mechanisms underpinning these alterations remain unclear. Cellular metabolism has been shown to be critical for immune cell function. Therefore, we investigated if obesity impacted DC metabolism using a murine model of obesity. We utilized two different stimulations, bacterial-derived lipopolysaccharide (LPS) and an active virus, Sendai virus (SeV), and demonstrate both can drive robust activation of DCs as measured by cytokine production and metabolic activity. We also map DC metabolism, highlighting glycolysis as a major metabolic pathway and demonstrate metabolic flexibility when exposed to alternative carbon sources, maintaining similar levels of cytokine between glucose, fructose, galactose, or glucose-deprived conditions. We show that DC cytokine production is significantly impacted in our obesity model, especially of IFNβ and IL-6. Finally, we demonstrate reduced cellular metabolism, as measured by protein synthesis in both LPS- and SeV-stimulated DCs from mice on the high fat diet. Collectively, our data supports the possibility that defects in DC function are linked to altered cellular metabolism, and this may underpin dysfunctional pathogen or vaccine responses.

Chapter One

General Introduction

1.1 Immune System

1.1.1 Overview of the Innate and Adaptive Immune systems

There are two key components of a functional vertebrate immune system: the innate and the adaptive immune systems. The innate immune system is non-specific and comprises of the cells which patrol the body and are the first responders upon initial pathogen encounter, along with the physical, mucosal, and physiologic barriers of the body (Marshall *et al.,* 2018; Medzhitov & Janeway, 1997). The initiation of the immune response is governed by the recognition of non-specific shared markers amongst pathogens, known as pathogen-associated molecular patterns (PAMPs), or host-derived damage-associated molecular patterns (DAMPs) (Matzinger, 2003; Medzhitov & Janeway, 1997). PAMPs are non-self-motifs, conserved within microorganisms. DAMPs can occur during tissue damage or cellular stress. PAMPs and DAMPs are recognised via pattern recognition receptors (PRRs) either on the surface of or within phagocytic cells, particularly dendritic cells (DCs) and macrophages (Li & Wu, 2021).

PRRs differ in their localisation (Janeway & Medzhitov, 2002). Certain PRRs are found on the cell surface to detect extracellular pathogens or their ligands (Hayashi *et al.,* 2001). Other PRRs can be found within the cell, on organelle membranes or in the cytosol (Alexopoulou *et al.,* 2001; Janeway & Medzhitov, 2002; Walsh *et al.,* 2013). Upon triggering of the PRRs, immune cells have varied functions. Some innate immune cells (such as natural killer (NK) cells) can kill infected cells through cytotoxic properties (Huntington, 2014). Phagocytes endocytose and digest pathogen or pathogen-infected cells via the process of phagocytosis (Nagl *et al.,* 2002). Certain phagocytes have another essential function which is to act as antigen-presenting cells (APCs) (Nagl *et al.,* 2002). APCs phagocytose pathogens or pathogen-infected cells and digest the pathogen to expose antigens (Ags) which are presented on the APC's cell surface via major histocompatibility complexes (MHC), hence their name (Medzhitov & Janeway, 1997). Antigen presentation to T cells by DCs is a key process required to activate the adaptive immune system (Banchereau *et al.,* 2003).

The main differences between the innate and adaptive immune systems are pathogen recognition specificity, the length of time to respond to a pathogen and the generation of a

memory response (Janeway & Medzhitov, 2002; Vivier & Malissen, 2004). The adaptive immune cells recognise specific antigen-MHC molecules presented by APCs via their specific cell receptor, compared to innate immune cells which recognise general non-specific pathogen markers (Janeway & Medzhitov, 2002). This causes a lag time between activation of the adaptive immune response compared to the rapid response of the innate (Janeway & Medzhitov, 2002). Daily, humans are exposed to a multitude of pathogens, but immunocompetent individuals are not continuously ill. When infected with a pathogen, the adaptive immune response generates a memory response which remains following pathogen clearance (Charles A Janeway *et al.,* 2001c). Hence, upon re-infection these individuals can clear the infection with rarely any symptoms. A brief overview of the generation of immune cells and the different subsets is included for the purpose of highlighting the origin of the cell of interest, DCs, and the cells with which DCs interact.

1.1.2 Myeloid vs Lymphoid – Generation of Immune cells

Immune cells and other blood cells originate from hematopoietic stem cells (HSCs) within the bone marrow, though a process known as haematopoiesis (Figure 1.1) (Hoggatt & Pelus, 2013). Immune cells circulate the body through blood and lymphatic vessels (Charles A Janeway *et al.,* 2001f). HSCs can remain quiescent or are proliferating and differentiating (Ho & Méndez-Ferrer, 2020). HSCs yield different cell types through a series of differentiation events. These events result in other multipotent progenitors, such as the common myeloid progenitor (CMP) or common lymphoid progenitor (CLP), which further differentiate into more specific unipotent progenitor cells and subsequently into their particular cell subtype (Hoggatt & Pelus, 2013). CMPs eventually give rise to megakaryocytes, erythrocytes, monocytes, and granulocytes. Granulocytes are granule-containing cells and are made up of the basophils, eosinophils, mast cells and neutrophils (Hoggatt & Pelus, 2013). Most DC subtypes are of myeloid origin, however, DC subtypes of lymphoid origin have also been described (Dress *et al.,* 2019; Sichien *et al.,* 2017). DC generation is described further in Section 1.2.2. CLPs eventually give rise to NK, B and T cells (Hoggatt & Pelus, 2013).

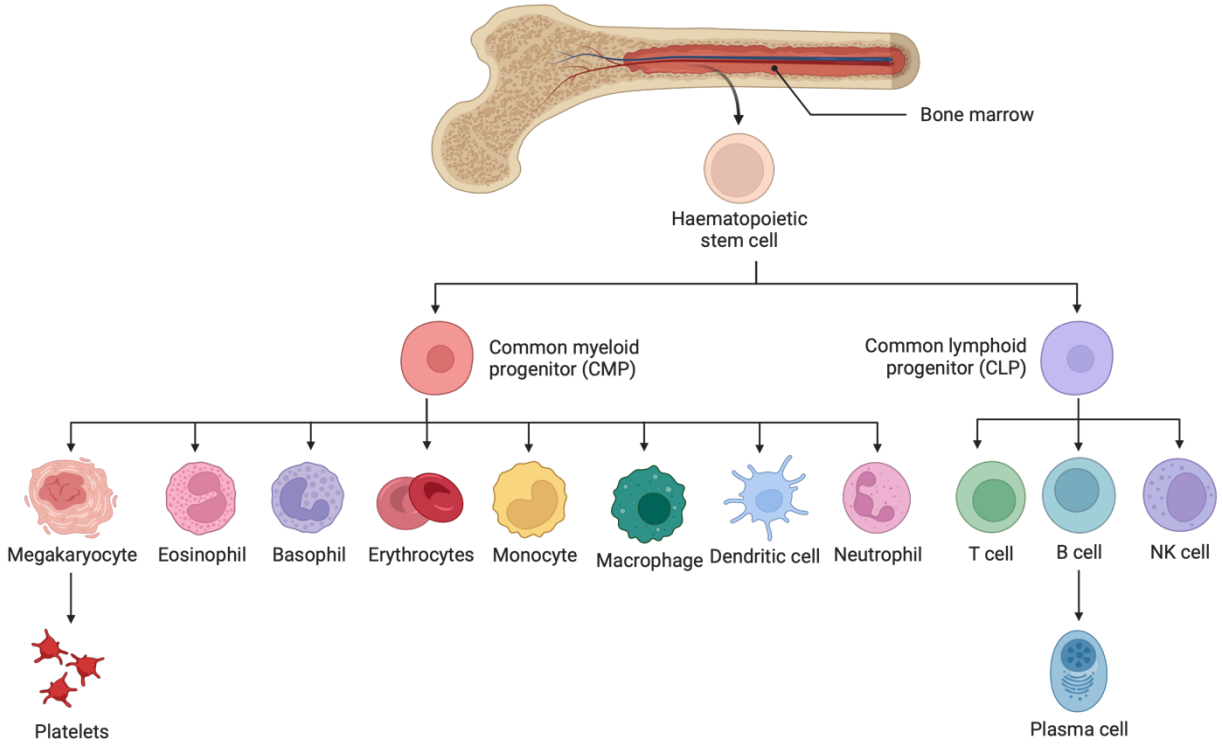


Figure 1.1 Haematopoiesis simplified Blood cells are derived from a pluripotent stem cell in the bone marrow. Haematopoietic stem cell (HSC) differentiation yields the diverse range of immune cells and other blood cells (adapted from Hoggatt & Pelus (2013); Lai & Kondo (2008)).

B and T cells comprise the adaptive immune response and confer humoral or cell-mediated immunity respectively (Charles A Janeway *et al.,* 2001d, 2001a). B cells are generated and develop in the bone marrow (Hardy & Hayakawa, 2003). T cells, on the other hand, are generated in the bone marrow but exit and develop in the thymus (Charles A Janeway *et al.,* 2001b). These cells undergo selection and differentiation within the thymus to ensure they display functional and non-self-reactive TCRs. Naïve cells are considered immature or in their resting state as they have not yet encountered their antigen in the periphery (Pennock *et al.,* 2013). Naïve lymphocytes encounter pathogens displayed on APCs in the peripheral lymphoid organs and undergo maturation (Charles A Janeway *et al.,* 2001g).

1.1.3 Innate Immune System

The innate immune response is commonly known as the first line of defence. This response also includes physical (the skin, epithelial linings) and mucosal barriers, protecting the host from the external environment (Marshall *et al.,* 2018; Medzhitov & Janeway, 1997). However, if pathogens manage to circumvent these barriers, innate immune cells and other soluble mediators are present at injured/damaged tissues or in the circulatory system to combat pathogen invasion (Marshall *et al.,* 2018; Medzhitov & Janeway, 1997). Many cell types are part of innate immunity, including the epithelial cells found at exposed barriers to importantly, the immune cells, including NK cells, innate lymphoid cells (ILCs) and phagocytes. Phagocytes are further subdivided into DCs, macrophages, monocytes, and the granulocytes (Marshall *et al.,* 2018; Medzhitov & Janeway, 1997). Some granulocytes (basophils and eosinophils) are weak inducers of phagocytosis (Geering *et al.,* 2013)

All these cells have particular functions and undergo pathogen-specific induction to elicit the appropriate responses. For example, neutrophils, a type of granulocyte, are the most abundant immune cell and are the first to be recruited to sites of inflammation. The cells contain enzymatic granules which lyse ingested particles (Kim & Luster, 2015; Rosales, 2018). Another granulocyte population, eosinophils, are vital during parasitic infections (Gounni *et al.,* 1994) but their interactions with basophils and mast cells can result in allergic responses (Stone et al., 2010). Monocytes and macrophages are phagocytes and are important for pathogen ingestion, tissue homeostasis and inflammation resolution (Ginhoux & Jung, 2014). Ag presentation is an essential function of DCs and DCs are referred to as professional APCs. DCs can present both self and foreign antigens, allowing either the induction of tolerance or immunity respectively (Banchereau & Steinman, 1998). Self-antigens are markers derived from the host. The targeting of self-antigens occurs during immune dysregulation and results in autoimmune diseases (Banchereau & Steinman, 1998), such as rheumatoid arthritis (Klimiuk *et al.,* 1999) or inflammatory bowel disease (Jodeleit *et al.,* 2020). DCs will be described in greater detail in Section 1.2. NK cells release cytotoxic granules containing perforin and granzymes that create pores within the cell membrane of infected cells and induce programmed cell death within these target cells (Brandstadter & Yang, 2011). NK cells are important during viral infection and in the detection of tumour cells (Brandstadter & Yang,

2011; Bukowski *et al.,* 1983; Kiessling *et al.,* 1975). ILCs are derived from the common lymphoid progenitor but lack antigen specificity. ILCs are important in tissue homeostasis and during inflammation, NK cells have been classified as a type of ILC (Nagasawa *et al.,* 2018). In addition to the innate cells, there are many different proteins which are essential for the innate immune response.

Soluble mediators released by innate cells, include soluble PRRs, cytokines, chemokines, or the complement system. These all serve to potentiate the inflammatory response (Marshall *et al.,* 2018; Medzhitov & Janeway, 1997; Smole *et al.,* 2020). The complement system is composed of multiple plasma proteins which are activated through an enzymatic cascade (Charles A Janeway *et al.,* 2001e). Their activation results in several effects, firstly, activated complement proteins can bind to pathogen which subsequently is recognised by phagocytes expressing complement receptors and are phagocytosed, a process known as opsonisation (Charles A Janeway *et al.,* 2001e). Complement proteins can opsonise pathogens directly or indirectly, alongside antibodies. Secondly, complement proteins can attract phagocytes to their target site. Additionally, complement proteins can create pores within bacterial membranes leading to pathogen death (Charles A Janeway *et al.,* 2001e). Other factors are cytokines released by cells at sites of injury/infection or immune cells and are a vital component of the innate response, including DC function, as cytokines can affect both innate and adaptive immune cells (Medzhitov & Janeway, 1997). Chemokines are important for cell migration (Medzhitov & Janeway, 1997).

Recently, a memory aspect to the innate immune response, termed trained immunity, has been observed. Trained immunity involves the reprogramming of innate cells, metabolically or epigenetically, following a primary stimulus. Secondary stimulus of the non-activated cells results in altered responses, either a stronger or weaker response (Netea *et al.,* 2020). However, trained immunity still differs from adaptive memory responses, as the latter involves gene recombination. A synergy exists between the innate and adaptive immune response, as despite the adaptive response needing activation by the former, both can regulate each other.

1.1.4 Adaptive Immune System

The main classes within the adaptive immune system are T and B lymphocytes, which confer cell-mediated and humoral immunity respectively (Charles A Janeway *et al.,* 2001a, 2001d). T cells precursors arise in the bone marrow and migrate to the thymus where they develop and mature (Charles A Janeway *et al.,* 2001b). Classical T cells have T cell receptors (TCRs) which are heterodimers of α- and β-chains (Charles A Janeway *et al.,* 2001g). Several signals are required for successful naïve T cell activation. TCRs interact with MHC-Ag complexes presented by APCs, known as signal 1 (Charles A Janeway *et al.,* 2001g). Simplistically, there are two main effector T cells, cluster of differentiation (CD)4^+ T helper (Th) lymphocytes or $CD8^+$ cytotoxic T lymphocytes (CTLs) (Charles A Janeway *et al.,* 2001g). MHC class I complexes interact with the TCR and CD8 coreceptor on CTLs. MHC class II complexes interact with the TCR and CD4 coreceptor on Th cells (Charles A Janeway *et al.,* 2001g). MHC class I molecule is expressed ubiquitously, whereas MHC class II molecules are present on APCs (Hewitt, 2003; Ting & Trowsdale, 2002). TCRs are specific for antigens due to the complex rearrangement of genes which form the TCR (Medzhitov & Janeway, 1997). However, signal 1 alone is insufficient to activate T cells. Signal 2 is the interaction of co-stimulatory molecules on the surfaces of APCs and T cells (Charles A Janeway *et al.,* 2001g). APCs express CD80 and CD86 which interact with coreceptors on T cells, such as CD28 (Charles A Janeway *et al.,* 2001g). Signal 2 potentiates T cell activation and promotes further DC maturation, the latter results in cytokine release (signal 3) which coordinates T cell proliferation and differentiation (Curtsinger *et al.,* 2005; Curtsinger & Mescher, 2010). The absence of signal 2 can lead to inactivation of T cells, as it can be indicative of T cells reacting to self-antigens (Charles A Janeway *et al.,* 2001g). T cells vary in function and phenotype. CTLs kill infected cells or cancer cells through their cytotoxic granules containing granzyme and perforin (Charles A Janeway *et al.,* 2001g, 2001d) and the release of effector cytokines interferon (IFN)-γ and tumour necrosis factor (TNF) α (Wohlleber *et al.,* 2012; Garnier *et al.,* 2022). There is a broad spectrum of Th cells which respond to different pathogens, these are categorised based on the transcription factors activating them and the cytokines Th cells release. The differentiation of Th cells into their respective subtypes is reliant upon the third signal during activation (Lee, 2014). APCs secrete specific cytokines, depending on their activation pathway, which in turn, dictates the T cell profile (Geginat *et al.,* 2001; Khayrullina *et al.,* 2008). For example, viral infections lead to APC production of interleukin (IL) -12 and IFN-γ which favours Th1 cell development (Dalod *et al.,*

2002; Schijns *et al.,* 1998). Parasitic infections can cause production of IL-4 from various cells, which influence Th2 cell development (Pelly *et al.,* 2016). Other extracellular pathogens, bacteria (e.g. *Staphylococcus aureus*) and fungi (e.g. *Candida albicans*), induce APC production of transforming growth factor (TGF) -β, IL-1β, IL-6 and IL-23 which favour Th17 cell development (Zielinski *et al.,* 2012). T helper cells can direct innate and adaptive responses such as increasing the phagocytic capacity of macrophages and activating B cells and CTLs (Alberts *et al.,* 2002). Another type of $CD4^+$ cell is regulatory T (Treg) cells. Tregs are important in inducing tolerance and suppressing the immune response, through dampening of effector functions of Th cells, CTLs and APCs (Chattopadhyay & Shevach, 2013; Mempel *et al.,* 2006; Pandiyan *et al.,* 2007). Following resolving of the infection, most T cells undergo apoptosis, however, some remain as memory T cells, allowing a quicker response upon subsequent re-infection (Pennock *et al.,* 2013).

B cells develop and mature in the bone marrow and express the B cell receptor (BCR) (Charles A Janeway *et al.,* 2001b). The BCRs are also specific for antigens due to the complex rearrangement of genes which form the BCR. The BCRs are membrane-bound antibodies (Abs) or immunoglobulins (Ig) (Wen *et al.,* 2019). An antibody is a Y-shaped protein consisting of 4 polypeptide chains. On each arm there are the antigen-binding sites (Fab, fragment antigen-binding), which vary between antibodies to allow antigen specificity. The tail of the antibody is known as the fragment crystallizable (Fc) region (Charles A Janeway *et al.,* 2001h). 5 classes of antibodies exist: IgA, IgD, IgE, IgG and IgM. Each class will have their specific Fc region, which is conserved within each class (Charles A Janeway *et al.,* 2001h). The Fc region allows interaction with other components of the immune response. Fc receptors are expressed on various cells, especially phagocytes, and facilitate the binding of the Fc region of the antibody (Takai, 2002) to enable Ab-dependent cellular phagocytosis (ADCP) (Kamen *et al.,* 2019). B cells require two signals for successful activation: firstly, recognition of the antigen by the BCR; secondly, an additional signal provided by T helper cells (Charles A Janeway *et al*., 2001a). T cell activation is required for long-lasting B cell responses (Turner *et al.,* 2020). Upon activation, B cells will proliferate and differentiate into either plasma cells which secrete vast quantities of antibodies against the antigen or into memory B cells which maintain long-lasting immunity against the specific antigen *(*Charles A Janeway *et al.,* 2001a). Activated B cells (following signal 1) interact with T cells in germinal centres in secondary lymphoid organs,

where Abs become highly specific for its respective antigen and these selected B cells differentiate into long-lived plasma cells and memory B cells (Turner *et al.*, 2020). T-independent responses have been observed against certain pathogens which are able to induce B cell responses through a strong signal 1 or with other co-stimulatory signals from certain innate or innate-like cells (Drake *et al.,* 2016; Liao *et al.,* 2017; Turner *et al.,* 2020).

The secretion of antibodies results in various scenarios. Firstly, antibodies can neutralise pathogens or their toxins, through binding of an antigen (epitope on the pathogen or its toxin) and prevent the toxin from binding to host cells or prevent pathogens from infecting cells (Forthal, 2015). Secondly, antibodies can opsonise pathogens and Fc-bearing phagocytes endocytose and digest the pathogen (Forthal, 2015). Thirdly, antibodies can activate the complement pathway and both function together to opsonise pathogens, or complement activation can result in pathogen lysis, as described previously (Forthal, 2015). Lastly, antibodies are required for antibody-dependent cellular cytotoxicity (ADCC). ADCC involves antibody binding to infected target cells and the antibody is recognised by Fc-bearing effector cells, the target cell is subsequently lysed or undergoes a type of cell death known as apoptosis (Forthal, 2015). The humoral response is a key component of a functional adaptive immune response, but antibodies can remain in the host for varied lengths of time (Antia *et al.*, 2018). Memory B cells are long-lasting, similar to memory T cells, highlighting a crucial difference between the innate and adaptive response. The memory capacity of the adaptive immune response confers a more rapid response in the host upon subsequent infection, a process that is reliant on antigen presentation – where DCs play a central role.

1.2 Dendritic cells

1.2.1 Dendritic cells overview

DCs were first described in 1973, following the observation of a new type of glass-adherent cell from mouse spleen which were able to form pseudopods or dendrites and differed from previously described macrophages (Steinman and Cohn, 1973, 1974). In the following years, DCs were identified as the key cells required to stimulate B and T cells (Steinman and Witmer, 1978; Nussenzweig and Steinman, 1980), including CTLs in mice and humans (Nussenzweig *et al.*, 1980; Inaba *et al.*, 1983). Since then, DCs have been recognised as professional antigen-

presenting cells which act as a bridge between the innate and adaptive immune response. DCs are important in both activating adaptive immune responses and inducing tolerance, as well as stimulating the differentiation of naïve T cells into memory T cells (Banchereau and Steinman, 1998), a key component to successful vaccination strategies (Clem, 2011).

DCs are known as innate immune sentinels, and circulate the body in an immature state, particularly mucosal tissues, the areas exposed to the external environment where pathogen entry is likely (Banchereau and Steinman, 1998; Iwasaki and Kelsall, 1999). In their immature state, DCs are highly phagocytic and have low expression of the antigen presentation molecule, major histocompatibility complex (MHC) II and costimulatory molecules (Banchereau and Steinman, 1998). Upon antigen exposure, DCs endocytose and present antigen on their antigen-MHC complexes, upregulate the expression of costimulatory molecules and chemokine receptors, produce and secrete cytokines and chemokines, and subsequently downregulate their phagocytic machinery (Banchereau and Steinman, 1998). A process known as the maturation of DCs. Antigen presentation occurs via complexes with MHC class I or class II molecules. DCs as professional APCs express both MHC classes (Banchereau and Steinman, 1998; Tel *et al.*, 2013). Soluble antigenic peptides can be endocytosed from the environment, pathogens and infected or damaged cells can be digested within endosomal-lysosomal organelles, to yield antigenic peptides (Sallusto and Lanzavecchi, 1994; Nagl *et al.*, 2002; Savina and Amigorena, 2007). These associate with MHC class II molecules and are exported to the cell surface for presentation to $CD4^+$ T cells, this is known as the exogenous pathway of antigen processing and presentation (Banchereau and Steinman, 1998; Veeraswamy *et al.*, 2003; Watts, 2004). Self-antigens or intracellular pathogens are digested by the proteasome in the cytosol to yield peptides which associate with MHC class I in the endoplasmic reticulum (ER) and these complexes are exported to the cell surface for presentation to $CD8^+$ T cells, this is known as the endogenous pathway of antigen processing and presentation (Lu *et al.*, 2012; Leone *et al.*, 2013; Garstka *et al.*, 2015). These pathways are illustrated in Figure 1.2. Additionally, DCs can cross-present exogenous peptides from phagosomes on MHC class I molecules (Joffre *et al.*, 2012). DC cross-presentation allows presentation of viral or tumour antigens to CTLs (Joffre *et al.,* 2012; Fessenden *et al.*, 2022).

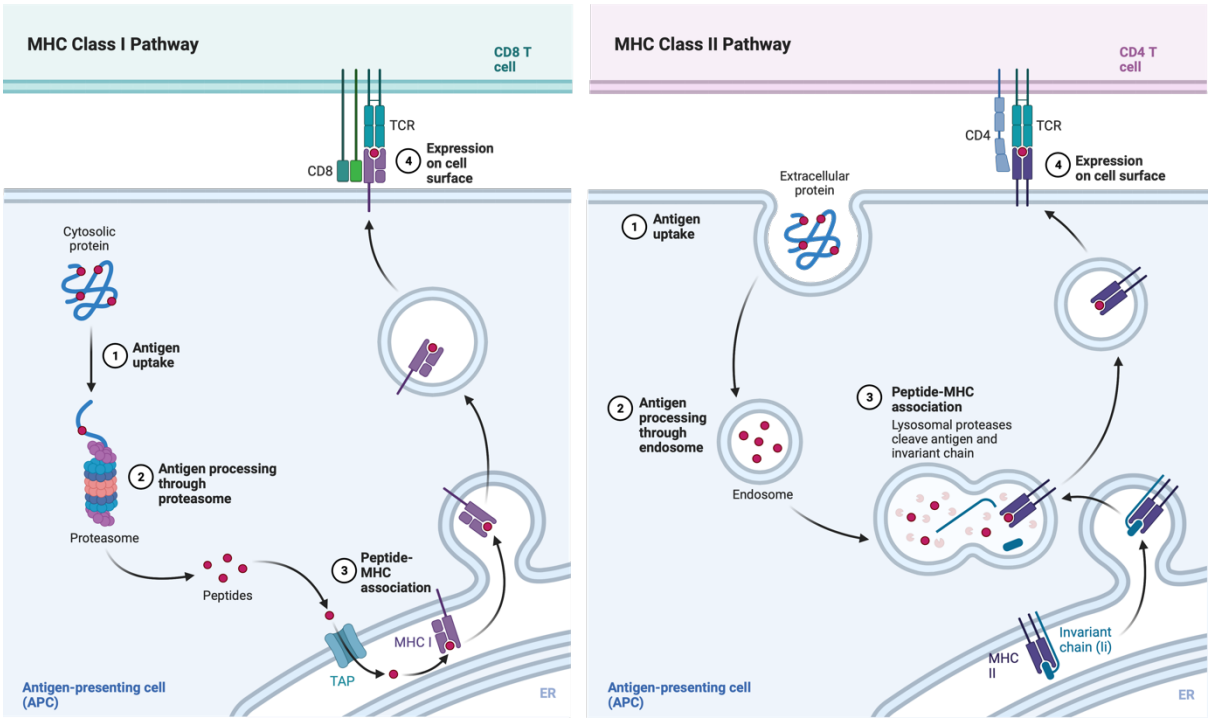


Figure 1.2 – Endogenous (MHC class I) and Exogenous (MHC class II) pathways of antigen presentation to T cells Self-antigens or antigens derived from intracellular pathogens are digested in the cytosol and resulting peptides associate with MHC class I molecules in the ER and presented to $CD8^+$ T cells on the APC cell surface. Extracellular pathogens or soluble antigens are endocytosed by the APC and subsequently degraded by lysosomes to generate antigens which associate with MHC class II molecules. Resulting complexes are presented to $CD4^+$ T cells on the APC cell surface (adapted from Murphy and Weaver (2017)).

Antigen uptake has traditionally been observed to occur through various processes of endocytosis in DCs: 1) receptor-mediated endocytosis 2) phagocytosis 3) macropinocytosis (Kamphorst *et al.*, 2010; Liu and Roche, 2015). Receptor-mediated endocytosis involves antigen binding to specific receptors on DC surface, such as binding of Fc regions to Fc receptors or complement to complement receptors (Platt *et al.*, 2010; Liu and Roche, 2015). This can allow the internalisation of pathogens coated with, using the same example, antibodies or complement respectively. These can be internalised in vesicles coated with clathrin or independent of clathrin (Liu and Roche, 2015). A range of antigens can be phagocytosed; these antigens can be relatively large, such as dying cells (Virella, 2007). These antigens are internalised in phagosomes. The development of phagosomes into phagolysosomes allows the degradation of the internalised antigen and the direct loading of the internalised antigen onto MHC complexes (Virella, 2007). Macropinocytosis is a key

function of DCs, also known as cell drinking, where non-specific soluble antigens are internalised into macropinosomes and downstream can be transferred to intracellular compartments where the antigen can be loaded onto MHC complexes (Sallusto *et al.*, 1995; Lim and Gleeson, 2011). Ag uptake in DCs can also occur via direct infection of the cell (Farzi *et al.*, 2022). In addition to these standard methods of antigen uptake, alternative processes have been described: gap junctions, tunnelling nanotubes and trogocytosis (Herbst, Harshyne and Igyártó, 2022). Gap junctions allow intercellular interactions through channels; however, antigen transfer is limited to small molecules (Neijssen *et al.*, 2005). Tunnelling nanotubes (TNTs) utilise the cytoskeletal machinery to connect between sessile cells (Herbst, Harshyne and Igyártó, 2022), DCs were observed to connect with each other (Zaccard *et al.*, 2015), where they signalled during detection of bacterial supernatants (Watkins and Salter, 2005). TNTs can be used for the transport of organelles or molecules (Watkins and Salter, 2005; Önfelt *et al.*, 2006). Trogocytosis is referred to as cell nibbling, where functional membrane fragments are transferred from the target cell to another (Harshyne *et al.*, 2003; Zhao *et al.*, 2022). Herbst *et al.* (2022) propose an alternative and novel process of intracellular monitoring, where DCs sample target cell cytosol, especially in confined tissues where morphological changes, such as those which occur during phagocytosis, are difficult. These mechanisms result in antigen uptake required for downstream adaptive immune cell activation.

Ag uptake triggers DC migration to lymphoid organs (draining lymph nodes) where they provide signal 1, 2 and 3 to Ag-specific T cells (Banchereau and Steinman, 1998). DCs begin maturation upon activation. Through DC-T cell interaction, DCs terminally mature and antigen-specific lymphocytes differentiate and expand (Banchereau and Steinman, 1998; Talay *et al.*, 2009). Chemokine association with its respective receptors cause activated cells to home to the sites of pathogen entry or injury (Hughes and Nibbs, 2018). Activated CTLs lyse infected or damaged cells (Charles A Janeway *et al.*, 2001). Helper T cells serve to further activate innate cells at the site of infection or injury through the secretion of cytokines, particularly enhancing the phagocytic capacity of macrophages (Alberts *et al.*, 2002). B cells can differentiate into antibody-producing plasma cells following activation via T cell and DC interactions (Alberts *et al.*, 2002). DCs interact with B cells in germinal centres where they provide cytokines which can activate naïve and memory B cells directly or in synergy with T

cells (De Silva and Klein, 2015; Heath *et al.*, 2019). Ag-specific $CD4^+$ T helper cells, Ag-presenting DCs and Ag-specific B cells can interact synergistically and promote a humoral immune response. Tissue-resident DCs, such as epidermal Langerhans cells (LCs), upon pathogen exposure at sites of injury, capture antigen and migrate to T cell niches within lymph nodes for T cell activation (Kissenpfennig *et al.*, 2005). The production of cytokines is a key effector function of DCs, as the production of TGFβ and/or IL-10 can impair B cell antibody production or favour the production of certain antibody subclasses from B cells during isotype switching (Malisan *et al.*, 1996; Tsuchida *et al.*, 2017). These cytokines also favour an anti-inflammatory or tolerogenic phenotype (Akbari, DeKruyff and Umetsu, 2001; Qian *et al.*, 2008). Other cytokines produced are, type I IFNs, which are essential for host responses against viruses (Kumagai *et al.*, 2009). DCs also interact with other innate cells, such as NK cells, enhancing the activity of both cells, this interaction has been exploited as potential cancer therapies (Pham *et al.*, 2010).

DCs have another essential role in inducing tolerance. Tolerance is an important part of the immune response, or lack thereof, as the high variation in TCRs could generate those which identify self-molecules as antigens (Hawiger *et al.*, 2001; Fucikova *et al.*, 2019). This is the basis of autoimmune diseases. Tolerance occurs within central lymphoid organs; the locations where immune cells develop. In the thymus, where T cells develop, DCs promote negative selection of T cells through the absence of co-stimulation (signal 2) (Gao *et al.*, 2002; Audiger *et al.*, 2017). Co-stimulatory molecules are upregulated through PRR stimulation, in the cases of self-antigens, this would not occur (Hawiger *et al.*, 2001; Audiger *et al.*, 2017). Therefore, T cells which recognise self-antigens displayed on MHC complexes are inactivated or targeted for deletion (Hawiger *et al.*, 2001). Importantly, DCs result in Treg cell development, key cells for dampening the immune response or inducing tolerance in peripheral tissues (Hilpert *et al.*, 2019). DC induction of tolerance is a potential process to be manipulated during transplantation to ensure an immune response is not elicited by the recipient (Fu *et al.*, 1996) or for the treatment of allergies or autoimmune conditions (Cifuentes-Rius *et al.*, 2020).

1.2.2 Dendritic cell formation; Mouse vs Human DCs

Dendritic cells were originally discovered in mice (Steinman and Cohn, 1973), and were later described in human blood by Van Voorhis *et al.* (1982). Mouse and human DCs share

similarities and murine models are widely used for studying DCs *in vivo*. However, there are tissue-specific differences between both species which express a range of markers and have varying phenotypes, described further next. The postnatal development of multipotent HSCs in the bone marrow is the initial step in the generation of DCs (Shortman and Naik, 2006). Figure 1.1 depicts the formation of a CMP from HSCs in the bone marrow. These progenitors can differentiate to form other progenitor precursors. DCs differentiate separately through an alternate lineage downstream in haematopoiesis to other innate immune cells. Transcription factors and cytokines are vital for commitment to specific differentiation lineages (Caux *et al.*, 1996; Karsunky *et al.*, 2003; Suzuki *et al.*, 2004; Becker *et al.*, 2012). Cytokines are also important for peripheral DC maintenance (Waskow *et al.*, 2008).

Simplistically, HSCs give rise to the CMP. CMPs differentiate into a granulocyte-macrophage progenitor (GMP) (D'Amico and Wu, 2003; Sichien et al., 2017). GMPs further differentiate into a common macrophage-dendritic cell-restricted precursor (MDP) (Sichien et al., 2017). MDP can in turn develop into a common dendritic cell progenitor (CDP) or common monocyte progenitor (cMoP) (Sichien et al., 2017). The former produces pre-conventional DCs (pre-cDCs) or pre-plasmacytoid DCs (pre-pDCs) and the latter produces monocytes (Naik et al., 2006; Sichien et al., 2017). Pre-cDCs exit the bone marrow and enter circulation (O'Keeffe et al., 2003; Sichien et al., 2017). Pre-pDCs can differentiate into pDCs, however, pDCs can also be derived from lymphoid progenitors (Dress *et al.*, 2019) (process illustrated in Figure 1.3).

Monocytes can differentiate into macrophages, and upon infection or during inflammation *in vivo*, monocytes have been shown to differentiate into inflammatory monocyte-derived dendritic cells (Mo-DCs) (Naik *et al.*, 2006). Monocytes can differentiate into Mo-DCs in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 *in vitro* (Wołkow, Gębska and Korbut, 2018). Langerhans cells are skin-resident cells which share characteristics with both macrophages and DCs (Wu *et al.*, 2016). Long-lived LCs in adults which can self-renew within the skin are mainly derived from foetal liver monocytes (Hoeffel *et al.*, 2012). However, following inflammation when there is loss of LCs, blood monocytes can replenish LC populations (Ginhoux *et al.*, 2006).

DCs are located in lymphoid organs and peripheral tissues in the steady state (Balan, Saxena and Bhardwaj, 2019). Pre-cDCs and pDCs are found in lymphoid organs and in circulation. Pre-cDCs mature into cDCs in lymphoid tissue or the periphery (Sichien et al., 2017). Murine and human DCs share similarities but express varying markers and are localised to different tissues. DC subtypes between both species are similar based on their transcriptional programming (Merad *et al.*, 2013). pDCs exist in both human and mice (Guilliams *et al.*, 2014). The master transcription factor for development and function of pDCs was identified as E2-2, also known as transcription factor 4 (TCF4) (Cisse *et al.*, 2008). Pre-cDC precursors differentiate into either cDC1 or cDC2 (Naik *et al.*, 2006). cDC differentiation is governed by distinctive transcription factors, those involved in cDC1 differentiation have been more clearly elucidated, compared to cDC2.

Pre-cDCs in circulation in humans were observed to be pre-committed to either a $CD141^+$ cDC1 or $CD1c^+$ cDC2 fate based on the expression of a marker CD172a (Breton *et al.*, 2016). CD172a is also known as signal regulatory protein α (SIRPα) and is a transmembrane glycoprotein which interacts with the broadly expressed CD47 protein. In *CD47* knockout mice, DC migration and T cell priming was impaired (Van *et al.*, 2006). Pre-cDCs were divided into $CD172a^+$, $CD172a^{int}$ or $CD172a^-$ populations, to varying frequencies in bone marrow, cord or peripheral blood (Breton *et al.*, 2016). $CD172a^+$ indicated a commitment to $CD1c^+$ cDCs and $CD172a^-$ indicated a commitment to $CD141^+$ cDCs (Breton *et al.*, 2016). Whereas, $CD172a^{int}$ had the capacity to yield either subset. Peripheral blood was observed to contain mostly $CD172a^+$ pre-cDCs (Breton *et al.*, 2016).

cDC1s in humans are similar to the lymphoid tissue-resident mouse $CD8\alpha^+$ $CD11b^-$ classical DC and the migratory $CD103^+$ $CD11b^-$ DCs (Amon *et al.*, 2020). All three can express the chemokine receptor XCR1 and the C-type lectin-like receptor, Clec9A (Seillet *et al.*, 2013; Tullett *et al.,* 2014). Additionally, the essential transcription factors for the survival and development of all three are: Interferon Regulatory Factor 8 (IRF8), Basic Leucine Zipper ATF-Like Transcription Factor 3 (Batf3) and Inhibitor of DNA Binding 2 (ID2) (Hacker *et al.*, 2003; Hildner *et al.*, 2008; Lança *et al.*, 2022) (see Figure 1.3). cDC2s in humans are similar to the lymphoid tissue-resident $CD11b^+$ classic DC and the migratory $CD11b^+$ $CD8\alpha^-$ $CD103^-$ $SIRP\alpha^+$ DCs (Merad *et al.*, 2013; Amon *et al.*, 2020), but $CD11b^+$ $CD103^+$ DCs have also been observed

in tissues (Persson *et al.*, 2013). Transcription factors required for the development of murine subsets include IRF4, Notch2 and RelB (Schlitzer *et al.*, 2013; Bajaña *et al.*, 2016; Briseño *et al.*, 2017) (see Figure 1.3). However, deletion of these transcription factors has been shown to not result in complete reduction of all CD11b$^+$ DCs, depicting only a partial reliance on them for cDC2 development (Bajaña *et al.*, 2016; Briseño *et al.*, 2017). A cDC2-specific transcription factor is yet to be discovered. The existence of these different subsets allows dendritic cells to elicit immune responses against a range of pathogens and their functions will be delineated next.

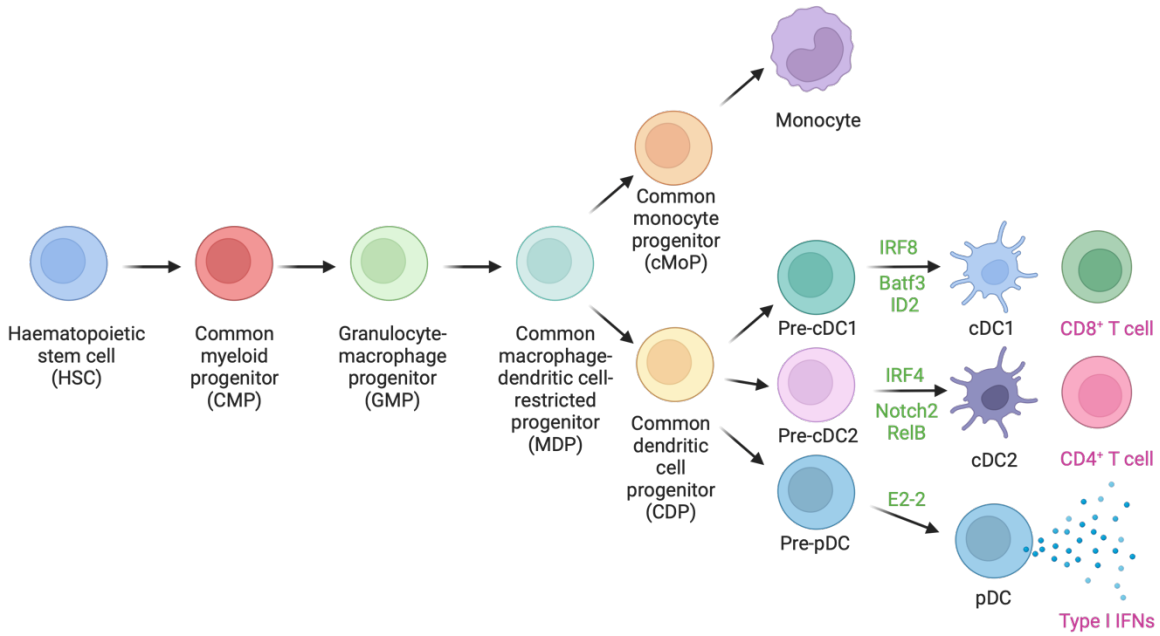


Figure 1.3 – DC generation and effector function Haematopoietic stem cells (HSCs) differentiate sequentially to generate different multipotent and unipotent progenitors which eventually yield the different dendritic cell subsets. Transcription factors required for each DC subset differentiation is highlighted in green. Effector function of the DC subset is highlighted in pink. CD141$^+$ cDC1s can activate CD8$^+$ T cells, CD1c$^+$ cDC2s can activate CD4$^+$ T cells and pDCs can secrete type I interferons (adapted from Tussiwand and Gautier (2015); Sichien et al. (2017)).

1.2.3 Dendritic cell functions

DCs express CD11c and MHC class II generally (Merad *et al.*, 2013). CD11c, also known as Integrin Subunit Alpha X (ITGAX), is an integrin protein involved in phagocytosis and cell adhesion (Vorup-Jensen *et al.*, 2007; Wu *et al.*, 2018). MHC class II is a marker of professional antigen-presenting cells required for antigen presentation to CD4$^+$ T cells (Banchereau and

Steinman, 1998; Watts, 2004). In human circulation, there are three main types of DCs, the conventional cDC1 and cDC2 and the plasmacytoid DCs (Merad *et al.*, 2013). Analysis of DC populations have more recently identified more DC subsets (Villani *et al.*, 2017), these could potentially be precursors which then differentiate into the aforementioned cDCs or are new DC subtypes. cDC2s were found to be a heterogeneous population with a division that yields a new DC3 subset (Villani *et al.*, 2017). DCs can display phenotypic plasticity, therefore, the defined subsets are less based on differences in phenotype but rather their developmental lineage and functional characteristics (Villani *et al.*, 2017).

These DC subtypes also have different phenotypes, different functions and are activated by different pathogens, and consequently activate specific effector cells equipped for the pathogen. The ability of T cells to expand and differentiate into their specific subtype depends on the cytokine milieu created by DCs based on their phenotype and level of maturation.

$CD141^+$ cDC1 induces immune responses against intracellular pathogens and tumours, cDC1s are better characterised by the expression of Clec9A, a C-type lectin receptor which detects actin exposed by necrotic cells (Huysamen *et al.*, 2008; Sancho *et al.*, 2008; Spranger *et al.*, 2017). Pre-cDC1s and mature cDC1s expressed the marker CXCR3, which allowed their migration to melanoma tumours (Cook *et al.*, 2018; Boltjes *et al.*, 2023). CXCR3 is not expressed on pre-cDC2 (Cook *et al.*, 2018), depicting the varied roles and migration of both. Human cDC1s are more enriched within tissues (Boltjes *et al.*, 2023) compared to low populations in the blood (Merad *et al.*, 2013). Both cDC subsets are observed in lymphoid tissues (e.g., spleen, tonsils, lymph nodes) and non-lymphoid tissues (e.g., liver, skin, lungs) and which subset is more enriched depends on the organ (Heidkamp *et al.*, 2016; Balan, Saxena and Bhardwaj, 2019). A key function of these cDC1s is their ability to cross-present exogenous antigen to $CD8^+$ T cells on MHC class I complexes (Sancho *et al.*, 2008; Joffre *et al.*, 2012; Silvin *et al.*, 2017). XCR1 is another key marker on cDC1 and this chemokine receptor interacts with its ligand XCL1 produced by $CD8^+$ T cells to promote DC-T cell interactions (Brewitz *et al.*, 2017). cDC1s can also elicit Th1 responses (Sulczewski *et al.*, 2020). Cross-presentation is a process more restricted to the cDC1-equivalent in mice (Schnorrer *et al.*, 2006), compared to humans (Klechevsky *et al.*, 2008). NK cell priming of cDC1s can enhance subsequent activation of CTLs and Th1 responses (Mailliard *et al.*, 2003). Additionally, cDC1s

also produce Type III IFNs, both of which are important in anti-tumour immune responses (Hubert *et al.*, 2020).

$CD1c^+$ cDC2 induce immune responses against extracellular pathogens, including parasites and bacteria (Satpathy *et al.*, 2013; Tussiwand *et al.*, 2015; Durai and Murphy, 2016). cDC2s present exogenous antigen to $CD4^+$ T cells on MHC class II complexes (Vander Lugt *et al.*, 2014). cDC2s can express and release a myriad of cytokines which govern the differentiation of T helper cells into the specific cell required for the response (Gao *et al.*, 2013; Satpathy *et al.*, 2013). DC2s tend to be the predominant subset of cDC population in the blood (Merad *et al.*, 2013). cDC2s are characterised by the expression of CD1c (Heger *et al.*, 2020), a transmembrane glycoprotein, similar to MHC molecules, and present lipid-based antigens (Moody and Porcelli, 2003; Schiefner and Wilson, 2009). CD1c is not solely restricted to cDC2s, therefore, cDC2s also express SIRPα and various other markers which allow cDCs subsets to be distinguished (Villani *et al.*, 2017; Collin and Bigley, 2018). cDC2s have more overlapping markers with monocytes (Backer, Probst and Clausen, 2023), compared to cDC1s. There are many transcription factors regulating cDC2 development, in humans and mice, possibly due to the heterogeneity found in this subset (Bosteels and Scott, 2020). cDC2s can result in the activation of many T helper cells: Th1, Th2 and Th17 (Tussiwand *et al.*, 2015; Leal Rojas *et al.*, 2017). cDC2s also a produce an array of cytokines with varied functions, e.g., IL-6 and IL-23 (Leal Rojas *et al.*, 2017). Additionally, human cDC2s can become cross-presenting cells, accompanied by large production of IL-12 (Nizzoli *et al.*, 2013).

Plasmacytoid cells are described as cytokine-producing cells (Ogata *et al.*, 2013). Due to the heterogeneity of DCs, there is constant speculation on the existence of distinct subtypes of DCs. A recent comment suggested that pDCs derived from lymphoid progenitors are ILCs due to their low antigen presentation capacity at steady state and their main function as an interferon-producing cell (Ziegler-Heitbrock *et al.*, 2022). However, a reply to this Nature comment (Reizis *et al.*, 2023), highlighted the transcriptional reprogramming which occurs in pDCs allowing increased antigen presentation and C-C motif chemokine receptor (CCR) 7 following activation, resembling cDCs. These comments showcase the ongoing debates on DC ontogeny. There are low levels of MHC class II and co-stimulatory molecules, the normal markers of a professional APC, expressed on pDCs (Chehimi *et al.*, 1989; Asselin-Paturel *et al.*,

2001; Colonna, Trinchieri and Liu, 2004). Additionally, these cells express fewer PRRs, predominantly toll-like receptor (TLR) 7 and TLR9 (Kadowaki *et al.*, 2001), essential intracellular cytosolic PRRs which detect viral antigens (Akira, Uematsu and Takeuchi, 2006). pDCs are vital cells during viral infection, as these cytosolic PRRs are activated and pDCs are the highest producer of type I interferons within the host (Fitzgerald-Bocarsly, Dai and Singh, 2008; Yun *et al.*, 2021).

DCs which reside in tissues, surveil for pathogens or damage signals (Mbongue *et al.*, 2014). One major subset is LCs. LCs are found in the skin of both humans and mice, representing less than 5% of skin cells (Schuler, Romani and Steinman, 1985b; Merad, Ginhoux and Collin, 2008). Upon recognition of pathogen and activation, LCs can migrate to lymph nodes for subsequent T cell activation (Stoitzner *et al.*, 2005). LCs are important in the maintenance of epidermal immune homeostasis (Romani *et al.*, 2003). Monocytes can differentiate to form inflammatory DCs that can act at the inflammatory sites to expand resident populations, these Mo-DCs are not present at steady state (Naik *et al.*, 2006). Production of IL-12 by monocyte-derived $CD11c^+$ DCs induce $CD4^+$ T helper cells to acquire a Th1 phenotype (León, López-Bravo and Ardavín, 2007). Mo-DCs have also been shown to activate $CD8^+$ T cells and contribute to anti-tumour immunity (Kuhn, Yang and Ronchese, 2015).

Therefore, in mice and humans, different DCs exist which coordinate the adaptive immune response through the differentiation of specific T cells. Along with effects on the innate immune response. DCs can exhibit plasticity due to their microenvironment, e.g., in the presence of pro- or anti-inflammatory mediators. DCs are short-lived cells and die following resolution of infection or inflammation (Sallusto and Lanzavecchia, 2002). Throughout DC development, maturation and activation, the cells require nutrients and energy to be metabolically active. The association between metabolism and immune cell function is a whole new field of immunology, entitled immunometabolism, which will be discussed next.

1.3 Immunometabolism

1.3.1 Immunometabolism Overview

Originally cellular metabolism was thought to be important for merely energy production and biomass. However, in the last couple of decades, the role of cellular metabolism on immune cell function has been subject to intense research activity (O'Neill *et al.,* 2016). The accepted consensus is that cellular metabolism controls and can dictate immune cell phenotype. Different immune populations, immune cells during different stages of development, or in different microenvironments utilize different metabolic pathways, to produce energy as adenosine triphosphate (ATP) and metabolites (Berod et al., 2014; Everts et al., 2014; Michalek et al., 2011; Thwe et al., 2017). These metabolites can enter different pathways to generate other macromolecules, alter signalling pathways, induce epigenetic changes, or alter post-translational modifications (Langer *et al.,* 2002; Bowman *et al.,* 2017; Huo *et al.,* 2021; He *et al.,* 2023). The overall finding of the field is that reprogramming of immune cell metabolism is vital for cell differentiation, fate, and function (O'Neill *et al.,* 2016).

The two main cellular metabolic pathways used to produce ATP by immune cells are glycolysis and oxidative phosphorylation (OXPHOS). Glycolysis is a series of 10 reactions which occur in the cytosol, where for each glucose molecule that is broken down, 2 pyruvate, 2 net ATP molecules and 2 nicotinamide adenine dinucleotide (NADH) coenzymes are generated (O'Neill *et al.,* 2016). When oxygen is available in the cell, pyruvate enters the mitochondrial matrix where it is converted to acetyl coenzyme A (acetyl CoA) by pyruvate dehydrogenase (PDH) (Kolobova *et al.,* 2001; O'Neill *et al.,* 2016). Acetyl CoA enters the citric acid cycle or tricarboxylic acid cycle (TCA cycle), where one of the objectives of this cycle is to replenish sources of the coenzymes NADH and flavin adenine dinucleotide ($FADH_2$) (O'Neill *et al.,* 2016). These coenzymes supply electrons to the electron train chain (ETC) in the inner membrane of the mitochondria. The ETC and oxygen is required to produce large quantities of ATP, a process known as OXPHOS. OXPHOS yields 36 net ATP per glucose molecule. These processes are depicted in Figure 1.4. These pathways are also sources of different metabolites. The TCA cycle links the catabolism of various macromolecules. Carbohydrates, lipids, and amino acids (such as glutamine) can be broken down and can enter at various stages of the TCA cycle, this allows different macromolecules or stores to be utilised (O'Neill *et al.,* 2016). In the absence of or

low oxygen, glycolysis is highly favoured, where pyruvate is converted to lactate and secreted into the microenvironment. Tumour cells utilise aerobic glycolysis, known as the Warburg Effect, where glycolysis is upregulated despite the presence of oxygen (Warburg, 1925). Normal host cells were observed to utilise Warburg metabolism (O'Neill *et al.,* 2016); activation of cells can upregulate aerobic glycolysis, e.g., TLR activation (Krawczyk *et al.,* 2010). The glycolytic pathway also yields various metabolic intermediates which can be shuttled into other pathways, such as the pentose phosphate pathway to generate nucleotides (O'Neill *et al.,* 2016). These allow DNA/RNA synthesis or amino acid production essential for growth, proliferation, or function of cells. Glycolysis is upregulated in highly proliferative cells (Michalek *et al.,* 2011; O'Neill *et al.,* 2016), whereas the TCA cycle is favoured by cells requiring more energy and longevity. Therefore, non-proliferative or quiescent cells tend to upregulate the TCA cycle and OXPHOS (Yao *et al.,* 2019). Metabolic pathways are linked within the cells, as products from one pathway may be used as a precursor in another. Additionally, the enzymes and metabolites from these pathways are of importance as many have roles in other pathways (Pan *et al.,* 2021). For example, glycolytic enzymes have been found in the nucleus, where they bind to DNA or act as cofactors for transcriptional regulation (Pan *et al.,* 2021). Cell stress induced modifications in the glycolytic enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which resulted in its translocation to the nucleus. Modified GAPDH eventually caused nuclear protein degradation through the stabilisation of an E3 ubiquitin ligase (Hara *et al.,* 2005). Chang *et al.* (2013) demonstrated that GAPDH influenced IFN-γ production in T cells due to its binding to the 3' untranslated region (UTR) of IFN-γ mRNA, intrinsically linking glycolysis to T cell function. This demonstrates the many roles of metabolites or metabolic enzymes in a range of pathways.

The environment of the cells and the nutrient availability within the cells are key considerations for steady state cell phenotypes and during stress or activation. Studies in the past deduced the high metabolic demands required by proliferating T cells and activated macrophages (Michalek *et al.,* 2011; Newsholme *et al.,* 1986). Two keys molecules which detect nutrient availability are mechanistic target of rapamycin (mTOR) and Adenosine Monophosphate-Activated Protein Kinase (AMPK) (O'Neill *et al.,* 2016). mTOR is a serine/threonine kinase, referred to as a central metabolic regulator and detects growth factors and amino acids (Hay & Sonenberg, 2004; Laplante & Sabatini, 2009; Sinclair *et al.,*

2013). mTOR signalling results in cell growth or proliferation and is highly activated during differentiation of immune cells, including T cells (Delgoffe *et al.,* 2009). mTOR increases anabolism whereas AMPK increases catabolism (Hay & Sonenberg, 2004; Kemmerer *et al.,* 2015). For example, excess lipids within cells can induce lipid droplet formation, a process which is regulated by mTORC1 and AMPK (Jarc & Petan, 2019). AMPK promotes lipolysis in specific cells (Hardie *et al.,* 2012; Muoio *et al.,* 1999), whereas mTORC1 promotes lipid biogenesis (Porstmann *et al.,* 2008). AMPK signalling re-establishes cell homeostasis by inhibiting mTOR (Hay & Sonenberg, 2004). Activation of AMPK occurs during nutrient deprivation (Egan *et al.,* 2011; Wu *et al.,* 2013), and AMPK represses activation of immune cells (Pandit *et al.,* 2022).

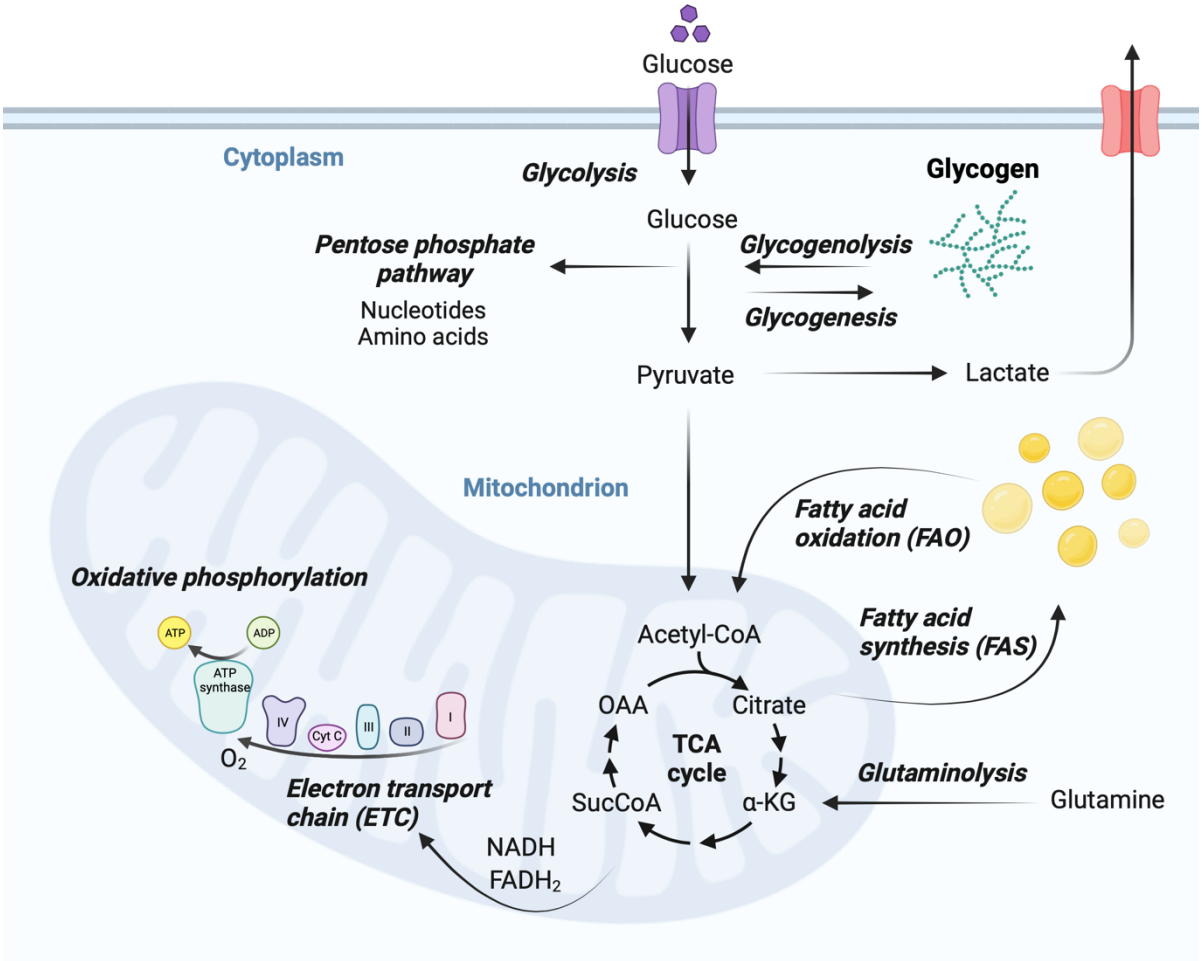


Figure 1.4 – Metabolic pathways within the cell Glucose can enter the cell and undergo glycolysis to yield pyruvate which can either undergo the TCA cycle and oxidative phosphorylation to yield TCA intermediates and high numbers of ATP or in the absence of oxygen or during cell activation, pyruvate is directed towards lactate production. Other pathways feed in, such as glycogenolysis or fatty acid and amino acid metabolism (adapted from Biswas *et al.* (2019); DeBerardinis & Chandel (2020)).

Cells contain lipid droplets which regulate lipid uptake, storage, distribution, and usage (Jarc & Petan, 2019). Lipid droplets are involved in the management of lipid metabolism and lipid-related cellular stress. Lipids are key: components of membranes of the cell or organelles; as precursors for signalling molecules, such as hormones; and as a source of high levels of ATP per fatty acid (Jarc & Petan, 2019; O'Neill *et al.,* 2016). Cells obtain fatty acids either from the circulation or from their intracellular lipid stores (Jarc & Petan, 2019). Fatty acid oxidation begins with the conversion of fatty acids within the cytosol to yield fatty acid acyl-CoA. Short-chain fatty acids can passively diffuse into the mitochondria, whereas medium- and long-chained fatty acids conjugate with carnitine by carnitine palmitoyl transferase I (CPT1) and are transported into the mitochondria (O'Neill *et al.,* 2016). CPT2 removes the carnitine and releases the fatty acyl-CoA which is subsequently oxidised to acetyl CoA and yields the cofactors NADH and $FADH_2$. These 3 molecules enter the TCA cycle and OXPHOS to generate ATP (O'Neill *et al.,* 2016). Fatty acid oxidation is downregulated when fatty acid synthesis is occurring due to the inhibition of the rate-limiting enzyme CPT1A by malonyl-CoA, an intermediate of lipid synthesis (O'Neill *et al.,* 2016).

Synthesis of fatty acids requires metabolites from other pathways, importantly glycolysis, the pentose phosphate pathway and the TCA cycle (Marin-Valencia *et al.,* 2012; O'Neill *et al.,* 2016). Pyruvate, generated from glycolysis, is converted to acetyl CoA and subsequently to citrate in the first step of the TCA cycle, through the combining of acetyl CoA and oxaloacetate (O'Neill *et al.,* 2016). Citrate can enter the next steps of the TCA cycle, or it is exported from the mitochondria to the cytosol where it is converted to acetyl CoA. Acetyl CoA is used in subsequent reactions with other products to yield fatty acids (O'Neill *et al.,* 2016). mTOR signalling activates sterol regulatory element binding protein (SREBP), a transcription factor controlling the expression of genes involved in lipid synthesis (Porstmann *et al.,* 2008). Cytosolic acetyl CoA is carboxylated by acetyl CoA carboxylase (ACC) to generate malonyl-CoA. The latter is subsequently elongated by fatty acid synthase (FASN) and several reactions may take place to generate fatty acids of varying chain lengths, unsaturated fatty acids, or branched chains fatty acids. The latter requires branched amino acids as substrates too (O'Neill *et al.,* 2016).

Amino acids act as substrates, in addition to their role in protein synthesis. Amino acids are involved in nucleotide synthesis and are linked to mTOR signalling as mTOR complexes can act as sensors for amino acid levels (Laplante & Sabatini, 2009; O'Neill *et al.,* 2016; Sinclair *et al.,* 2013). The amino acid glutamine is the most abundant, it can enter the TCA cycle by conversion to glutamate then to α-ketoglutarate and is utilised for ATP production or citrate production for fatty acid synthesis (Baquet *et al.,* 1993; Cruzat *et al.,* 2018; O'Neill *et al.,* 2016). Glutamine and aspartate are substrates in the synthesis of purine and pyrimidines (O'Neill *et al.,* 2016). Therefore, metabolism of these different fuels are interlinked. The main objective for the cell is to synthesise products for cell survival and/or growth, where production is governed by a complex interplay between external and internal signals and cellular metabolic pathways (O'Neill *et al.,* 2016). Immune cell metabolism alterations ensure there is adequate energy or biointermediates required for their specific function.

1.3.2 Dendritic cell metabolism

Simplified, the primary function of DCs is pathogen or danger recognition. This initiates a series of signalling events resulting in expression of genes and increased biosynthesis required for DC activation and effector function (Clark *et al.,* 2000). Resting DCs have a distinct metabolic phenotype compared to activated DCs (Krawczyk *et al.,* 2010). Therefore, signalling pathways required for and following activation must be linked with DC metabolism. Harnessing DC metabolism to influence their function is a potential therapeutic avenue for diseases. For example, active vitamin D3 induced tolerance in human Mo-DCs, with increased glycolysis and oxidative metabolism (Ferreira *et al.,* 2015). Tolerogenic DCs can be utilised for the treatment of autoimmune diseases (Willekens *et al.,* 2019).

Fatty acid synthesis is vital for DC development. During DC differentiation from monocytes both peroxisome proliferator-activated receptor-γ (PPARγ) and PPARγ co-activator 1α (PGC1α) expression are upregulated (Rehman *et al.,* 2013; Zaccagnino *et al.,* 2012). PPARγ is the transcription factor which regulates lipid metabolism and PGC1α, is the master regulator of mitochondrial biogenesis. Mitochondrial biogenesis increases following upregulation of both proteins; increasing mitochondrial numbers, which correlates with greater OXPHOS capacity (Wculek *et al.*, 2019a; Zaccagnino *et al.,* 2012). DC differentiation is abrogated upon inhibition

of mitochondrial respiration in monocytes (Del Prete *et al.,* 2008). DC differentiation or activation has also been found to correlate with citrate synthase activity (Everts et al., 2014). Citrate production is required in the TCA cycle but is also exported into the cytosol for fatty acid synthesis. Fatty acid synthesis is also linked to DC differentiation *in* vivo in lymphoid organs and peripheral tissues (Rehman *et al.,* 2013). Additionally, mTORC1 is essential for DC differentiation and effector function (co-stimulatory molecule expression, cytokine production and T cell stimulatory capacity). Rapamycin-mediated inhibition of mTORC1 decreased DC numbers *in vivo* and diminished *in vitro* development of DCs from bone marrow precursors cultured with FMS-like tyrosine kinase 3 ligand (FLT3L) and impaired Mo-DC survival (Hackstein *et al.,* 2003; Haidinger *et al.*, 2010). These studies depict the role of mTORC1 signalling in development and survival of DCs. Fatty acid oxidation was required for murine cDC1 differentiation and correlated with the larger mitochondrial mass and membrane potential compared to cDC2 (Du et al., 2018; Kratchmarov et al., 2018; Wculek et al., 2019). Murine cDC2 differentiation requires reactive oxygen species (ROS) (Kratchmarov *et al.,* 2018) produced by complexes within the ETC during mitochondrial oxidation. These studies demonstrate the role of metabolic regulators and different metabolic pathways, especially fatty acid metabolism, in DC development and activation.

Resting GM-CSF cultured BM-DCs (GM-DCs) use fatty oxidation and glutaminolysis to supply OXPHOS to meet the metabolic demands of this quiescent state (Krawczyk et al., 2010; Pearce & Everts, 2014; Wculek et al., 2019). Following activation, notably TLR stimulation, DCs upregulate their rate of glycolysis and lactate production (Krawczyk *et al.*, 2010). However, the metabolic profile changes depending on the length of time after stimulation (Everts et al., 2012; Wculek et al., 2019). Lipopolysaccharide (LPS)-activated GM-DCs maintain upregulated glycolysis long-term, via inhibition of oxidative phosphorylation (Everts *et al.*, 2012). DC activation involves antigen presentation, migration, cell-cell interactions and cytokine/chemokine secretion (Banchereau & Steinman, 1998), hence, the cell requires more nucleotides and amino acids for RNA and protein synthesis respectively. Glycolytic intermediates can enter the pentose phosphate pathway to generate these. Mitochondrial pyruvate carrier 1 (MPC1) transport of pyruvate into the mitochondria is required for early activation of DCs (Everts *et al.,* 2014). The early increase in glycolysis serves to provide pyruvate for TCA cycle function (Everts *et al.,* 2014). DCs must increase their intrinsic

metabolism for their energy- and metabolite-demanding activated phenotype. The increase in TCA cycle correlates with an increase in spare respiratory capacity detected early following LPS stimulation and an increase in citrate (Everts *et al.,* 2014) which is shuttled into the cytosol. Similar to DC development, fatty acid synthesis is a requisite for DC activation and is promoted by the citrate shuttle, providing the building blocks required for increased biogenesis of protein production-related organelles, Golgi apparatus and ER (Everts *et al.,* 2014). Additionally, some DC subsets, for example GM-DCs, express inducible nitric oxide synthase (iNOS) which mediates nitric oxide (NO) production (Everts et al., 2012). NO plays a role in inflammation, it can act as a signalling molecule and can trigger apoptosis (Lu et al., 1996). NO can also inhibit the electron transport chain in the mitochondria, and thus, inhibit OXPHOS, which results in the commitment of GM-DCs to glycolysis and allows their survival (Everts et al., 2012; Lawless et al., 2017a). This occurs between 18 to 48 hours after stimulation via a mTOR/hypoxia-inducible factor 1-alpha (HIF1α)/iNOS axis (Lawless et al., 2017). HIF1α is downstream of mTOR, promotes glycolysis and is activated during low or lack of oxygen (Dodd *et al.,* 2015; Lum *et al.,* 2007). This supports T cell stimulation at this timepoint (Everts et al., 2012). iNOS is absent from cDCs (Wculek et al., 2019) but these cells could potentially be affected by NO in the microenvironment (Lawless et al., 2017). Mo-DCs also display HIF1α signalling following activation (Spirig *et al.,* 2010) . However, the use of glucose to fuel the cell was shown to affect GM-DC stimulation of T cells long term (Lawless *et al.,* 2017). Between 72 to 96 hours, GM-DCs can either utilise glucose resulting in inefficient T cell stimulation; or glucose deprivation or inhibition of mTOR results in OXPHOS upregulation and greater T cell stimulation and increased GM-DC life span (Lawless et al., 2017). Greater T cell stimulatory capacity would be beneficial in tumour microenvironments (TME) or sites of inflammation where DCs can be deprived of glucose, due to glucose-hungry tumour cells and T cells.

As highlighted, glucose is an important fuel for the cell, as many cells are reliant upon glycolysis. A common feature amongst most DC subsets is an initial glycolytic burst (Krawczyk *et al.,* 2010). Cells can increase their glucose uptake through increased expression of glucose transporters on the cell surface. Expression tends to be governed by surrounding glucose concentration; insulin binding to its receptor on cell surfaces also results in GLUT4 translocation to the cell surface (Dugani & Klip, 2005). Glucose can be stored within the cells as glycogen. Recently, glycogen breakdown was demonstrated to be important during the first

few hours following TLR activation (Thwe *et al.,* 2017). Glycogenolysis provides glucose-1-phosphate which can enter the glycolytic pathway (Adeva-Andany *et al.,* 2016; Burke *et al.,* 1987). Carbons provided from this pathway enter the TCA cycle and following LPS stimulation, promoted citrate synthesis (Thwe *et al.,* 2017). Initial metabolic reprogramming is facilitated by the breakdown of glycogen which correlates with the upregulation of glucose transporters hours after TLR stimulation. Inhibition of glycogen breakdown resulted in diminished DC maturation and effector function (Thwe *et al.,* 2017).

Fatty acid metabolism has been demonstrated to be important for DCs. In certain diseases, such as obesity, a high lipid content within immune cells can have deleterious roles (Michelet *et al.,* 2018). The field of immunometabolism studies the complex interaction between metabolism and other pathways within the cell to ensure effective immune cell function. We sought to determine how a metabolism-altering disease, like obesity, impacts DC function.

1.4 Obesity

1.4.1 Obesity overview

Obesity can be defined as a chronic, progressive, and relapsing disease (Bray, Kim and Wilding, 2017). WHO defines obesity as an excessive or abnormal accumulation of fat, which can impair an individuals' health (WHO, 2021). Global estimates generated in 2016 found more than 1.9 billion adults to be overweight (Body Mass Index (BMI) ≥25 kg/m^2). Over 650 million of these same adults were classified as living with obesity (BMI ≥30 kg/m^2) (WHO, 2021). In the WHO European Region, approximately 60% of adults and almost 1 in 3 children are overweight or living with obesity ('WHO EUROPEAN REGIONAL OBESITY REPORT 2022', 2022). Obesity has been strongly associated with the development of chronic non-communicable diseases (NCD), including; Type 2 Diabetes Mellitus (T2DM), cardiovascular disease (CVD) and cancer (Haslam and James, 2005; Horn, Almandoz and Look, 2022).

Obesity has long been associated with only physical inactivity and nutrient overload; however, obesity is a complex multifactorial disease involving environmental, genetic, psychological, behavioural, and social factors which affect the intake of food and energy expenditure (Bray, Kim and Wilding, 2017; Anekwe *et al.*, 2020). Various organs act together to ensure energy

balance, such as the central nervous system (particularly, the hypothalamus), the gut, liver, pancreas, adipose tissue (AT). Hormones are released based on hunger and satiety to regulate energy metabolism. Dysfunction in these organs and hormones occur during the onset of obesity and contribute to the worsening of the disease (Xu, 2013; Uranga and Keller, 2019; Geng *et al.*, 2022; Ylli *et al.*, 2022).

A chronic, low-grade inflammatory state is characteristic of obesity (Fantuzzi, 2005), it is intrinsically linked to the inflammatory state of the host and is often referred to as metabolic inflammation (Xu, 2013). One of the defining factors is the localisation of myeloid cells to organs involved in metabolism, including the white adipose tissue (WAT) (Fujisaka *et al.*, 2013). AT comprises of a heterogenous composition of cells, including adipocytes, stromal cells, and resident immune cells (Lenz *et al.*, 2020). AT serves as an endocrine organ, in addition to its role as an energy store, it can produce cytokines, known as adipokines, which can influence metabolism, and promote or dampen inflammation (Chan *et al.*, 2017). Obesity involves an increase in both the volume and function of AT, causing changes in local and systemic metabolism and the resident cells resulting in AT inflammation (Chan *et al.*, 2017). Dysregulation of adipocytes and immune cells disrupts AT homeostasis, and the resulting localised inflammatory state can spread systemically and eventually lead to dysregulated homeostasis of other cells and tissues, underpinning the development of various chronic co-morbidities (Chan *et al.*, 2017), illustrated in Figure 1.5. For example, hyperinsulinemia has been observed in people with obesity (PWO) (Kim *et al.*, 2015). Insulin target organs (e.g., liver) become resistant to the actions of insulin due to chronic inflammation, resulting in increased insulin production from the pancreas (Bazotte, Silva and Schiavon, 2014). Insulin resistance ultimately leads to hyperglycaemia, when the body is resistant to insulin and is unable to normalise blood glucose levels due to reduced glucose uptake, signalling the start of T2DM (Bazotte, Silva and Schiavon, 2014). Hyperlipidaemia, another characteristic of obesity, can lead to plaque formation within blood vessels and the heart, resulting in hypertension and CVD (Jung and Choi, 2014).

Underpinning its classification as a chronic, progressive, and relapsing disease, a major factor is that obesity-driven defects in metabolism, hormones, and cells, can persist despite weight loss (De Barra *et al.*, 2023; Frasca *et al.*, 2023) and can support the regaining of weight (Vink

et al., 2016). The most effective treatment for people with obesity is invasive bariatric surgery, however, this is reserved for a minority of patients with the most severe and complicated obesity (Sjöström *et al.*, 2012; Wolfe, Kvach and Eckel, 2016). In the past 10 years, a new generation of pharmacological therapies have been developed (e.g. glucagon-like peptide-1 analogues) which are resulting in clinically significant weight loss (10-20%) (Chakhtoura *et al.*, 2023), and have noted positive impacts on immune cells and associated co-morbidities (De Barra *et al.*, 2023). The impact of obesity in the context of immunity will be discussed further.

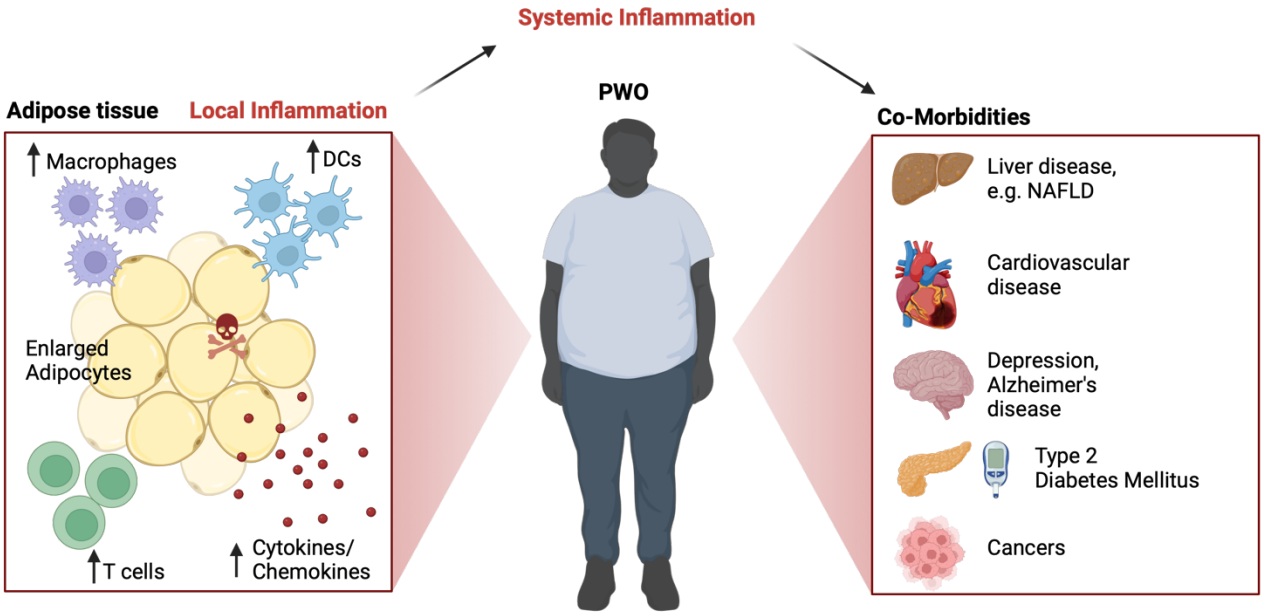


Figure 1.5 - Impact of obesity on adipose tissue driving local and systemic inflammation & associated co-morbidities. Obesity can induce adipocyte hypertrophy, and adipocyte cell death which can recruit immune cells with pro-inflammatory properties (e.g., macrophages/ DCs/T cells). Adipocytes secrete chemokines & cytokines (e.g., MCP-1, TNF-α, IL-6) which promote immune cell infiltration and activation respectively. Local inflammation can spread inducing the chronic, low-grade inflammation observed in obesity, which is linked to an increased risk of multiple co-morbidities (e.g., non-alcoholic fatty liver disease (NAFLD), cancers, depression) (adapted from Barakat and Almeida (2021); Lim and Boster (2023)).

1.4.2 Obesity Immunology

Significant alterations have been reported in the immune system of PWO, with a chronic, low-grade systemic inflammatory profile observed (Fantuzzi, 2005). Obesity drives several pro-inflammatory mechanisms including increased adipocyte stress, increased free fatty acid (FFA) levels, increased reactive oxygen species and altered metabolic processes (Haslam and James,

2005; Wolowczuk *et al.*, 2008; Minihane *et al.*, 2015). These alterations lead to dysregulation of the immune system in both adipose tissue and systemically, resulting in increased levels of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α (Trayhurn and Wood, 2004; Chan *et al.*, 2017). These inflammatory mediators underpin the development of obesity-related diseases.

The initial trigger of inflammation remains unclear. Increased uptake of lipids within AT results in both hypertrophy and hyperplasia of adipocytes (Chan *et al.*, 2017). Enlarged adipocytes can upregulate stress signals, signalling to the immune system for action, resulting in an inflammatory response (Lumeng, Bodzin and Saltiel, 2007; Jiao *et al.*, 2011). Unchecked these enlarged adipocytes can become necrotic, initiating a high-inflammatory state (Cinti *et al.*, 2005). Limited angiogenesis, inflammation and hyperplasia can produce a hypoxic environment within AT (Chan *et al.*, 2017). Elevated levels of HIF1α have been reported in obesity (Rausch *et al.*, 2008). A hypoxic environment can trigger adipocyte death, promoting infiltration of macrophages and free FFA release into the blood (Yin *et al.*, 2009; Chan *et al.*, 2017). FFA can activate TLRs, TLR4 in particular, and downstream signalling results in the production of cytokines (Milanski *et al.*, 2009). Greater adipocyte death correlated with increased expression of cytokines such as TNF-α, IL-6 and monocyte chemoattractant protein (MCP-1) (Strissel *et al.*, 2007). MCP-1 is also known as C-C motif chemokine ligand (CCL) 2 (Deshmane *et al.*, 2009). MCP-1 attracts monocytes, including macrophages, to the site of inflammation, the adipose tissue (Strissel *et al.*, 2007; Deshmane *et al.*, 2009). The pro-inflammatory cytokines, TNF-α and IL-6, can promote exacerbation of inflammation from local to systemic (Chan *et al.*, 2017). In addition to this systemic inflammatory profile, obesity is associated with major alterations to immune cell populations responsible for host-pathogen defences, with both innate and adaptive populations affected (Milner and Beck, 2012; Carolan *et al.*, 2015; Michelet *et al.*, 2018).

'Stressed' macrophages were observed in AT, where during obesity, a pro-inflammatory M1-like polarisation of AT-resident macrophages was favoured as opposed to the anti-inflammatory M2-like state (Lumeng, Bodzin and Saltiel, 2007). Other chemokines such as RANTES, have higher expression levels in AT of diet-induced obesity (DIO) in mice or PWO, influencing cell migration and accumulation (Baturcam *et al.*, 2014; Chan *et al.*, 2022; Zhou *et*

al., 2023). An innate-like unconventional T cell, Mucosal associated invariant T (MAIT) cell, important during microbial infections, has been found to contribute to chronic inflammation via an IL-17 producing phenotype in obesity and insulin resistance (Carolan *et al.*, 2015; Bergin *et al.*, 2022). NK cells are an important innate effector population with potent anti-viral activities. Obesity-associated defects in NK cell functions (decreased cytotoxicity and effector molecule production) has been reported extensively in PWO, with defects starting early in childhood (Lynch *et al.*, 2009; Tobin *et al.*, 2017; Michelet *et al.*, 2018; O'Shea and Hogan, 2019). Neutrophils were observed to be increased in circulation of children and adults living with obesity (Dixon and O'Brien, 2006; Rodríguez-Rodríguez *et al.*, 2022). Children with obesity are at 80% risk of having obesity in adulthood (Simmonds *et al.*, 2016). Therefore, the dysfunctional properties conferred by obesity have lifelong consequences.

Dysregulation of the immune system is a significant consequence of obesity, as shown in relation to certain innate immune cells. DCs have been implicated in metabolic dysfunction and their roles and impact on adaptive immune cells will be elucidated next.

1.4.3 Obesity and Dendritic cells

DCs act as a bridge between the innate and adaptive immune response. Alterations have previously been observed in dendritic cells, in the context of obesity. CD83 is marker for DC maturation and membrane-bound CD83 is essential for the induction of T lymphocyte responses (Prechtel *et al.*, 2007). The CD83 marker was significantly downregulated on DC surface from PWO, compared to controls (O'Shea *et al.*, 2013), basally and following TLR stimulation. The most intriguing finding of the study was that following immature DC stimulation from controls and PWO, significantly elevated IL-10 levels were generated by the DCs from PWO compared to the control DCs (O'Shea *et al.*, 2013). DCs not only act as APCs to adaptive immune cells but are also involved in the polarisation of T lymphocytes, e.g., to either a pro-inflammatory Th1 pathway or an immunosuppressive Th2 pathway (Banchereau and Steinman, 1998). Consequently, LPS-stimulated DCs from PWO and controls were then co-cultured with T cells and IL-4, a Th2 cytokine, was significantly elevated from PWO compared to controls (O'Shea *et al.*, 2013). PWO have reduced numbers of DCs (O'Shea *et al.*, 2013), which may contribute to the impaired T lymphocyte response associated with PWO following influenza infection or vaccination (Smith *et al.*, 2009; Sheridan *et al.*, 2011). Less DCs and a

downregulation of their necessary markers would affect the subsequent activation of T cells. Additionally, the increase of immunosuppressive cytokine expression upon TLR stimulation, corroborates findings from DIO mice where reduced DC numbers were observed in the lungs, with diminished cytokine mRNA expression, and subsequent impaired T lymphocyte response following influenza infection (Smith *et al.*, 2009). Obesity has led to the impairment of immune responses either directly or indirectly and this has had significant implications for infection, as PWO are at a greater risk of being infected and of greater infection severity (van Kerkhove *et al.*, 2011; Popkin *et al.*, 2020).

The effects of obesity have been studied in both human and mice. Bertola *et al.* (2012) found an increase in DCs in AT of both humans and mice. The murine $CD11c^{high}$ $F4/80^{low}$ DCs from mice on a high fat diet (HFD) induced naïve T cells to differentiate into Th17 cells. The human $CD11c^{+}$ $CD1c^{+}$ DCs correlated with increased *IL17* expression from AT $CD3^{+}$ T cells and induced Th17 differentiation from naïve T cells *in vitro* (Bertola *et al.*, 2012). DC accumulation was also based on different chemokines, particularly, CCR7. In *CCR7* knockout mice, there were less adipose tissue dendritic cells (ATDCs), the mice expressed lower levels of inflammatory genes in epididymal white adipose tissue (eWAT) and were more protected from insulin resistance compared to wild-type (WT) mice, when both were on a HFD (Cho *et al.*, 2016). A recent paper from vanderZande *et al.* (2023) found that percentages of cDCs in eWAT or the liver between mice on a low-fat diet (LFD) or HFD were similar. However, HFD DCs from both metabolic tissues had increased activation marker expression, such as MHC class II and CCR7 in both tissues compared to LFD (vanderZande *et al.*, 2023). Higher CCR7 expression would suggest increased migratory capacity, which could result in greater activation of inflammatory T cells in the draining lymph nodes, where the DCs would home to (Guak *et al.*, 2018). DCs from eWAT in HFD mice expressed increased MHC Class I and CD86 compared to LFD. DCs from the liver in HFD mice expressed increased CD40 compared to LFD (vanderZande *et al.*, 2023). Consequently, alterations were observed in T cell populations within the same tissues. In eWAT from HFD mice, there was increased IFN-γ^{+} T cells, representing Th1 cells, and decreased IL-5^{+} and $FOXP3^{+}$ cells, representing Th2 and Treg cells respectively, compared to eWAT from LFD mice (vanderZande *et al.*, 2023). In the liver of HFD mice, there were increased IFN-γ^{+}, $FOXP3^{+}$ and IL-$17A^{+}$ T cells (vanderZande *et al.*, 2023). The latter represents Th17 cells, these cells produce IL-17A, a pro-inflammatory cytokine implicated in many inflammatory diseases,

including obesity (Bergin *et al.*, 2022). As mentioned previously, IL-17 production by MAIT cells, from children with obesity, was found to reduce glucose uptake and strongly correlated with insulin resistance in these children (Bergin *et al.*, 2022). The observed increase in activation markers were on DCs in metabolic organs and was not observed in other organs, i.e., the spleen (vanderZande *et al.*, 2023), which suggests the activated DCs are caused by changes in these metabolic environments. The increase in IL-17-producing cells correlates with the increase of IL-17 associated with obesity and the detrimental effects this cytokine can exert (Sumarac-Dumanovic *et al.*, 2009; Winer *et al.*, 2009; Bergin *et al.*, 2022). The impact of obesity on DC function in turn affects their activation of the adaptive immune response, affecting the host's ability to respond appropriately to pathogens and vaccination, the latter will be discussed in detail next.

1.4.4. Obesity and Vaccination

Vaccination relies on the stimulation of both the innate and adaptive immune response (Clem, 2011), issues arise when underlying conditions, such as obesity, already dampen the immune response (Karlsson, Sheridan and Beck, 2010a; Sheridan *et al.*, 2011). Obesity results in immune dysfunction in various ways, affecting the innate immune system, humoral and T-cell mediated immune responses (Smith *et al.*, 2009; O'Shea *et al.*, 2013; Frasca *et al.*, 2023), which are crucial for effective vaccination programmes. Particularly, the T cell-mediated immune response. Growing evidence depicts the inability of PWO to mount an adequate immune response to vaccination compared to controls (Sheridan *et al.*, 2011). Vaccination relies on the ability of the immune system to generate a memory response, which would protect the vaccinated individual upon re-exposure to the causative agent (Clem, 2011). T-cell mediated responses are essential as these would target internal proteins, which may be common amongst the viral strains, e.g., observed amongst influenza strains (Thomas *et al.*, 2006), whereas the humoral Ab-based response would target surface proteins which may differ between viral strains (Carrat and Flahault, 2007). Karlsson *et al.* (2010a) demonstrated a significant reduction in influenza-specific memory T cell function and efficiency in mice with DIO. A follow-up study demonstrated a reduction in influenza-specific $CD8^+$ memory T cell maintenance in mice with DIO (Karlsson, Sheridan and Beck, 2010b). 84 days post-infection, mice on a HFD had a significant reduction in influenza-specific effector memory T cells in their

lungs compared to the mice on a standard diet (SD), these cells are essential as they are located in the peripheral sites targeted by the pathogen (Karlsson, Sheridan and Beck, 2010b). The ability to confer protection upon secondary infection correlates with the number of these specific memory T cells within the target tissue (Karlsson, Sheridan and Beck, 2010b), in this case, the lungs. Maintenance of these Ag-specific $CD8^+$ memory T cells is regulated by two vital cytokines, IL-7 and IL-15 (Karlsson, Sheridan and Beck, 2010b). The mechanisms by which obesity affects memory T cell maintenance have been hypothesised. Obesity is characterised by hyperleptinaemia which results in leptin resistance (Berger and Polotsky, 2018); leptin is a hormone shown to link metabolism and the immune system (Pérez-Pérez *et al.*, 2017). Leptin signalling has been associated with IL-15 function, despite an increase in *Il15* mRNA expression observed in these HFD mice, the bystander proliferation of memory T cells still diminishes over time (Karlsson, Sheridan and Beck, 2010b). The impairment of leptin signalling was given as a possible explanation for reduction in memory T cell numbers. As previously mentioned, obesity can affect DC function (O'Shea *et al.*, 2013) and these cells are important APCs for the maintenance of memory T cells by trans-presentation of IL-15 on its respective receptor (Stonier *et al.*, 2008). Therefore, obesity can result in the reduction of these important memory T cells, required for a rapid immune response upon re-exposure to their specific Ag. Sheridan *et al.* (2012) studied the effect of BMI on adaptive immune responses in humans following influenza vaccination. PWO had a decrease in percentage of influenza-activated $CD8^+$ T cells and a significant decrease in activity compared to controls (Sheridan *et al.*, 2011). $CD8^+$ T cell activity was determined by the percentage of cells expressing IFN-γ and Granzyme B. Therefore, these cells had a reduced ability to inhibit viral replication and a reduced ability to lyse virally infected cells (Russell and Ley, 2002; Teijaro *et al.*, 2010). A study in mice revealed that obesity induced alterations to T cell metabolism, which correlated with the memory T cell response impairment to influenza (Rebeles *et al.*, 2019). Importantly, this impairment of memory T cells and their associated metabolic dysfunction was not reversed following weight loss (Rebeles *et al.*, 2019). These findings have significant implications for public health as weight loss which previously was shown to reverse certain metabolic consequences of obesity (Lawrence and Kopelman, 2004), may not adequately reverse certain immune dysfunction (Rebeles *et al.*, 2019; De Barra *et al.*, 2023), causing these individuals to remain susceptible to future re-infections and maintains a diminished efficacy of vaccines.

Sheridan *et al.* (2011) also demonstrated a significant reduction in influenza-specific Ab titres as BMI increased, a year after vaccination. Significant reductions of Abs were also present in PWO after receiving the Hepatitis B (HBV) vaccine, compared to controls (Mckee *et al.*, 1990; Kwon and Jeong, 2019). Non-responsiveness to the HBV vaccine correlated with increasing BMI (Kwon and Jeong, 2019). Consequently, obesity would affect the susceptibility of individuals to infection by these specific viruses and affect the severity of the infections. Obesity has been shown to impact vaccinations against other pathogen types, such as bacteria, where overweight children had a significant decrease in anti-tetanus Abs in comparison to controls (Eliakim *et al.*, 2006). Obesity may affect the efficacy of vaccines in several ways, firstly, the dysfunction of the immune system can weaken the response to the vaccine and affect the generation of memory responses required for protection against re-exposure to the specific Ag. Secondly, the increased body mass associated with obesity may affect the vaccine uptake, absorption, and distribution within PWO following inoculation (Eliakim *et al.*, 2006). These are all factors which must be accounted for during development of a vaccine, as obesity can affect the pharmacokinetics of drug development and impair the desired immune response. As adults with obesity, despite vaccination, were found to be twice at risk of developing influenza or influenza-like infections, in comparison, to vaccinated controls (Neidich *et al.*, 2017), portraying PWO as an at-risk group for increased susceptibility to infection and greater infection morbidity and mortality (van Kerkhove *et al.*, 2011; Popkin *et al.*, 2020).

DCs are essential for the activation of the adaptive immune response, through pathogen recognition and presentation to T cells. Importantly, DCs induce adaptive memory responses and can induce tolerance. DCs are found to be altered in obesity, therefore, this study sought to investigate the impact of obesity on GM-DCs, a widely used model to study DC function, from mice on a high fat diet compared to those on a standard diet. We hypothesised that obesity-driven changes in metabolism would affect DC function. Various techniques were employed to determine the metabolic and functional properties of DCs.

Chapter Two

Materials & Methods

2.1 Ethics

Full ethical approval for all aspects of the studies presented was granted by both the Maynooth University Research Ethics Committee and by the Health Products Regulatory Authority.

2.2 Study design & diet-induced model of obesity

Male C57BL6/J mice were purchased from Charles River Laboratories at 6-8 weeks of age. Mice were acclimatised for 1 week prior to the initiation of experiments. Following acclimatisation, mice were fed either the standard diet (18% kcal of fat, T2918, Envigo) or the high fat diet (60%kcal of fat, TD.06414, Envigo) *ad libitum*. Mice were on their respective diets for 16 weeks (Figure 2.1). Two 16-week high fat diet models were performed to obtain adequate numbers for experiments. During the first HFD model, 5 representative mice were weighed from the SD or HFD group each week from the start of the diet, week 0. Mice were culled via cervical dislocation. All mice were weighed following sacrifice. Epididymal adipose tissue was extracted and weighed. Control WT mice between 8 to 16 weeks were used to optimise experiments prior to diet-induced models of obesity.

2.3 Generation of murine bone marrow-derived dendritic cells

Mice were sacrificed and sterilised with 70% ethanol (Sigma-Aldrich) prior to entry into a laminar flow hood to maintain aseptic conditions. The femur and tibia were removed and were flushed with a 27¾ gauge needle containing 10mls of Dulbecco's phosphate buffered saline (DPBS, Sigma Aldrich) for each bone. Cells were washed by centrifugation at 400 x *g* for 5 minutes, supernatants were discarded, and cells were re-suspended in complete medium (cRPMI): RPMI-1640 medium supplemented with GlutaMAX (Thermo Fisher Scientific), 10% v/v premium foetal bovine serum (FBS) (Thermo Fisher Scientific), penicillin (100 I.U./ml, Sigma-Aldrich) and streptomycin (100µg/ml, Sigma-Aldrich). During bone marrow cell precursor growth and differentiation, media was supplemented with murine recombinant GM-CSF (20ng/ml, BioLegend), as previously described (Inaba *et al.*, 2001). Cells were grown in T175 flasks. Fresh complete medium was added on Day 3 and Day 7. On Day 6, supernatants were discarded, and complete culture medium entirely replaced. On Day 10, culture supernatant containing non-adherent and loosely adherent cells were harvested, washed by

centrifugation at 600 x *g* for 5 minutes, supernatants were discarded and resulting cell pellet resuspended in fresh medium of RPMI-1640 supplemented with GlutaMAX, 10% (v/v) dialysed foetal bovine serum (FBS, Gibco, ThermoFisher Scientific), penicillin (100 I.U./ml, Sigma-Aldrich), streptomycin (100µg/ml, Sigma-Aldrich) and murine recombinant GM-CSF (10ng/ml, BioLegend) for all subsequent experiments, except experiments involving a different carbon source. Figure 2.1 is a schematic of the workflow, from the diet-induced model of obesity to the subsequent experiments performed.

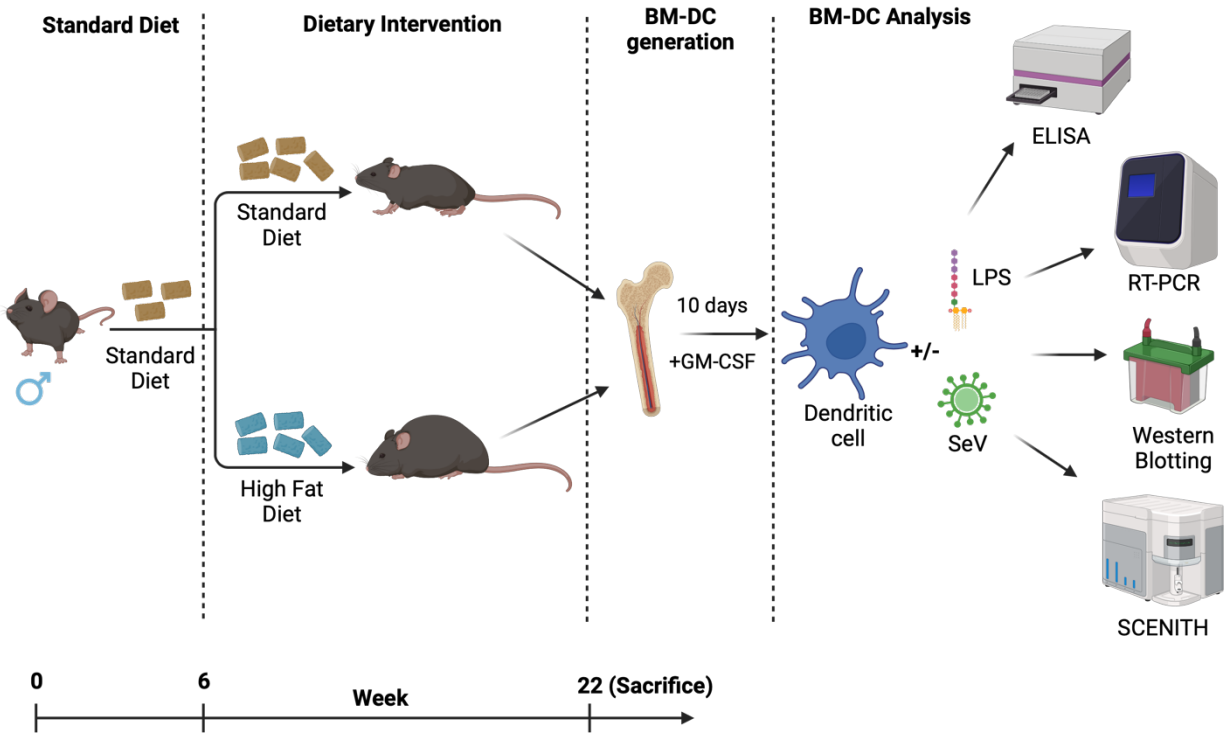


Figure 2.1 - Workflow of DIO model of obesity and GM-DC generation and subsequent experiments

2.4 Cell enumeration

Viable cells were counted through a dye exclusion test. Prior to cell plating for experiments, cells were stained using trypan blue solution (Sigma-Aldrich) using 1:5, 1:10 or 1:20 dilution, depending on pellet size. 10µl of stained cell suspension was added to a haemocytometer and visualised under a microscope. Dead cells appear blue due to the uptake of the dye, whereas viable cells appear white due to an intact cell membrane and no dye uptake. These viable cells were counted in the opposite upper left and bottom right squares of the haemocytometer and the average taken of these two squares (see Figure 2.2).

The following formula was used to calculate the number of cells in the original suspension per 1ml: **(Average of two squares x dilution factor)/100 = X x10^6 cells per ml**

For subsequent experiments, cells were resuspended in media at 1x10^6/ml, unless specified otherwise.

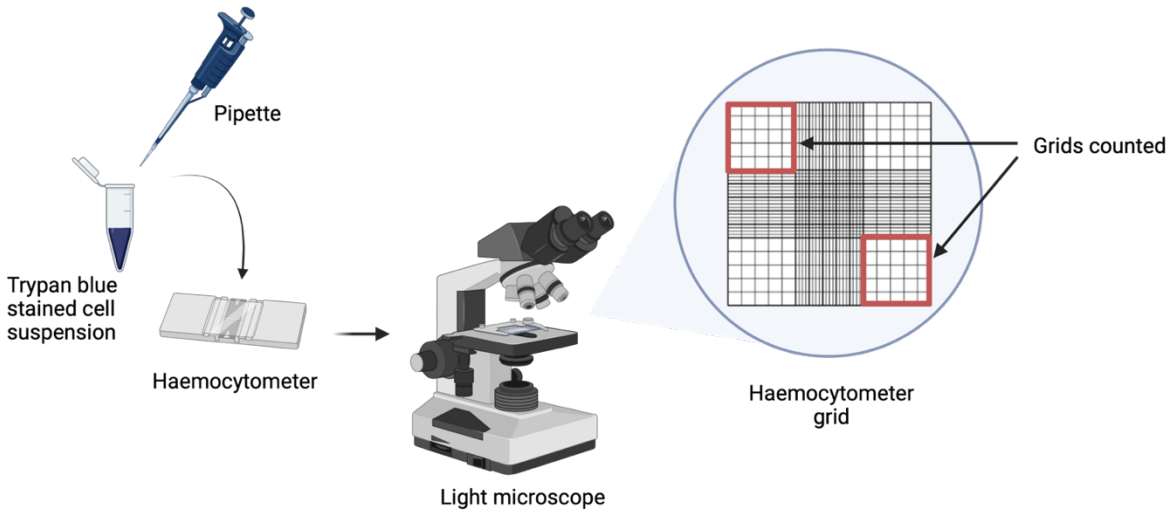


Figure 2.2 Cell counting Cells stained with trypan blue solution were visualised and counted using a haemocytometer and light microscope. The average of the cell counts from the two labelled squares were used for the subsequent calculation of cell number per ml.

2.5 Cell culture media

For experiments involving an alternative carbon source to glucose, RPMI 1640 no glucose (ThermoFisher Scientific) was used, this was supplemented with 10% dialysed FBS (ThermoFisher Scientific), penicillin (100 I.U./ml, Sigma-Aldrich) and streptomycin (100µg/ml, Sigma-Aldrich). The respective sugar was weighed Fructose (10mM; molecular weight of 180.16 g/mol, Sigma-Aldrich), Galactose (10mM; 180.16 g/mol, Sigma-Aldrich) and Low Glucose (1mM; 180.16 g/mol, Sigma-Aldrich) and were added to the appropriate volume of the aforementioned media under sterile conditions to obtain the correct concentration. Each media was vortexed to ensure a homogenous solution, followed by filtration through a 0.2µm filter and 20ml syringe. No glucose media was the aforementioned media with no addition of

glucose. Glucose & fructose containing media was produced through a similar method but weighing fructose and glucose to obtain a final concentration of 5mM each in the media solution.

As mentioned previously, for all other experiments which did not involve the carbon flexibility study, the normal culture media was used which was RPMI 1640 GlutaMax supplemented with 10% dialysed FBS, penicillin (100 I.U./ml) and streptomycin (100µg/ml), referred to as cRPMI. All medias contained 10ng/ml of murine recombinant GM-CSF for cell plating and subsequent experiments.

2.6 GM-DC stimulation

2.6.1 Gifts

Sendai virus (SeV) Cantell Strain was gifted from Professor Paul Moynagh (Maynooth University, Co. Kildare, Ireland). The Sendai Cantell strain is also known as Murine Parainfluenza 1. SeV was obtained at a hemagglutination (HA) tube titer of 4000 per ml.

2.6.2 Stimulations

For all SeV stimulations, a dilution factor of 1000 was used. Cells were stimulated for varying lengths of time depending on the experiment and is explicitly stated in each section. For all lipopolysaccharide (LPS) (Enzo Life Sciences) stimulations, LPS was used at a concentration of 100ng/ml. Cells were stimulated for varying lengths of time depending on the experiment and is explicitly stated in each section. In instances where working concentrations had to be made beforehand, the stimulant was diluted in the appropriate media. For carbon source experiments, the diluent was no glucose media and for all other experiments, the diluent was cRPMI.

2.7 Metabolic inhibitors

Various metabolic inhibitors were utilised to investigate the metabolic pathways in GM-DCs. Inhibitors were added based on the literature or through the generation of dose curves, in the case of the 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3 (PFKFB3) inhibitor AZ67. Inhibitors were reconstituted based on manufacturer's instructions. The hexokinase

inhibitor 2-Deoxy-D-glucose (2-DG) (Sigma-Aldrich) was reconstituted in molecular grade water (Sigma-Aldrich) and used at concentration of 1mM. The adenosine triphosphate (ATP) synthase inhibitor oligomycin (Sigma-Aldrich) was reconstituted in dimethylsulfoxide (DMSO) (Sigma-Aldrich) and used at a concentration of 1 µM. The Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) inhibitor heptelidic acid (Abcam) was reconstituted in molecular grade water and used at a concentration of 5µM. The PFKFB3 inhibitor AZ67 (Tocris Bioscience) was reconstituted in DMSO and used at a concentration of 10µM. The glycogen phosphorylase inhibitor CP91149 (Sigma-Aldrich) was reconstituted in DMSO and used at a concentration of 100µM. In instances where working concentrations had to be made beforehand, the reconstituted inhibitors were diluted in the appropriate media. For carbon source experiments, the diluent was no glucose media and for all other experiments, the diluent was cRPMI.

2.8 GM-DC molecular analysis

2.8.1 mRNA experimental set up

GM-DCs were generated as per section 2.3 and re-suspended in cRPMI at $1x10^6$ per ml. GM-DCs were plated 3mls per well in a 6-well plate. Cells were rested for 2-3 hours. Inhibitors were added to their appropriate wells, 2-DG (1mM) or Oligomycin (1µM). Cells were stimulated for 3 hours with LPS (100ng/ml). For SeV stimulations (1:1000), cells were stimulated overnight and the following morning, cells were stimulated with SeV for the 3-hour and 6-hour timepoints. At the end of the stimulation period, the cell extraction process was carried out on ice, the plates were centrifuged at 400 x *g* for 5 minutes. Supernatants were discarded and 500µl of TRIzol™ Reagent (ThermoFisher Scientific) was added to each well. The cells were briefly scraped using disposable scrapers (Biotium) as the reagent should result in cell dissociation from the well and the resulting liquid with cells were transferred into their respective labelled 1.5ml Eppendorf tubes (ThermoFisher Scientific). Samples were stored at -70°C.

2.8.2 mRNA isolation

mRNA isolation was performed using ThermoFisher Scientific's protocol. Frozen samples of cells in TRIzol™ Reagent were allowed to equilibrate to room temperature and incubated for

5 minutes to allow nucleoprotein complex dissociation. 100µl of chloroform (Sigma-Aldrich) was added to each sample and vortexed. Samples were incubated at room temperature for 2-3 minutes. Samples were then centrifuged for 15 minutes at 12,000 x *g*. The mixture forms layers, proteins concentrate in the lower phase, DNA concentrates within the interphase and RNA concentrates within the colourless upper aqueous phase. Approximately 100 to 200µl of the upper aqueous phase, depending on its size, is transferred into new labelled tubes. 250µl of isopropanol (ThermoFisher Scientific) was added to each sample and incubated for 10 minutes, to precipitate the RNA. Following incubation, the samples were centrifuged at 12,000 x *g* for 10 minutes. Due to the small size of the RNA pellet obtained following isolation, resulting supernatants were discarded via blotting on clean paper towels to ensure thorough removal of excess liquids. 1mL of 75% ethanol was added to each tube to wash the pellets, the tubes were vortexed briefly then centrifuged at 7500 x *g* for 5 minutes. Supernatants were once again discarded via blotting. The RNA pellets were air dried for 10 minutes then resuspended in 20µl of RNAse-free molecular grade water each. Samples were then placed on a heating block at 60°C for 10 minutes to ensure solubilisation of the RNA. RNA samples were quantified as per section 2.8.3 then stored at -70°C or used for subsequent cDNA synthesis or for RNAsequencing.

2.8.3 mRNA quantification

RNA was quantified using the Nanodrop™ 2000 spectrophotometer (ThermoFisher Scientific). RNA nucleic acid settings were used on the Nanodrop software and absorbance values were measured at 260 nm and 280 nm. A blank of 1µl of molecular grade water was required beforehand to set up subsequent readings. 1µl of each sample was added to the Nanodrop and RNA concentration (ng/µl) was measured. All RNA samples were diluted with molecular grade water to obtain a final concentration of 100ng/µl. Diluted samples were used for subsequent applications or RNA samples were stored at -70°C.

2.8.4 cDNA synthesis

Complementary DNA (cDNA) synthesis was performed by reverse transcription of isolated RNA using qScript cDNA Synthesis kit (QuantaBio). cDNA synthesis was performed following RNA equalisation and performed according to manufacturer's instructions. Each reaction was

20µl in volume and was prepared in sterile 0.2ml RNA-ase free tubes (Sarstedt), the reaction components are found in Table 2.1. Samples were run on a G-Storm Thermal Cycler (Gene Technologies Ltd) and three cycles were performed, outlined in Table 2.2. The 1st cycle promoted annealing of primers, the 2nd cycle enabled cDNA synthesis and the 3rd cycle terminated the reaction. The samples were then held at 4°C for storage until removed from the Thermal Cycler. cDNA samples were diluted with 80µl of molecular grade nuclease-free water (1:5 dilution, Sigma-Aldrich) and either amplified by Real Time-Polymerase chain reaction (RT-PCR) or kept at -20°C for long-term storage.

Table 2.1 – Reaction mix for cDNA synthesis

Reagent	**Volume (µl)**
RNA sample (equalised to 100ng/µl)	5
5X qScript Reaction Mix	4
qScript Reverse Transcriptase	1
Molecular grade nuclease-free water	10

Table 2.2 – Cycling conditions for cDNA synthesis

Cycle	**Temperature (°C)**	**Time (minutes)**
1	22	5
2	42	30
3	85	5
Holding	4	Indefinite

2.8.5 Real time PCR

Prior to RT-PCR, KiCqStart primer sets (Sigma-Aldrich) were reconstituted in molecular grade water to obtain 100µM stock solutions of each. RT-PCR uses primers to amplify specific target regions. RT-PCR analyses were performed using PerfeCTa SYBR Green FastMix ROX Reaction Mix (QuantaBio) and primer sets, according to manufacturer's instructions. Primers were diluted further in molecular grade water to obtain 4µM working solutions. Master mixes consisted of forward and reverse primers, molecular grade water and SYBR green enzyme mix

per sample, made as per Table 2.3. Forward and reverse primer sequences for each target gene are detailed in Table 2.5. 18µl of the master mix was added to the appropriate wells in a labelled MicroAmp Fast 96-well reaction plate (0.1mL, ThermoFisher Scientific). Followed by the addition of 2µl of cDNA template added to the appropriate wells. Samples were plated in duplicates for both target gene and treatment. The plate was sealed with MicroAmp Optical Adhesive film (ThermoFisher Scientific) and pulsed in the centrifuge for 10 seconds to ensure the solution settled to the bottom of wells. The plate was loaded onto a StepOnePlus™ Real-Time PCR System (Applied Biosystems).

Table 2.3 – Reaction mix for RT-PCR

Reagent	**Volume (µl)**
Forward primer working solution (4µM)	2.5
Reverse primer working solution (4µM)	2.5
Molecular grade nuclease-free water	3
SYBR green enzyme mix	10
cDNA template	2

The cycling conditions of RT-PCR is outlined in Table 2.4. Samples were initially denatured, followed by 45 cycles of 3 steps, denaturation, primer annealing to cDNA template and extension of DNA product. Annealing temperature was varied depending on the lowest melting temperature of the primer used. A melt curve was generated following the cycling, by heating to 90°C incrementally, with fluorescent readings obtained every 2° temperature increase, to determine integrity of the primers.

Table 2.4 – Real time-PCR cycling conditions

Stage	Step	Temperature (°C)	Time (seconds)
Initial denaturation	Initial (occurs once)	95	30
Denaturation	45 cycles	95	5
Primer Annealing		57	15
DNA Extension		72	10
Melt Curve	Final (occurs once)	Incremental increase to 90	-

Target gene expression was evaluated based on relative quantification to a control gene, using the ΔΔCrossing Threshold (CT) method. The control or housekeeping gene used was either hypoxanthine phosphoribosyltransferase (*Hprt*), required for purine recycling (Seegmiller, Rosenbloom and Kelley, 1967) or actin beta (*Actb*), involved in cell motility and structure (Hofmann and De Lanerolle, 2006), specified where appropriate. The average of the gene of interest was normalised to the average of the specific housekeeping gene. Treatment results obtained from SD GM-DCs were normalised to each sample's basal expression. Treatment results obtained from SD and HFD GM-DCs were normalised to a SD sample with median gene expression to facilitate comparison of SD and HFD gene expression. However, for inhibitor studies in both SD and HFD GM-DCs, the results were normalised to each sample's basal expression.

Table 2.5 – Primer sequences for target genes utilised for RT-PCR

Target Gene	Forward Primer Sequence 5' – 3'	Reverse Primer Sequence 5' – 3'
Actb	GATGTATGAAGGCTTTGGTC	TGTGCACTTTTATTGGTCTC
Hprt	AGGGATTTGAATCACGTTTG	TTTACTGGCAACATCAACAG
Ifnb1	CCTATGGAGATGACGGAGA	CCCAGTGCTGGAGAAATTGT
Il6	AAGAAATGATGGATGCTACC	GAGTTTCTGTATCTCTCTGAAG
Il10	CAGGACTTTAAGGGTTACTTG	ATTTTCACAGGGGAGAAATC
Il12b	CATCAGGGACATCATCAAAC	CTCTGTCTCCTTCATCTTTTC
Il27	AATCTCGATTGCCAGGAG	CTCAGAGTCAGAGAGGTG
Oas2	CTGGTACAAACAGTATGAAAGG	GTAATTGACTGTCCAGAAGATG
Ptgs2	GAAGAACTTACAGGAGAGAAG	AGGAGAACAGATGGGATTTC
Slfn4	GATTCCAGGACAAGAATCTG	GACACTTGACTTGAACAGAG
Tnf	CTATGTCTCAGCCTCTTCTC	CATTTGGGAACTTCTCATCC

2.8.6 RNA sequencing

Transcriptome analysis of RNA isolated from basal and 18-hour SeV-stimulated GM-DCs from 3 standard diet mice and 3 high fat diet mice was performed by Novogene (UK) Company Limited. Firstly, the Agilent 5400 Fragment Analyzer system was used to quantify RNA in each sample and to determine sample integrity and purity for sample quality control. Followed by the preparation of the RNA library through mRNA enrichment, RNA fragmentation and reverse transcription to synthesise cDNA. Quality control of the generated library was performed using Qubit and real-time PCR. Quantified libraries were then sequenced on an Illumina sequencing platform. The raw data was obtained following sequencing and data quality control was performed through examination of the sequencing error rate, determination of the GC content distribution and filtering of the sequencing data to obtain clean reads. Cleaned data was aligned to the reference mouse (*Mus musculus*) genome (ensembl_mus_musculus_grcm38_p6_gca_000001635_8) using the alignment program HISAT2. Alignment was proceeded by quantification of gene expression. The read counts are directly proportional to the level of gene expression, gene length and sequencing depth (Liao, Smyth and Shi, 2014). Fragments Per Kilobase of transcript per Million mapped reads (FPKM) normalises read counts to determine gene expression levels by accounting for gene length and sequencing depth (Mortazavi *et al.*, 2008). Downstream analysis of gene counts and differential expression analysis was performed with the assistance of Dr. Cathriona Foley (University College Cork). The gene counts were inputted into edgeR and normalised using the Voom R package. The Log_2FoldChange, p-values and false discovery rate (FDR; to correct for multiple hypothesis testing) were calculated. Differential expression analysis was performed between two conditions, each with three biological replicates: standard diet GM-DCs untreated vs SeV-stimulated; standard diet GM-DCs untreated vs high fat diet GM-DCs untreated; and standard diet GM-DCs SeV-stimulated vs high fat diet GM-DCs SeV-stimulated. Gene Set Enrichment Analysis (GSEA) was performed on normalised gene counts and mouse hallmark gene sets from the Molecular Signatures Database (MSigDB) were used to determine differences in genes within specific cellular pathways. Differential gene statistics can also be obtained through comparison of each group to determine the number of genes upregulated or downregulated. Results from RNA sequencing data were displayed as heatmaps of differentially expressed genes. Heatmaps were produced using Morpheus (https://software.broadinstitute.org/morpheus). Differentially expressed gene IDs were

inputted into the PANTHER classification system (https://www.pantherdb.org) and gene ontology (GO) enrichment analysis was performed, and pie charts were produced of the different biological processes altered between conditions.

2.9 Enzyme-Linked Immunosorbent Assay (ELISA)

2.9.1 ELISA experimental set-up

GM-DCs were generated as described in section 2.3. Cells were resuspended in their respective media depending on the experiment, at a concentration of $1x10^6$ cells/ml. GM-DCs were seeded onto 96-well plates (Corning Inc.) at 200µl per well. Cells were rested for 2-3 hours. To determine the metabolic requirements for GM-DC function, various metabolic inhibitors were added, as per section 2.7, 2-DG (1mM), Oligomycin (1µM), Heptelidic acid (5µM), AZ67 (10µM) or CP91149 (100µM). GM-DCs were stimulated overnight (18 hours), with either LPS or Sendai virus, as per section 2.6. Following overnight stimulation, the plate was centrifuged at 400 x *g* for 5 minutes and approximately 200µl of supernatant was collected and moved to a fresh 96-well plate and frozen at -20°C.

2.9.2 ELISA protocol

Concentrations of proteins were determined using mouse DuoSet ELISA kits (R&D systems), cytokines measured were: TNFα, IL-12p70, IL-6, IL-10 or IFNβ. Reagents were reconstituted or made up according to the manufacturer's instructions prior to the assay, see Table 2.6. Day 1 involved dilution of reconstituted capture antibody in PBS to its specified working concentration. 25µl of diluted capture antibody per well was coated onto 96-well Half Area Clear Flat Bottom High Bind Microplate (Corning Inc.). The plate was sealed and slowly rocked for incubation at room temperature overnight. The following day, the plate was washed with 100µl per well of wash buffer (see Table 2.6) and the plate was inverted to dispense liquids. The wash process was repeated twice, for a total of three washes. After the final wash, the plate was inverted to remove liquids then blotted against clean paper towels to ensure thorough removal of fluids out of wells. Subsequent ELISA wash steps were performed exactly as described here. The plate was blocked by addition of 75µl of reagent diluent per well. The plate was slowly rocked and incubated for a minimum of one hour at room temperature. Blocking prevents non-specific antibody binding. During this blocking step, the standards and

samples were prepared. Samples were thawed and any required dilutions were performed in reagent diluent in 96-well plates. The reconstituted standard was diluted in reagent diluent to obtain the highest standard concentration. A serial dilution was performed of the top standard in reagent diluent to obtain 6 subsequent diluted standard concentrations and a blank of reagent diluent only was used, these dilutions and blank were used to generate a standard curve of the specific recombinant protein. Dilutions and blank are added in duplicates to the plate. Following the blocking step, the plate was washed as described and 25µl of samples (diluted or neat) were added in triplicates, and standards and blank were added to respective wells. The plate was incubated for two hours at room temperature with slow rocking. During the incubation, reconstituted detection antibody was diluted in reagent diluent to its specified working concentration. Following the two-hour incubation, the plate was washed once again and 25µl of diluted detection antibody was added to the wells for another two-hour incubation at room temperature with slow rocking. Following the final two-hour incubation, the plate was washed. Steptavidin-horse radish peroxidase (Strep-HRP) was diluted in reagent diluent to its specified working concentration and 25µl was added per well following wash, the plate was incubated at room temperature for 20 minutes, with slow rocking and protected from light. After 20 minutes, the plate was washed, 25µl of Substrate solution (see Table 2.6) was added per well. The plate was incubated at room temperature for a further 20 minutes, with slow rocking and protected from light. The reaction was stopped with the addition of 12.5µl of the stop solution (see Table 2.6) per well. The plate was gently tapped to ensure thorough mixing. A microplate reader (ThermoFisher Scientific) set to 450nm, with wavelength correction set to 595nm, was used to measure the optical density of each well on the plate. Data was exported and analysed using Microsoft Excel and Graph Pad Prism 9 or 10 software.

Table 2.6 Reagents utilised for ELISAs

Reagent	Formulation
PBS	137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 µm filtered
Wash Buffer	0.05% TWEEN® 20 (Sigma-Aldrich) in PBS, pH 7.2-7.4
Reagent Diluent	1% Bovine Serum Albumin (BSA, Sigma-Aldrich) in PBS, pH 7.2-7.4, 0.2 µm filtered
Capture Antibody	Reconstituted as per manufacturer's instructions. Diluted in PBS to specific kit concentration for assay
Standard	Reconstituted as per manufacturer's instructions. Serial dilution of standard in reagent diluent, specific to kit concentration for assay
Detection Antibody	Reconstituted as per manufacturer's instructions. Diluted in reagent diluent to specific kit concentration for assay
Steptavidin-horse radish peroxidase (Strep-HRP)	In solution in kit, diluted 1:40 with reagent diluent for assay
Substrate solution	1:1 mixture of tetramethyl benzidine (TMB) substrate A and TMB substrate B (BioLegend)
Stop solution	2 N H_2SO_4, 5.6 mls of sulphuric acid (Sigma-Aldrich) added to 94.4 mls of distilled water

2.9.3 ELISA data analysis

ELISA optical readings are obtained in a Microsoft Excel format. Using the raw data in excel, the absorbance values obtained at 595nm are subtracted from the absorbance values obtained at 450nm. The average of duplicate blank well absorbance values was obtained and the average value was subtracted from all wells to remove any additional background. These values were then transferred into Graph Pad Prism into XY tables, where a simple linear regression was performed to generate the standard curve. Followed by the intrapolation of sample absorbance values to obtain their respective concentrations of the particular protein. The average of the triplicate sample concentrations were calculated. If sample dilutions were performed, the calculated value was multiplied by the dilution factor to obtain the original protein concentration.

2.10 Immunoblotting

2.10.1 Immunoblotting experimental set-up

GM-DCs were generated as per section 2.3 and re-suspended in cRPMI at $1x10^6$ per ml. GM-DCs were plated 3mls per well in a 6-well plate. Cells were rested for 2-3 hours. Cells were stimulated for 6 hours with SeV (1:1000). At the end of the stimulation period, a protein extraction process was carried out on ice, the cells were scraped using disposable scrapers (Biotium) and the entire volume of each well was transferred to sterile labelled 15ml tubes (Sarstedt) and centrifuged at 400 x *g* for 5 minutes. Supernatants were discarded and the pellets were resuspended in 1ml of DPBS and the contents were transferred to labelled 1.5ml Eppendorf tubes. Samples were centrifuged again, and the supernatants were removed and the pellets were re-suspended in 80µl of 2X Laemmli sample buffer (see Table 2.7). Samples were then placed on a heating block at 100°C for 10 minutes. Tubes were kept at -20°C for storage until the day of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) protein separation.

2.10.2 Protein separation using SDS-PAGE

SDS-PAGE was performed using protocols from Laemmli (1970) and Bio-Rad's instructions. All reagents were made as per Table 2.7. Samples in 2X Laemmli sample buffer were boiled for 5 mins prior to loading onto SDS-PAGE gel. Bio-Rad gel rig (consisting of glass plates) was

assembled and SDS-PAGE gel was made using a 4% acrylamide stacking gel buffer above a 12% acrylamide resolving gel buffer (see Table 2.7 for ingredients of each buffer). The percentage of the resolving buffer was based on the molecular size of the target protein. Following the solidification of the gel, the plates were assembled into the running rig and the gel was immersed in 1X SDS Laemmli running buffer. 10µl of samples and 2µl of pre-loaded molecular weight ladder (10-250kDa, ThermoFisher Scientific) were loaded into the wells of the gel. Electrophoresis was performed at 80V and increased to 110V following observation of protein migration further through the resolving gel. Once electrophoresis was complete, the next step was the transfer of separated proteins to a nitrocellulose membrane for subsequent immunoblotting.

2.10.3 Immunoblotting

Following protein separation, the proteins were transferred from the gel to the nitrocellulose membrane through electrophoresis using a tank transfer system (Bio-Rad Laboratories). The transfer sandwich was assembled, constantly immersed in transfer buffer, as per Figure 2.3. 3 layers of pre-cut (7cm x 9cm) filter paper (Cytiva Life Sciences) followed by the resolving gel, then a pre-cut (7cm x 9cm) layer of nitrocellulose membrane (Amersham Biosciences) and another 3 layers of pre-cut filter paper, sandwiched between supporting grids and the electrodes. Electrical current flows from the negative electrode (cathode) to the positive electrode (anode), therefore, the sequence ensured protein migration from the gel to the membrane. Gentle rolling of the transfer sandwich occurred to ensure the absence of air bubbles. The transfer unit was closed and placed in the unit in the correct orientation. Transfer buffer was added to the unit and transfer tank, and an ice block was placed in the tank. Transfer was performed at 100V for 0.6h. Following the completion of protein transfer, the nitrocellulose membrane was removed and washed briefly with TBST. After wash, Immunoblotting Blocking Buffer (see Table 2.7) was added for 45 minutes incubation at room temperature with gentle rocking. Following blocking, the membrane was washed three times with TBST for 5 minutes each at fast rocking. Primary antibody solution was made, phospho-IκBα Rabbit mAb (1:1000; Cell Signaling Technologies) in Primary Antibody dilution buffer (see Table 2.7) and added to the membrane after washing and incubated overnight at 4°C with gentle rocking.

Following overnight incubation, the membrane was once again washed 3 times with TBST for 5 minutes each with fast rocking. Secondary antibody solution was made, Goat anti-rabbit IgG HRP-linked antibody (1:2500; Abcam) in Immunoblotting Blocking Buffer (see Table 2.7), and added to the membrane after washing and incubated for 1 hour at room temperature, with gentle rocking and protected from light. Following incubation, the membrane was washed 3 times in TBST for 5 minutes each with fast rocking. For the detection of the target proteins, Pierce™ Enhanced Chemiluminescence (ECL) Western Blotting substrate (ThermoFisher Scientific) was made (see Table 2.7) and 1-2mls was added to the membrane for 1 minute. Excess of the substrate was removed from the membrane and the membrane was transferred to transparency film pre-cut on an x-ray film cassette. The membrane was visualised in a dark room.

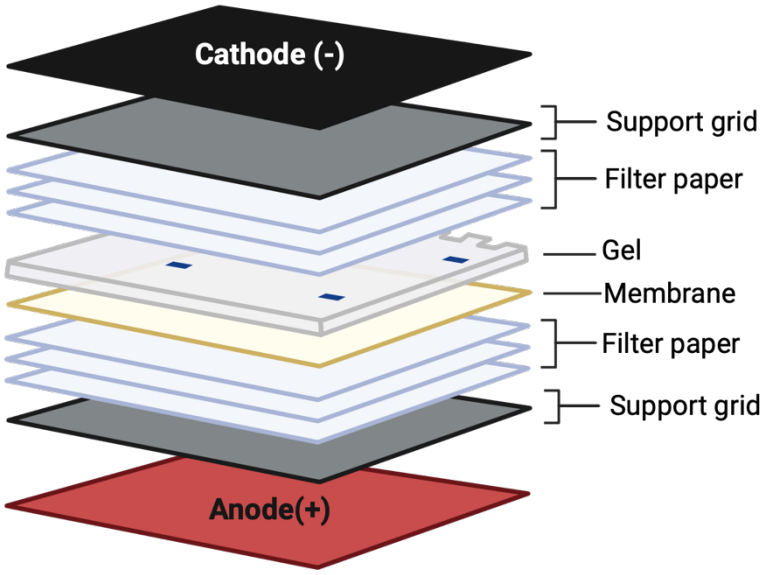


Figure 2.3 – Schematic depicting the sequence of the transfer sandwich during immunoblotting (adapted from Biorender.com)

An x-ray film (Santa Cruz Technologies) was placed on the membrane and was sealed in the cassette for 5-10 minutes (exposure time varied depending on the length of time required for band formation). The x-ray film was removed and immersed in developing solution (Scientific Laboratory Supplies) and agitated to ensure thorough immersion. Followed by a rinse step in

water then immersion in fixing solution (RG Universal) and a final rinse step in water. The protein bands could then be visualised on the x-ray film.

Following visualisation, the membrane was washed once again 3 times with TBST. β-actin was used as the loading control for the samples. Primary antibody solution was made, β-actin Mouse mAb (1:10,000; Sigma-Aldrich) in Immunoblotting Blocking Buffer, and added to the membrane after washing and incubated at room temperature for 1 hour with gentle rocking. Following primary antibody incubation, the same steps occurred as above, except the secondary antibody solution used was Goat anti-mouse IgG HRP-linked antibody (1:2500; Abcam) in Immunoblotting Blocking Buffer.

2.10.3 Densitometry analysis

Developed x-ray films were scanned as TIF files with a resolution of 600 dots per inch (DPI) on a HP Photosmart C4180 scanner and densitometry analysis was performed using ImageJ software. The band intensity was determined and the loading control protein, β-actin, was used to normalise the target protein, p-IκBα, levels.

Table 2.7 - Reagents utilised for Immunoblotting

Reagent	Ingredients
4X Laemmli Upper Tris	0.5M Tris-base (121g/mol; Sigma-Aldrich), 0.4% SDS in dH_2O, pH 6.8
4X Laemmli Lower Tris	1.5M Tris-base, 0.4% SDS in dH_2O, pH 8.8
4X Laemmli Sample Buffer	4X: 0.25M Tris-base (pH 6.8), 6% (w/v) SDS, 40% (w/v) glycerol (Sigma-Aldrich), 0.04% Bromophenol blue (Sigma-Aldrich), 20% 2-mercaptoethanol (Sigma-Aldrich) in dH_2O 2X: 1 in 2 dilution of 4X in dH_2O
Tris-Buffered Saline (TBS)	10X TBS: 100mM Tris-base, 1.5M NaCl (Sigma-Aldrich), pH 7.5, in distilled water (dH_2O)
Tris-Buffered Saline with Tween® 20 Detergent (TBST)	1X TBS: 1 in 10 dilution of 10X TBS in dH_2O and 0.1% Tween® 20
10X SDS Laemmli Running Buffer	10X: 0.25M Tris Base, 1.92M Glycine (75.07g/mol; Sigma-Aldrich), 1% SDS in dH_2O, pH 8.3 1X: 1 in dilution of 10X running buffer in dH_2O
Transfer Buffer	25mM Tris-base, 200mM Glycine, 20% (v/v) methanol (Sigma-Aldrich) in dH_2O
Immunoblotting Blocking Buffer	5% (w/v) non-fat dry milk (Sigma-Aldrich) in TBST
Ammonium Persulphate (APS) solution	20% (w/v) ammonium persulphate (Sigma-Aldrich) in dH_2O
Stacking Gel Buffer (Upper, 4% acrylamide)	2.5 ml 4X Upper Tris Buffer, 1ml 40% Acrylamide:Bis (Sigma-Aldrich), 50µl 20% APS, 10µl TEMED (Sigma-Aldrich) in 6.4ml dH_2O

Resolving Gel Buffer (Lower, 12% acrylamide)	3.125ml 4X Lower Tris Buffer, 3.75ml 40% Acrylamide:Bis, 65µl 20% APS, 7µl TEMED in 5.625ml dH_2O
Primary Antibody dilution buffer	5% BSA (Sigma-Aldrich) in 1X TBST
Pierce™ Enhanced Chemiluminescence (ECL) Western Blotting substrate (ThermoFisher Scientific)	1:1 mixture of Detection Reagent 1 (Peroxide solution) and Detection Reagent 2 (Luminol Enhancer solution)

2.11 Flow Cytometry

2.11.1 Antibody incubation

For flow cytometric analysis, the antibodies against mouse targets in Table 2.8 were used to detect GM-DCs. GM-DCs were harvested from the culture, between 0.2-2x10^6 cells depending on the experiment, and transferred to appropriate wells in a 96-well round bottom plate (Corning Inc.). Cells were washed in 200µl of flow buffer (DPBS with 1% (v/v) FBS), at 400 x *g* for 5 minutes. A master mix was made of flow buffer with the fluorochrome-conjugated surface antibodies against the mouse targets (in Table 2.8 from the GM-DC gating experiments) as per manufacturer recommendations. Supernatants were discarded and cell pellets were resuspended in 50-100µl of master mix and incubated at 4°C for 20-30 minutes, protected from light. Following incubation, cells were washed in 150-200µl of flow buffer as previously described and cells resuspended in 200µl of flow buffer and ran on the Attune Nxt Flow Cytometer (Life Technologies). Fluorescence minus one (FMO) controls were generated and control cells (no antibodies) were run with every experiment. Data analysis was performed using FlowJo (Treestar) software.

Table 2.8 -Antibodies used in flow cytometry analyses

Target	Fluorophore	Manufacturer	Clone	Experiment	Laser	Excitation laser (nm)	Emission filter (nm)
Anti-Puromycin	AlexaFluor488	Sigma-Aldrich	12D10	SCENITH	BL1	480	530/30
Anti-CD11c	FITC	BioLegend	N418	GM-DC gating	BL1	480	530/30
Anti-MHC II	VioBlue	Miltenyi	M5/114.15.2	GM-DC gating/SCENITH	VL1	405	440/50
Viability Dye	eFluor506	Invitrogen		Viability	VL2	405	512/25
Anti-CD40	PE	BioLegend	FGK45	GM-DC gating/SCENITH	YL1	516	585/16
Anti-CD86	Pe-Cyanine7	BioLegend	GL-1	GM-DC gating/SCENITH	YL4	516	780/60

2.11.2 Gating of GM-DCs

Following antibody incubation, as per section 2.11.1, GM-DCs were gated using flow cytometry using the gating sequence in Figure 2.4. Monocytes were gated using forward and side scatter. Single cells were selected, allowing exclusion of doublet cells, using FSC-area and FSC-width parameters. Using antibodies bound to fluorochromes which bind to specific cell markers (see Table 2.8), GM-DC populations were identified. Major histocompatibility (MHC) class II (MHCII), are present on antigen-presenting cells (APCs) and are required for presenting antigen. The MHCII positive cells were identified and gated, followed by the integrin CD11c positive cells, another key marker of APCs.

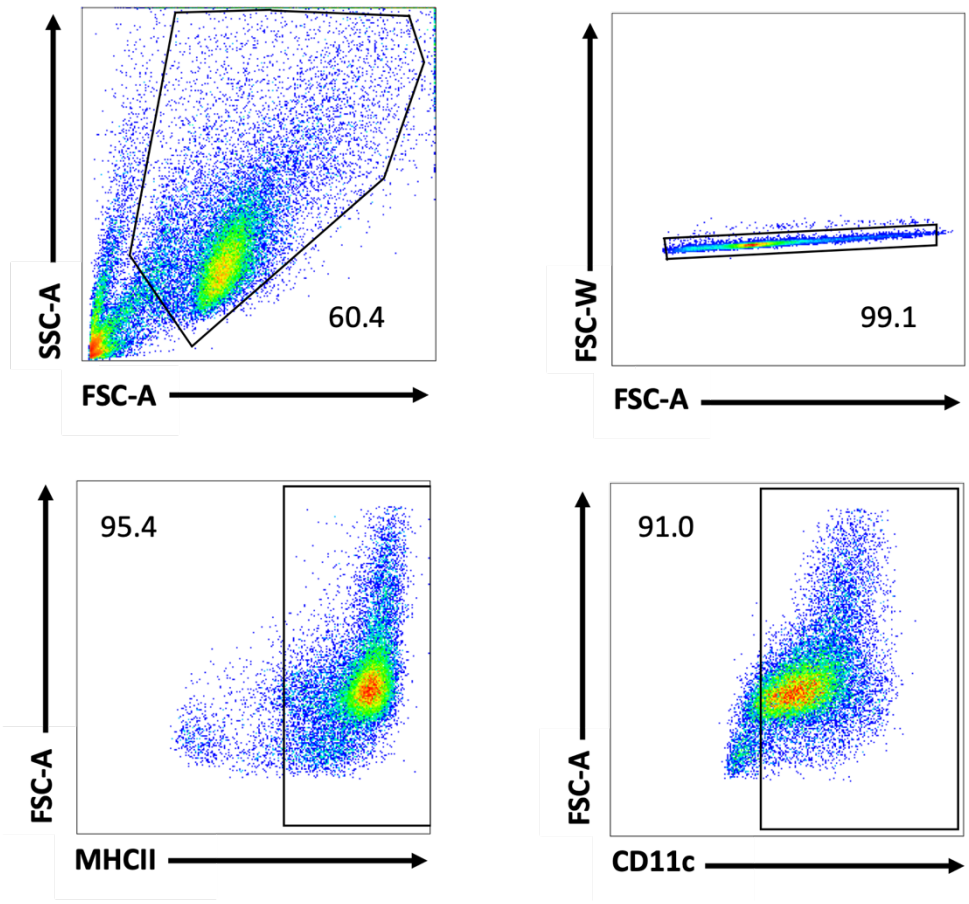


Figure 2.4 – GM-DC gating strategy using flow cytometric antibodies

2.11.3 Viability staining

Experiments where cell viability was determined, cells were stained with a viability dye eFluor™506 (eBioscience, Invitrogen). Cells ($0.2x10^6$) were harvested and moved to a new 96-well round bottom plate and centrifuged at 400 x *g* for 5 minutes to remove supernatants. The cell pellet was resuspended in 200µl flow buffer to wash, centrifuged at 400 x *g* for 5 minutes. This step was repeated for a total of two washes. Viability dye was diluted 1 in 1000 in DPBS and following the wash steps, cells were re-suspended in 100µl of the dye working solution. The plate was incubated at 4°C for 30 minutes, protected from light. Following incubation, 150µl of flow buffer was added and the cells were washed by centrifugation at 400 x *g* for 5 minutes. Cell pellets were re-suspended in 200µl of flow buffer and samples were transferred to their respective labelled 5ml FACS tubes (BD Falcon). 100µl of flow buffer was added to each tube to ensure a final volume of 300µl. Samples were then run on the flow cytometer. A live/dead control was made by killing half of the cells from one control sample by heating the cells at 60°C for 5 minutes then placed immediately on ice. The dead cells were combined with the other half of the live cells. An unstained control and this live/dead control were also run with each experiment.

2.11.4 SCENITH

Single Cell ENergetIc metabolism by profilIng Translation inhibition (SCENITH) is an assay which allows the global metabolic dependency and capacities of cells to be determined through the addition of metabolic inhibitors and the use of a protein translation inhibitor, puromycin (Argüello *et al.*, 2020). Approximately half of total cellular energy is utilised for protein synthesis, therefore, levels of protein synthesis can be used as a surrogate for cellular metabolic activity. Protein translation levels is determined through the levels of puromycin incorporated in the cells.

GM-DCs were seeded ($1x10^6$ cells/ml; 2mls/well) in 24-well plates (Corning Inc.), rested for 2-3 hours and stimulated with SeV (1:1000) or LPS (100ng/ml) for 18 hours. Following stimulation, cells were washed with fresh cRPMI (centrifuged at 400 x *g* for 5 minutes) and each sample was divided amongst 5 wells in a 96-well plate for the SCENITH assay: control, 2-DG, Oligomycin, 2-DG & Oligomycin, and no puromycin wells. Cells were resuspended in 173µl

of warm cRPMI. Inhibitors were added in the appropriate wells: 2-DG (100mM; 22µl/well) and/or oligomycin (1µM; 2µl/well). Final concentration of inhibitors within the wells was based on the final well volume, including puromycin addition. Inhibitors were incubated for 15 minutes at 37°C. Following the incubation, puromycin (final conc. 11µM; Sigma-Aldrich) was added for 25 minutes at 37°C to all wells except no puromycin wells. 100µl of ice-cold DPBS was added to stop puromycin incorporation and cells were centrifuged at 400 x *g* for 5 minutes. Supernatants were discarded and cells were incubated with antibodies, as per section 2.11.1. Following incubation, cells were fixed (1X; 100µl/well) using the True-Nuclear Transcription Factor Buffer set (BioLegend) for 30 minutes at room temperature, protected from light, following manufacturer's instructions. Following fixing, cells were washed and re-suspended in flow buffer and incubated at 4°C overnight. The following day, the plate was centrifuged at 400 x *g* for 5 minutes and cells re-suspended in perm solution (1 in 10 of molecular grade water; True-Nuclear Transcription Factor Buffer set) (100µl/well) and centrifuged at 400 x *g* for 5 minutes as a perm wash. Anti-puromycin antibody (see Table 2.8) was diluted 1 in 200 in perm solution and added 25µl/well, cells were incubated at 4°C for 1 hour, protected from light. Anti-puromycin was not added to the anti-puromycin FMO or control (no antibody) wells. Following incubation, 100µl of perm solution was added per well to wash, the plate was centrifuged at 400 x *g* for 5 mins. Supernatants were discarded and samples and controls were re-suspended in 200µl of flow buffer. Flow cytometry was performed using the Attune Nxt Flow Cytometer, and results analysed using FlowJo software (Treestar).

For SCENITH analysis, cells were gated and the geometric mean fluorescence intensity (MFI) of the anti-puromycin antibody was obtained for each sample. For all samples, the anti-puromycin MFI value of the respective sample's no puromycin well was subtracted from the anti-puromycin MFI value of each of the other treatments of the same sample. The resulting MFI's for each sample's treatment was inputted into the formulas in Table 2.9 to obtain the metabolic dependencies and capacities of the GM-DCs.

Table 2.9 - Formulas to calculate the metabolic dependencies and capacities of the cells following the SCENITH assay

Metabolic Monitoring	Formula
Glucose Dependency (%)	100x((Control Puro MFI-2DG only Puro MFI)/(Control Puro MFI-2DG&Oligo Puro MFI))
Fatty acid oxidation (FAO) and amino acids oxidation (AAO) capacity (%)	100-Glucose dependency
Mitochondrial Dependency (%)	100x((Control Puro MFI-Oligo only Puro MFI)/(Control Puro MFI-2DG&Oligo Puro MFI))
Glycolytic capacity (%)	100-Mitochondrial dependency

2.12 Statistical analysis

Graph Pad Prism 10 software was used for data visualisation and statistical analysis. Data is expressed as mean ± standard error mean (SEM). Outliers were excluded based on the Robust regression and Outlier removal (ROUT) method. Normality tests were performed on the data to determine Gaussian distribution, Shapiro-Wilk test was used. For comparisons of two paired groups, with normal distribution, a paired student's t-test was used. Two paired groups, where one or both sets of data was not normally distributed, Wilcoxon test was used. For comparisons of two unpaired groups, with normal distribution, an unpaired student's t-test was used. Analysis of three or more groups was determined using Analysis of Variance (ANOVA). Assuming normal distribution, Tukey's multiple comparisons test was performed to compare means between groups. In the event of one or more sets of data being not normally distributed, Kruskal-Wallis test was applied along with Dunn's test for multiple comparisons. For comparison of standard diet and high fat diet, where multiple treatments occurred for both groups, two-way ANOVA was performed with Tukey's multiple comparisons test. Statistical significance is denoted as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$. No significance is $p > 0.05$.

2.13 Figures

Graph Pad Prism 10 software was used for visualisation of all data. All other figures were created with BioRender.com.

Chapter Three

Results

Characterisation of murine dendritic cell functional responses

3.1 Introduction

3.1.1 Dendritic cells

Dendritic cells are innate immune sentinels which circulate the body, particularly mucosal tissues, where pathogen encounter is most likely (Banchereau *et al.*, 2003; Steinman, 2003). DCs circulate in an immature state primed for antigen capture (Banchereau *et al.*, 2003). Upon pathogen encounter and uptake, DCs undergo maturation to perform their main function, as a professional antigen presenting cell (Banchereau *et al.*, 2003). DCs bridge the innate and adaptive immune response, as they present antigen to T cells to activate the adaptive arm of the immune response (Banchereau *et al.*, 2003). Immature DCs are highly phagocytic and express low levels of the antigen presenting molecule MHC II and co-stimulatory molecules (Albert *et al.*, 1998; Ackerman and Cresswell, 2003; Kim and Kim, 2019). Upon antigen exposure, DCs endocytose, process then present antigen on antigen-MHC complexes and undergo maturation where their phagocytic machinery is downregulated and co-stimulatory molecules (Kim and Kim, 2019), cytokines and chemokines are expressed and upregulated instead (Nagorsen, Marincola and Panelli, 2004).

Interaction of chemokines and their respective chemokine receptors are important in coordinating the migration of cells around the body (Hughes and Nibbs, 2018). Immature DCs express high CCR6 (Greaves *et al.*, 1997; Yang *et al.*, 1999) and low CCR7 (Wu, Hu and Kaiser, 2011), the CCR6 ligand is Macrophage Inflammatory Protein (MIP)-3α (also known as CCL20) which is expressed in tissues (Fleming *et al.*, 2003). MIP-3α is a strong chemoattractant for DCs to the non-lymphoid tissues (Dieu *et al.*, 1998; Fleming *et al.*, 2003). When immature DCs encounter a pathogen, pathogen products, or cytokines such as TNFα, any of these events can cause DC maturation (Dieu *et al.*, 1998), causing DCs to migrate from non-lymphoid tissues or organs and into T cell rich lymphoid organs through the afferent lymph (Brocker, 1997; Banchereau and Steinman, 1998). One of the key components of DC maturation, is the downregulation or desensitisation of CCR6 and the upregulation of a different chemokine receptor, CCR7 (Yanagihara *et al.*, 1998; Wu, Hu and Kaiser, 2011). CCR7 ligands include 6Ckine (also known as CCL21) and MIP-3β (also known as CCL19), which attract the mature DCs to the lymphatic vessels and the draining lymph nodes respectively (Alt, Laschinger and Engelhardt, 2002; Scandella *et al.*, 2004). These two chemokines act on both mature DCs and

T cells (Kaiser *et al.*, 2005), ensuring the Ag-presenting DCs meet the Ag-specific T cells. T cell encounters can also occur at the site of injury or infection (Broz *et al.*, 2014; Krummel, Bartumeus and Gérard, 2016). DC-T cell interactions further DC maturation through receptor-ligand stimulation, such as CD40-CD40L, 41BBL-41BB and OX40L-OX40, between the cells respectively (Futagawa *et al.*, 2002; O'Sullivan and Thomas, 2002; Dannull *et al.*, 2005).

Several signals are required for effective T cell activation by DCs. DC express both MHC class I and MHC class II molecules which form the antigen-MHC complexes and interact with TCRs on $CD8^+$ cytotoxic T lymphocytes or $CD4^+$ T helper cells respectively (Banchereau and Steinman, 1998). The presentation of the antigen-MHC complex on DCs to TCRs is signal 1 (Alberts *et al.*, 2002). Triggering of PRRs, by conserved structures on pathogens, results in a series of signalling cascades, which culminates in the activation and translocation of transcription factors that upregulate the expression of genes important for DC function, such as co-stimulatory molecules and cytokines (Akira, Uematsu and Takeuchi, 2006). Signal 1 is antigen-specific based on the TCR. Signal 2 is non-specific, and co-stimulatory molecules on the surface of DCs such as CD80/CD86 interact with CD28 on the surface of T cells (Reis E Sousa, 2006). Signal 1 and Signal 2 are required for T cell activation and survival (Reis E Sousa, 2006). Signal 3 is the production and secretion of cytokines by DCs, which can induce T cell proliferation and polarisation into specific effector T cells (Reis E Sousa, 2006). The cytokines released determines the T cell subset polarisation. Key characteristics of DCs are their ability to induce the differentiation of naïve T cells into effector and memory subsets (Sallusto and Lanzavecchia, 2002), which are important for both initial adaptive immune responses and rapid responses upon subsequent infection with the same pathogen or following immunisation.

3.1.2 DC generation & subtypes

DC generation is relatively conserved between humans and mice and results from haematopoiesis in the bone marrow, albeit certain differences in the pathway (Parekh and Crooks, 2013). Briefly, haematopoiesis is the differentiation of HSCs into multipotent and unipotent progenitors. DCs arise from a CDP which produces pre-cDCs and pre-pDCs (Naik *et al.*, 2007; Onai *et al.*, 2007). Pre-cDCs exit the bone marrow and enter circulation where they mature in circulation or tissues (Liu *et al.*, 2009). Similiarly, pDCs differentiate from precursors,

referred to as pre-pDCs (Grouard *et al.*, 1997; McKenna, Beignon and Bhardwaj, 2005). The main subsets of DCs are the conventional DCs and plasmacytoid DCs. There are two main cDCs: cDC1 and cDC2. cDC1 are referred to as $CD141^+$ cDC1 (Dzionek *et al.*, 2000; Ziegler-Heitbrock *et al.*, 2010) and are important during immunity against intracellular pathogens and tumours (Imperato *et al.*, 2020; Lee *et al.*, 2021). cDC2 are referred to as $CD1c^+$ cDC2 (Dzionek *et al.*, 2000; Ziegler-Heitbrock *et al.*, 2010) and are important during immunity against extracellular pathogens (Gao *et al.*, 2013; Deckers *et al.*, 2017). Murine and human DCs share similarities but express varying markers and are localised to different tissues (Shortman and Liu, 2002). $CD141^+$ cDC1 resemble the $CD8\alpha^+$ DCs found in murine lymphoid tissues, both cells are important for cross-presentation (Bachem *et al.*, 2010). $CD1c^+$ cDC2 resemble the $CD11b^+$ DCs found in murine lymphoid tissues (Robbins *et al.*, 2008; Schlitzer *et al.*, 2013). DCs can coordinate both innate and adaptive immune responses. Plasmacytoid DCs is one of the main producers of type I interferons, especially IFNα, upon viral infection (Cervantes-Barragan *et al.*, 2012). cDCs are also able to produce type I interferons but not to the same extent as pDCs (Hervas-Stubbs *et al.*, 2014). The production of type I interferons and other cytokines, such as IL-12 and IL-15, by DCs and their subsequent activation of T helper cells, can activate NK cells and macrophages, promoting their effector function (Charles A Janeway *et al.*, 2001; Mattei *et al.*, 2001a; Hervas-Stubbs *et al.*, 2014; Huntington, 2014).

Due to difficulties in studying DCs in humans, e.g., limited numbers in circulation/tissues (Orsini *et al.*, 2012), DC function and metabolism have been studied extensively *in vitro* by inducing their differentiation from murine bone marrow precursors using growth factors (Krawczyk *et al.*, 2010; Everts *et al.*, 2012, 2014). Bone marrow-derived dendritic cells are widely used. Bone marrow precursors can be cultured *in vitro* with FLT3L, referred to as FL-DCs, and give rise to cDC1, cDC2 and pDCs (Gilliet *et al.*, 2002; Onai *et al.*, 2007; Zhang *et al.*, 2018). Bone marrow precursors cultured with GM-CSF, referred to as GM-DCs, have an inflammatory phenotype similar to *in vivo* Mo-DCs (Naik *et al.*, 2006; Zhang *et al.*, 2018). Mo-DCs were a group of cells which were observed to differentiate from monocytes upon infection or during inflammation *in vivo* (Naik *et al.*, 2006). These cells display an inflammatory phenotype. Zhang *et al.* (2018) observed that GM-DCs were more phagocytic and survived for longer *in vitro* compared to FL-DCs. GM-DCs activated with LPS favoured Th2 and Th17 polarisation, depicted by an increased production of IL-4 and IL-17A when co-cultured with

$CD4^+$ T cells, compared to LPS-activated FL-DCs (Zhang *et al.*, 2018). Whereas the activated FL-DCs favoured Th1 polarisation when co-cultured with $CD4^+$ T cells, based on the greater production of IFN-γ by the T cells (Zhang *et al.*, 2018). The differences in T cell polarisation were due to differences in cytokines released by either the GM-DCs or FL-DCs in the presence of LPS. LPS-treated FL-DCs produced significantly greater Th1 polarising cytokines, IL-12 p35 and p40 subunits (Zhang *et al.*, 2018). Whereas LPS-treated GM-DCs produced greater IL-1β and IL-6, compared to FL-DCs, these cytokines are essential for Th17 polarisation (Zhang *et al.*, 2018).

3.1.3 Pathogen recognition by DCs

Dendritic cells are innate cells which recognise antigen non-specifically (Banchereau and Steinman, 1998). Pathogen recognition occurs via PRRs which recognise conserved PAMPs (Medzhitov and Janeway, 1997). There are four families of PRRs: TLRs, Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), Nucleotide oligomerization domain (NOD)-like receptors (NLRs) and C-type lectin receptors (CLRs) (Walsh *et al.*, 2013). These PAMPs would be non-self, but PRRs can also detect DAMPs, which can be inflammatory markers released from damaged cells, first proposed by Matzinger (2003). PRRs are located on the cell surface and/or in the cytosol (Medzhitov and Janeway, 1997). Cells can express a myriad of PRRs or some cells express only specific PRRs, for example, pDCs are important for viral immunity and express only intracellular TLR7 and TLR9 which recognise viral RNA and DNA respectively (Kadowaki *et al.*, 2001). In humans 10 TLRs and in mice 12 TLRs have been identified (Bryant and Monie, 2012). TLRs which recognise extracellular PAMPs are expressed on the cell surface, e.g., TLR5 detects flagellin, a key component of bacterial flagella which allows their motility (Hayashi *et al.*, 2001). TLRs which recognise intracellular components of a pathogen, i.e., nucleic acids, are expressed on organelle membranes in the cytosol, e.g. TLR3 detects double-stranded RNA (Alexopoulou *et al.*, 2001). TLR engagement results in downstream signalling processes that ultimately results in transcription factor activation (Janeway and Medzhitov, 2002), particularly, nuclear factor-κB (NF-κB) and the IRFs (Bender *et al.*, 2020). These transcription factors translocate to the nucleus and induce cytokine, chemokine, and co-stimulatory molecule gene expression and protein production (Krawczyk *et al.*, 2010; Bender *et al.*, 2020). CLRs are located on the cell membrane and detect glycans or polysaccharides in fungal or some bacterial cell wall (Silva-Martín *et al.*, 2014; Drickamer and Taylor, 2015). NLRs are

located in the cytosol and detects PAMPS from bacteria, viruses or fungi (Franchi *et al.*, 2009; Zhang *et al.*, 2014). NLRs can detect damage signals, e.g. ATP or cholesterol crystals (Rajaṁaki *et al.*, 2010; Ayna *et al.*, 2012). Additionally, NLRs can form complexes called inflammasomes which results in IL-1β and IL-18 production (Schroder and Tschopp, 2010). RLRs are located in the cytosol and detect viral RNA (Yoneyama *et al.*, 2004). RLRs function similarly to TLRs, where upon ligand binding, signalling cascades result in transcription factor (particularly IRFs) activation and subsequent expression of interferons and interferon-stimulated genes (ISGs), vital for the antiviral immune responses (Yoneyama *et al.*, 1996, 2004). Different DC subtypes express different PRRs (Kadowaki *et al.*, 2001). Our cells of interest, GM-DCs, were stimulated with two different stimuli, LPS and SeV, which activate different PRRs within GM-DCs and these pathways will be highlighted next.

3.1.4 LPS stimulation

Lipopolysaccharide is an exogenous antigen found in the outer membrane of Gram-negative bacteria (Rietschel *et al.*, 1994). LPS consists of 3 components: the O-specific chain (referring to an oligosaccharide matrix), the core region (another polysaccharide region) and lipid A (phosphorylated glucosamines with fatty acids attached) (Raetz and Whitfield, 2002). Lipid A is detected and elicits an immune response (Harberts *et al.*, 2022). LPS is extremely toxic within the blood and can result in sepsis (Janosevic *et al.*, 2021). It has been extensively used in *in vitro* cell cultures (Krawczyk *et al.*, 2010) and *in vivo* (Tough, Sun and Sprent, 1997) to study inflammation. LPS-binding proteins (LBP) are synthesised in the liver and released into the bloodstream (Schumann *et al.*, 1996), where they can recognise LPS on the outer membrane of bacteria or free LPS (Pålsson-McDermott and O'Neill, 2004). LPS bound by LBP can bind to CD14 (Wright *et al.*, 1990), a glycoprotein which either exists membrane bound on the surface of myeloid cells or in a soluble form (Pålsson-McDermott and O'Neill, 2004). This complex is subsequently recognised by TLR4 (Ryu *et al.*, 2017), expressed on the surface of mostly myeloid immune cells, including DCs, and certain non-immune cells (Vaure and Liu, 2014). Another glycoprotein, MD-2, is required for LPS signalling in association with TLR4 (Ryu *et al.*, 2017). Binding of LPS results in dimerization of TLR4/MD-2 (Park *et al.*, 2009). The intracellular domain of the complex consists of adaptor proteins which are consequently activated and result in downstream transcription factor activation, particularly NF-κB, and inflammatory gene expression (Wan *et al.*, 2016). CD14-dependent TLR4 internalisation can

occur subsequently, and this process results in activation of the IRF3 transcription factor which induces type I IFN responses (Rajaiah *et al.*, 2015). The integrin CD11b was shown to be important for DC responses to LPS. CD11b promoted endocytosis of LPS-bound TLR4 and its subsequent signalling (Ling *et al.*, 2014). Triggering of TLR4 results in the activation of NF-κB (Küper, Beck and Neuhofer, 2012) and downstream transcription and translation of pro-inflammatory genes (illustrated in Figure 3.1), such as pro-inflammatory cytokines TNFα and IL-1 (Eltom *et al.*, 2014; Lin *et al.*, 2015), which function to stimulate and recruit immune cells (Scheurich *et al.*, 1987; Rider *et al.*, 2011). Cytoplasmic LPS can activate murine caspase-11 (Hagar *et al.*, 2013) or the human caspase-4 and caspase-5 (Viganò *et al.*, 2015). Activation of caspase-11 resulted in the non-canonical inflammasome activation, which induced pyroptosis (Hagar *et al.*, 2013). Caspase-11 activation is associated with septic shock in mice (Hagar *et al.*, 2013). Due to the capacity of LPS to stimulate an inflammatory response, it was used as a stimulant in this thesis.

3.1.5 SeV stimulation

Sendai virus, also known as murine parainfluenza 1, of the Paramyxoviridae family, is a negative sense single-stranded RNA virus (Brabb *et al.*, 2012). Similar to other RNA viruses, SeV can activate the PRR retinoic acid-inducible gene I (RIG-I) within cells, leading to the downstream activation of type I interferons, to induce an antiviral state in an autocrine and paracrine manner (Kato *et al.*, 2006; Baum, Sachidanandam and García-Sastre, 2010). RIG-I is a member of the RLR family of PRRs (Loo and Gale, 2011). However, this is strain-dependent, as some strains activate the immune response, and other SeV strains can be immunosuppressive. Notably, in terms of IFN-β production (López *et al.*, 2006). The Cantell strain of SeV can induce high production of IFN-β (López *et al.*, 2006). Interferons block, partially or completely, infection in infected cells or can act on neighbouring uninfected cells to induce a protective state to hinder their infection (Liu *et al.*, 2012). Additionally, type I interferons modulate the responses of innate and adaptive immune cells, for example, facilitating NK cell activation (Mattei *et al.*, 2001b; Huntington, 2014) or regulating T cell responses (Fuertes *et al.*, 2011). Binding of RNA to RIG-I, leads to its polyubiquitination and oligomerisation, and results in activation of mitochondrial antiviral signalling protein (MAVS) on the membrane of the mitochondria (Seth *et al.*, 2005; Hou *et al.*, 2011). MAVS dimerization leads to its association with various adaptor proteins which consequently activate kinases that

phosphorylate IRF3 and 7 (Seth *et al.*, 2005; Cheng *et al.*, 2019). IRF3 and IRF7 are transcription factors which homodimerize (heterodimers have also been observed) (Lin, Mamane and Hiscott, 1999, 2000) and translocate to the nucleus to induce the expression of interferon genes (Wathelet *et al.*, 1998; Crotta *et al.*, 2013), depicted in Figure 3.1.

SeV has been studied as a potential vaccine adjuvant as a RIG-I agonist (Martínez-Gil *et al.*, 2013) or as a viral vector (Russell *et al.*, 2017) for several reasons. SeV is non-pathogenic to humans (Matveeva *et al.*, 2015). SeV replicates within the cytoplasm and its genome does not fuse with that of the host, nor does it result in genetic recombination (Iida and Inoue, 2013). SeV also replicates with its own RNA polymerase (Tapparel *et al.*, 1997; Holmes and Moyer, 2002). SeV is a potent activator of dendritic cells (DCs) (Zheng *et al.*, 2021) and can induce DC maturation (Yount *et al.*, 2006). SeV can be used in tumour-targeting strategies, e.g., SeV particles in conjunction with *IL2* promoted innate and adaptive immune responses in a murine model of angiosarcoma (Takehara *et al.*, 2013). Due to the capacity of SeV to stimulate an antiviral response, it was used as a stimulant in these studies.

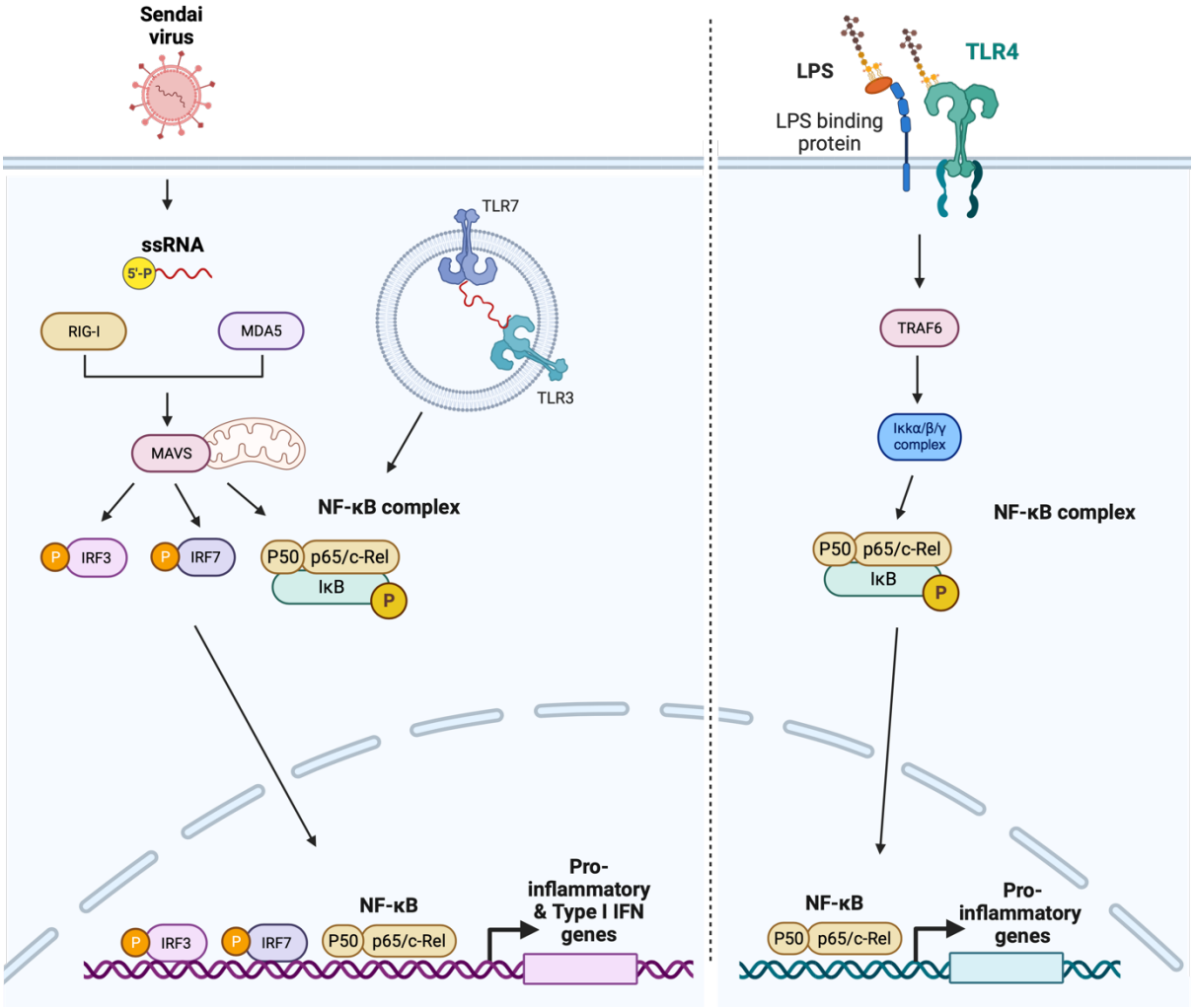


Figure 3.1. – Simplified overview of Sendai virus (SeV) and LPS activation and signalling within immune cells SeV is a single-stranded RNA (ssRNA) virus which can activate RIG-I and MDA5, along with certain TLRs. These result in a signalling cascade which induces genes involved in an inflammatory and antiviral response. LPS activates TLR4, in association with adaptor molecules, which results in a signalling cascade which induces an inflammatory response (Adapted from Medzhitov and Janeway (2000); Liu, Olagnier and Lin (2017); Firmal, Shah and Chattopadhyay (2020); Singh, Koury and Kaul (2021)).

We wanted to study the metabolism and function of GM-CSF stimulated bone marrow-derived dendritic cells (GM-DCs), as well as the impact of obesity on GM-DC function. However, prior to the elucidation of these factors, we stimulated GM-DCs from wild type C57BL6/J mice with LPS or SeV to ensure the cells performed as it has been previously described in the literature.

3.2 Specific aims of Chapter 3

The specific aim of this chapter was to characterise GM-DC responses following stimulation from mice on the standard diet, and therefore to:

1. Characterise transcriptional changes in SeV-infected DCs by RNA sequencing.
2. Define the functional characteristics of GM-DCs following LPS stimulation.
3. Define the functional characteristics of GM-DCs following SeV stimulation.

3.3 Results

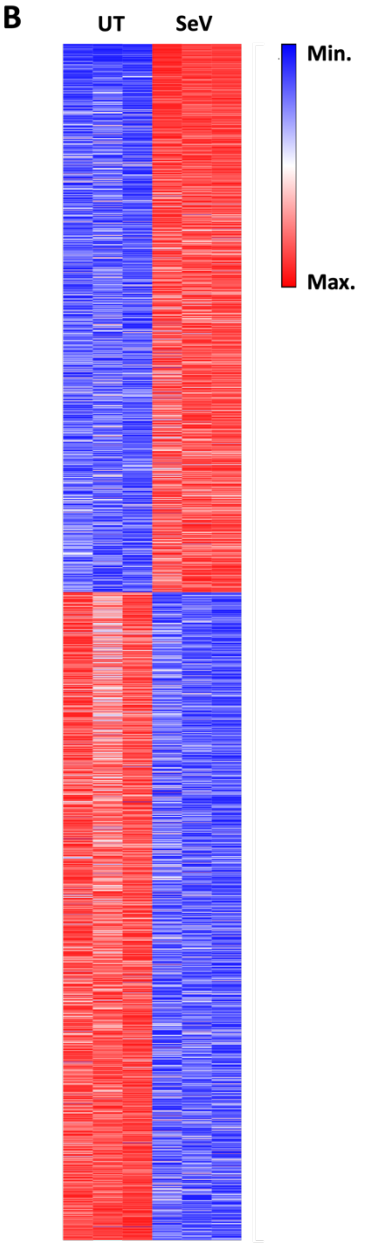
3.3.1 Differential gene expression following 18-hour SeV stimulation

First, GM-DCs were stimulated with SeV for 18 hours, after which RNA was isolated and RNA sequencing was performed (see section 2.8). Viral stimulation resulted in transcriptional remodelling of GM-DCs, as displayed by changes in their RNA expression (Figure 3.2). The total number of genes differentially expressed following 18-hour SeV stimulation was calculated (Figure 3.2A), where 1293 genes were increased in expression and 1528 genes were decreased in SeV-stimulated GM-DCs compared to untreated GM-DCs. All the 2821 significantly differentially expressed genes were displayed as a heatmap (Figure 3.2B). Next, we performed pathway analysis of all differentially expressed genes, and highlight the key processes the genes are involved in (Figure 3.2C). In summary, the major pathways altered by SeV stimulation include cellular processes, metabolic processes, regulation and an evident pathway, response to stimulus.

A

	Untreated vs SeV stimulation
Total differentially expressed genes	2821
Increased	1293
Decreased	1528

B



C

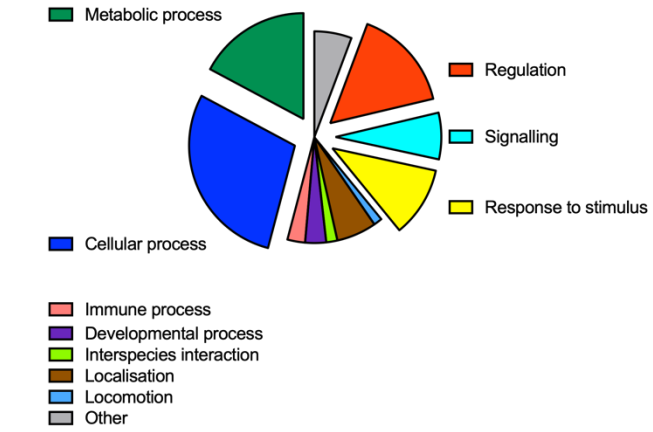


Figure 3.2 RNA sequencing revealed differential gene expression following 18-hour SeV stimulation of GM-DCs (A) Table outlining the total number of genes differentially expressed following an 18-hour SeV stimulation. (B) Heat map of normalised gene counts of all significantly different genes with FDR<0.1 (n=3). (C) Pathway analysis of biological processes affected by 18-hour SeV stimulation.

3.3.2 RNA sequencing revealed alterations in inflammatory and signalling pathways following 18-hour SeV stimulation of GM-DCs

Gene Set Expression analysis (GSEA) was performed, where gene counts (untreated and SeV stimulated GM-DCs) were compared to hallmark gene sets, available on the Molecular Signatures Database (MSigDB) (Figure 3.3). First, we show an enrichment in genes involved in the inflammatory responses in SeV stimulated GM-DCs compared to untreated controls (Figure 3.3A). These included genes encoding cytokines (e.g. *Il6*, *Il15*) or chemokines (e.g. *Cxcl9*, *Cxcl10*). We also noted decreased expression of *Myc* following 18-hour SeV stimulation (Figure 3.3A).

An enrichment of genes involved in the interferon alpha (IFNA) response was observed (Figure 3.3B). SeV stimulation increased expression of genes encoding transcription factors (e.g. *Stat2*, *Irf7*) and ISGs (e.g. *Mx2*, *Isg15*). ISGs are part of the anti-viral response of cells and increased expression is common following viral activation of cells (Liu *et al.*, 2012).

Genes involved in the IL6-JAK-STAT signalling pathway were enriched (Figure 3.3C). Here, SeV stimulation increased expression of key transcription factors (e.g. *Stat2*), cytokines (e.g. *Il6*) and chemokines (e.g. *Cxcl9*, *Cxcl10*, *Cxcl11*).

An enrichment of genes involved in the TNFA signalling pathway via the transcription factor NFKB was also observed (Figure 3.3D). These different pathways constitute of overlapping genes, therefore, there was increased expression of similar genes mentioned in the previous pathways. There was an increase of *Prostaglandin-endoperoxide synthase 2* (*Ptgs2)*, also known as *Cyclooxygenase 2* (*Cox2)*, involved in prostaglandin production, a normal inflammatory response following cell activation (Díaz-Muñoz *et al.,* 2010).

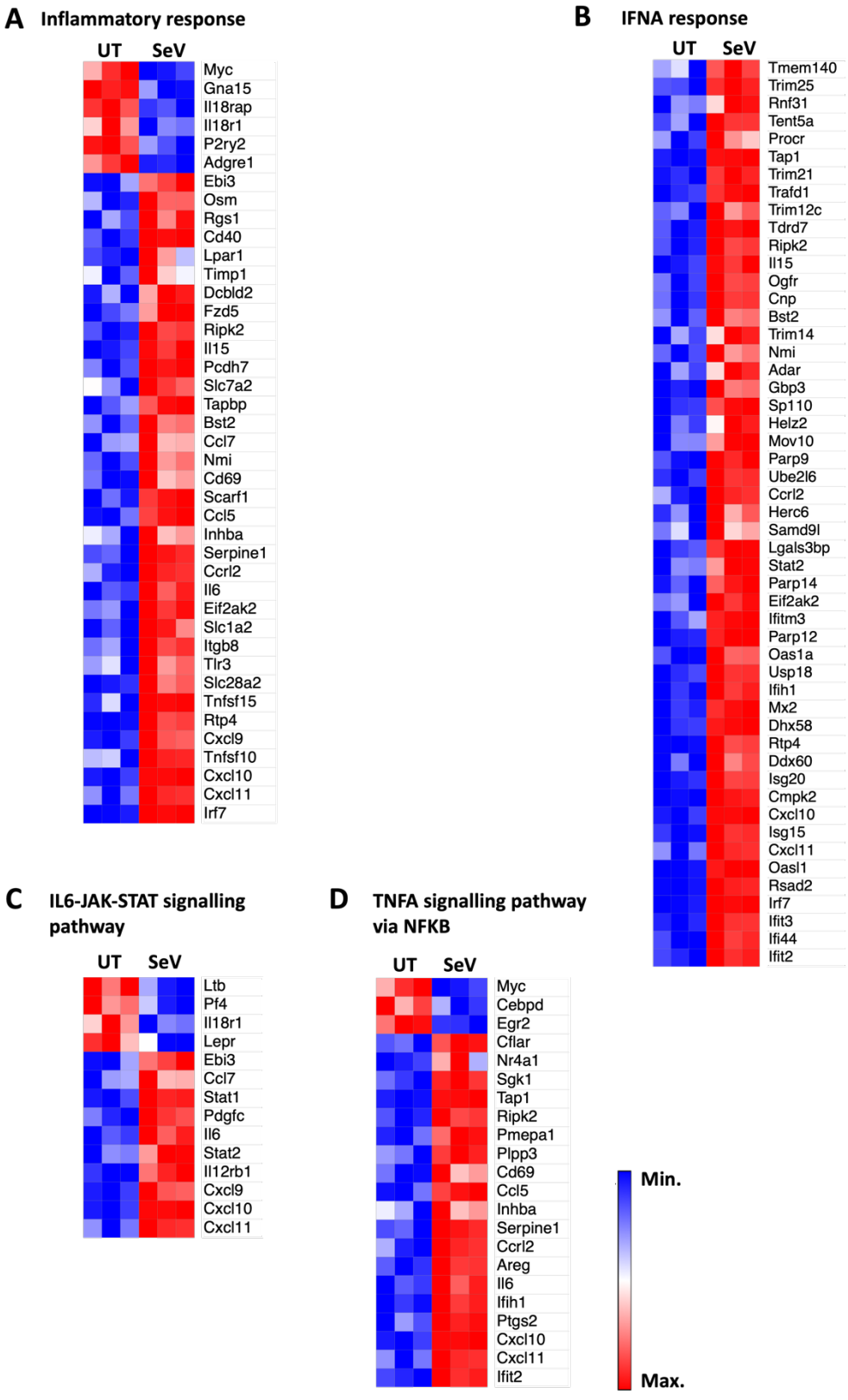


Figure 3.3 Inflammatory and signalling pathways affected following 18-hour SeV stimulation Heat map of significantly different genes involved in (A) inflammatory response, (B) interferon alpha response, (C) IL6 JAK STAT signalling pathway, (D) TNFA signalling via NFKB. All gene counts are normalised with a FDR<0.1 and $\log_2$fold change either ≥1.5 or ≤-1.5 (n=3).

3.3.3 RNA sequencing revealed alterations in metabolic pathways following 18-hour SeV stimulation of GM-DCs

Similar to section 3.3.2, gene counts (untreated and SeV stimulated GM-DCs) were compared to hallmark gene sets, available on the Molecular Signatures Database (MSigDB) (Figure 3.4). First, we show differentially expressed genes involved in fatty acid metabolism in SeV stimulated GM-DCs compared to untreated controls (Figure 3.4A). FA metabolism is important for normal DC function during a quiescent state and early during activation (Everts *et al.*, 2014). FA oxidation provides fatty acyl-CoA which enters the mitochondria and the TCA cycle to subsequently provide energy through OXPHOS (Lim *et al.*, 2018). FA synthesis provides substrates to enlarge the ER or Golgi apparatus to support increased synthesis and transport of proteins which occurs after DC activation (Rehman *et al.*, 2013; Everts *et al.*, 2014). Similar to cholesterol synthesis, an important component of biological membranes (Lange, 1991). We also show differentially expressed genes involved in cholesterol homeostasis (Figure 3.4E). A key gene in both pathways, is *Fasn,* which is decreased in expression in 18-hour SeV stimulated GM-DCs compared to untreated (Figures 3.4A & E).

mTORC1 is a master regulator of cellular metabolism. We demonstrate differentially expressed genes involved in mTORC1 signalling (Figure 3.4B). MYC is another metabolic regulator which converges with mTORC1 in different pathways, such as protein synthesis (Pourdehnad *et al.,* 2013). We observed decreased MYC target genes (Figure 3.4C). 18-hour SeV stimulation decreased the expression of *Myc* which would presumably in turn decrease levels of the *Myc* protein, therefore, affecting its target genes.

Genes involved in OXPHOS were decreased (Figure 3.4D). Key genes decreased in expression following SeV stimulation is *Isocitrate dehydrogenase (Idh) 2* and *Idh3a*, the encoded enzymes are involved in the third reaction of the TCA cycle, the oxidation of isocitrate to α-ketoglutarate.

Genes involved in glycolysis were differentially expressed (Figure 3.4F). A key gene decreased in expression is *lactate dehydrogenase (Ldh) b*. *Ldh* gene encodes enzymes which convert pyruvate to lactate, or vice versa. The *Ldhb* encoded enzyme preferentially converts lactate to pyruvate (Shibata *et al.*, 2021). Interestingly, *Ldhb* is decreased in expression following SeV

stimulation. As GM-DC metabolism changes depending on the time following activation, the differences observed in metabolic pathways are potentially timepoint-dependent and at earlier or later timepoints, other genes or pathways may be altered.

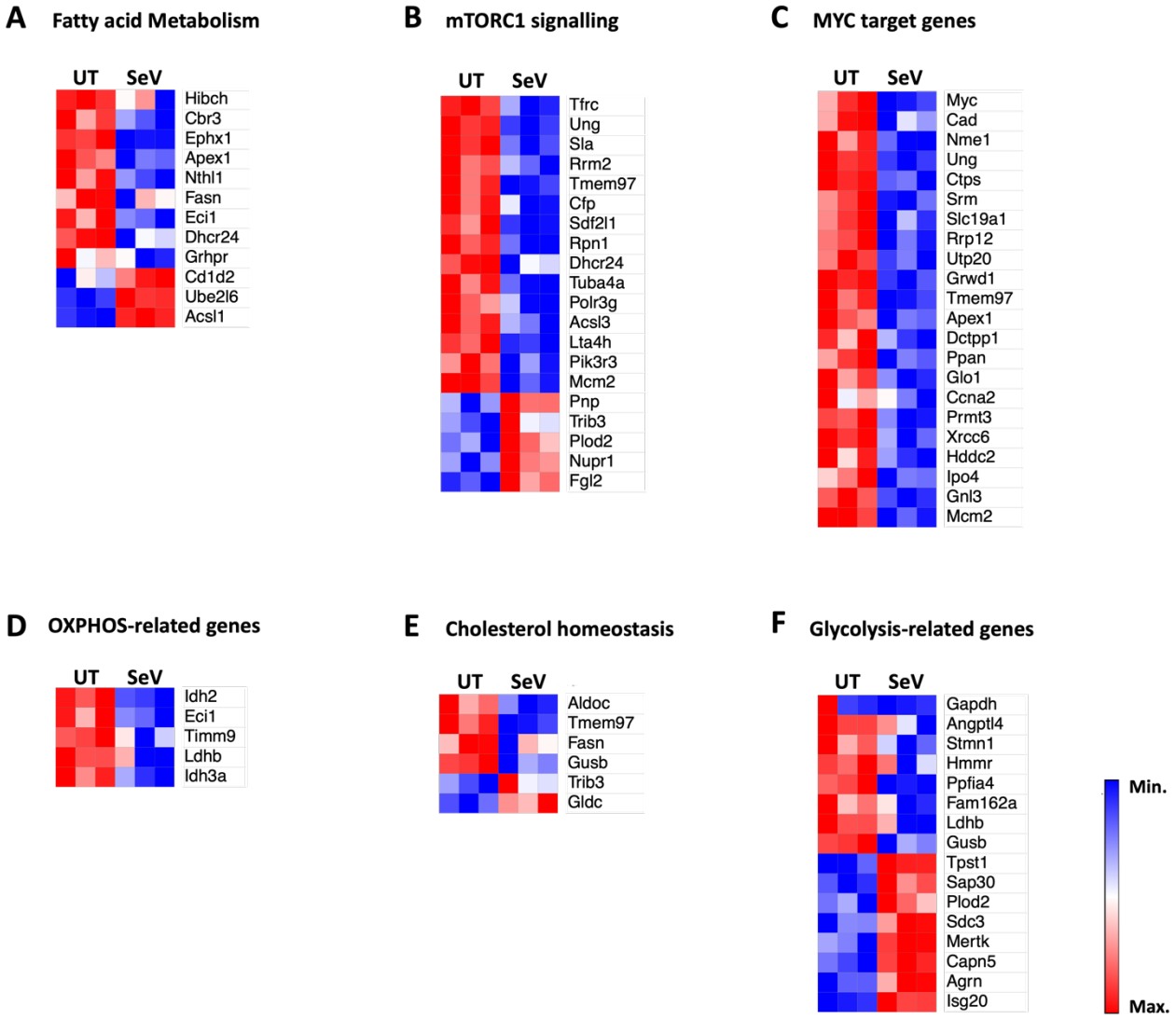


Figure 3.4 Metabolic pathways affected following 18-hour SeV stimulation Heat map of significantly different genes involved in (A) fatty acid metabolism, (B) mTORC1 signaling, (C) MYC target genes, (D) oxidative phosphorylation, (E) cholesterol homeostasis, (F) glycolysis. All gene counts are normalised with a FDR<0.1 and log_2fold change either ≥ 1.5 or ≤-1.5 (n=3).

3.3.4 Selected differentially expressed genes required for GM-DC activation

Out of the 2821 differentially expressed genes, we selected specific enriched genes (Figure 3.5) which encode key molecules important for GM-DC activation or their anti-viral response. *DEAD Box Protein 58* (*Ddx58)* (also known as Rig-I), *Interferon Induced with Helicase C Domain 1 (Ifih1)* (also known as Melanoma differentiation-associated protein 5 Mda-5) and *TLR3* are important for detecting viral nucleic acids within the cells and *Irf7* is a transcription factor downstream which is vital for inducing anti-viral responses. Increased expression of these genes is expected following viral stimulation. RIG-I has previously been shown to be important for SeV action of immune cells (Martínez-Gil *et al.*, 2013). A main component of the anti-viral response is the induction and production of type I interferons, there are various subtypes, and murine and human genome may encode different subtypes. Importantly, there is only one IFN-β in both human and murine genome, whereas different IFN-α exist. 14 *IFNA* genes exists in mice, compared to 13 in humans (van Pesch *et al.*, 2004; Kuruganti, Accavitti-Loper and Walter, 2014). We show enrichment of some of these Type I IFNs following SeV stimulation (Figure 3.5). The release of interferons from cells results in binding of interferons to their receptor, receptor signalling leads to downstream upregulation of interferon-stimulated genes (ISGs). Certain ISGs were also upregulated following 18-hour SeV stimulation. Upon stimulation, metabolic pathways are altered and signalling pathways are activated. Different enzymes or proteins the cell requires will be upregulated, the corresponding genes would be transcribed and translated to ensure appropriate GM-DC functions are elicited. Chemokines are required for the migration, or chemotaxis, of cells. *Cxcl9*, *Cxcl10* and *Cxcl11* encode chemokines which lead to the homing of immune cells to sites of inflammation and bind to the CXCR3 receptor and are involved in immune cell differentiation and activation (Cole *et al.*, 1998). SeV stimulation also results in the increased expression of genes, encoding cytokines which are involved in activation of other immune cells by GM-DCs, or proteins involved in antigen presentation, a key function of DCs.

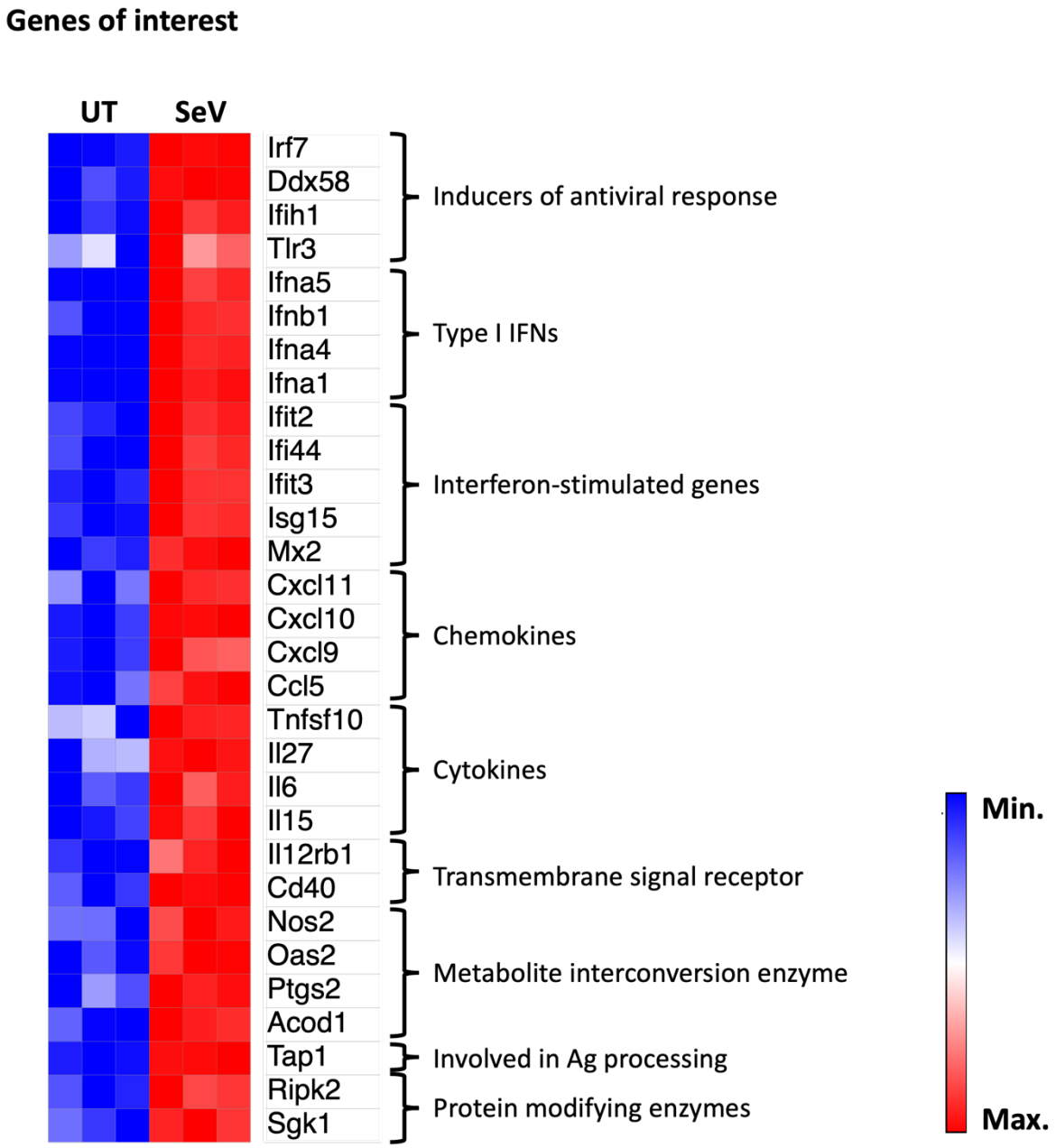


Figure 3.5 Key genes of interest upregulated following 18-hour SeV stimulation
Heat map of significantly different genes involved in GM-DC viral activation. Gene counts are normalised with a FDR<0.1 and $\log_2$fold change either ≥1.5 or ≤-1.5 (n=3).

3.3.5 LPS stimulation of GM-DCs induced cytokine mRNA expression

Prior to experiments with SeV, GM-DCs were first characterised by stimulation with LPS, the best-defined ligand, to ensure appropriate responses were elicited from our cells of interest. LPS stimulation has been shown extensively to induce proinflammatory responses in immune cells. GM-DCs were stimulated with LPS for 3 hours and RNA was extracted, and the expression of various inflammatory or anti-inflammatory genes was determined (Figure 3.6). *Tnf* and *Il6* encode TNFα and IL6 respectively, important pleiotropic cytokines. *Il12b* encodes the p40 subunit of IL-12 and IL-23, both cytokines are involved in activation and differentiation of T lymphocytes by DCs (Heufler *et al.*, 1996; Khayrullina *et al.*, 2008). *Il10* encodes IL10, which is normally associated with dampening inflammation as an anti-inflammatory cytokine. mRNA levels of all the genes are increased following LPS stimulation of GM-DCs (Figure 3.6 A-D), corroborating previous findings (Everts *et al.*, 2014)

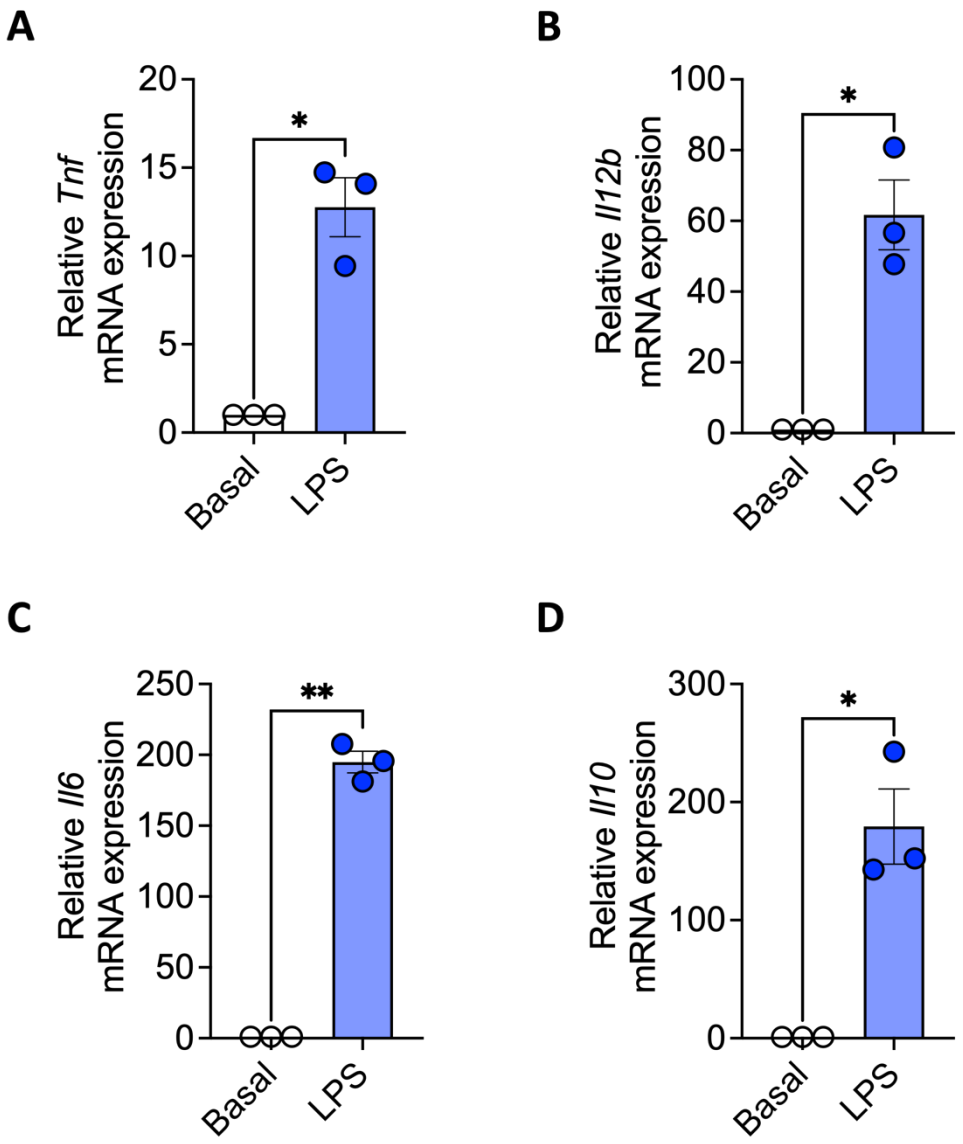


Figure 3.6 LPS stimulation induced GM-DC cytokine mRNA expression
Scatter plot of (A) *Tnf* mRNA expression relative to *Hprt* following 3-hour LPS stimulation. (B) *Il12b* mRNA expression relative to *Hprt* following 3-hour LPS stimulation. (C) *Il6* mRNA expression relative to *Hprt* following 3-hour LPS stimulation. (D) *Il10* mRNA expression relative to *Hprt* following 3-hour LPS stimulation. Statistical analysis performed using paired student's t-test, *p≤0.05, **p≤0.01 (n=3).

3.3.6 SeV stimulation altered *Hprt* mRNA expression

HPRT is an enzyme required in salvage synthesis, specifically the salvaging of purine nucleotides released during DNA breakdown (Stout and Caskey, 2003). Due to its presence at low levels in somatic tissue, *Hprt* has been used as a housekeeping gene, especially to compare relative gene expression of genes of interest to this housekeeper for RT-PCR experiments (Townsend *et al.*, 2019). *Hprt* was used as the housekeeping gene when we measured mRNA levels of target genes following LPS-treated GM-DCs, (Figure 3.6). No significant differences were observed in the Ct values of *Hprt* between basal and LPS-treated GM-DCs for each sample (Figure 3.7A). However, upon SeV stimulation, at different timepoints, we observed that the *Hprt* Ct values changed, and this was consistent between samples (Figures 3.7B-D). Therefore, we concluded *Hprt* was an inadequate housekeeping gene to use for SeV experiments, as this would alter the relative gene expression calculations between treated conditions and samples. Other studies have demonstrated the unsuitability of *Hprt* as a housekeeping gene (Townsend *et al.*, 2019). An alternative housekeeping gene was required and based on other studies, *Actb*, which encodes beta-actin, a key structural protein, was used for all subsequent SeV RT-PCR experiments. We show *Actb* Ct values at the same timepoints (Figure 3.7E) as for *Hprt* (Figures 3.7B-D), and no significant differences in *Actb* Ct values were observed. We determined relative gene expression of *Hprt* in relation to *Actb* (Figure 3.7F), and this clearly demonstrates the significantly decreased levels of *Hprt* mRNA following SeV stimulation at various timepoints.

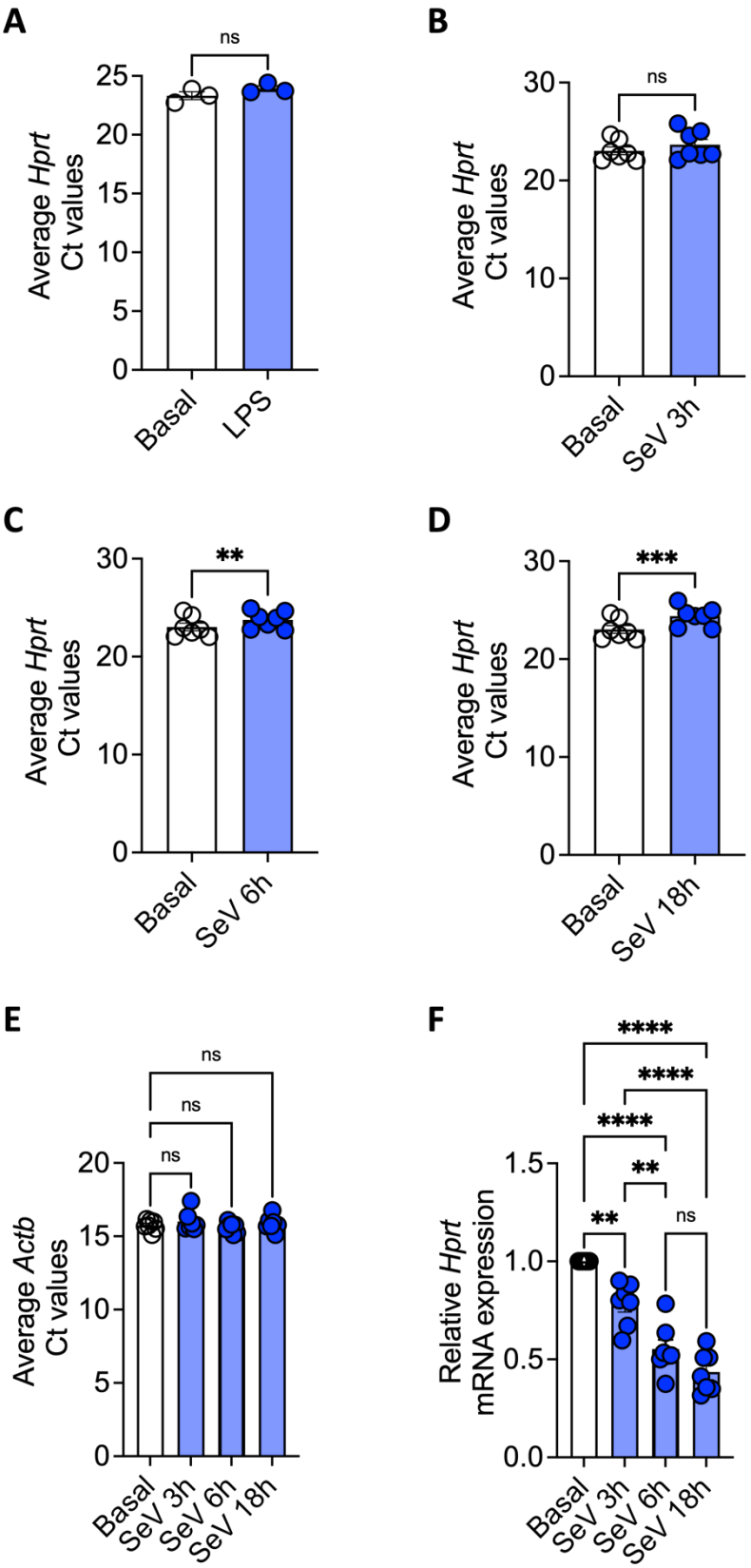


Figure 3.7 (Figure legend on the next page)

Figure 3.7 SeV stimulation reduced *Hprt* mRNA expression affecting its use as a housekeeping gene Scatter plot of (A) average *Hprt* Ct values following 3-hour LPS stimulation (n=3). (B-D) average *Hprt* Ct values following SeV stimulation at different timepoints (n=7). (E) average *Actb* Ct values following SeV stimulation at different timepoints (n=7). (F) *Hprt* mRNA expression relative to *Actb* following SeV stimulation at different timepoints (n=7). Statistical analysis performed using paired student's t-test or Kruskall-Wallis with Dunn's test or Ordinary one-way ANOVA with Tukey's correction where appropriate, ns=$p>0.05$, *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.01$, ****$p<0.0001$.

3.3.7 SeV stimulation of GM-DCs induced cytokine mRNA expression

Viral infection causes GM-DC activation and elicits anti-viral responses, the latter occurs to limit viral spread. Anti-viral responses include warning neighbouring cells of viral presence (Song *et al.*, 2023). Another vital function of DCs is to present antigen and necessary co-stimulatory factors to activate T lymphocytes (Inaba *et al.*, 1983). GM-DCs were stimulated with SeV at different timepoints and mRNA levels of genes encoding specific cytokines were measured (Figure 3.8). We measured *Tnf, Il12b* and *Il6* mRNA levels. We show that *Tnf* begins to increase at 3-hour SeV stimulation (Figure 3.8A) but is only significantly increased at 6 hours, where there is approximately double the level of *Tnf* compared to untreated GM-DCs (Figure 3.8B). At 18 hours, levels of *Tnf* partially decreased (Figure 3.8C).

Next, we investigated *Il12b* mRNA levels, and show similar kinetics to *Tnf,* where peak mRNA levels of *Il12b* is observed at 6-hour SeV stimulation (Figures 3.8D-E) and levels of *Il12b* are comparable at 18-hour stimulation to untreated (Figure 3.8F). We demonstrated *Il6* mRNA levels are significantly increased at all timepoints (Figures 3.8G-I). Peak *Il*6 mRNA levels was observed at the 6-hour timepoint (Figure 3.8H). We measured *Ifnb1* mRNA levels which encodes a key antiviral type I interferon, IFN-β. Levels of *Ifnb1* drastically increased during the timepoints (Figures 3.8J-L), with peak mRNA levels also at 6-hour SeV stimulation (Figure 3.8K).

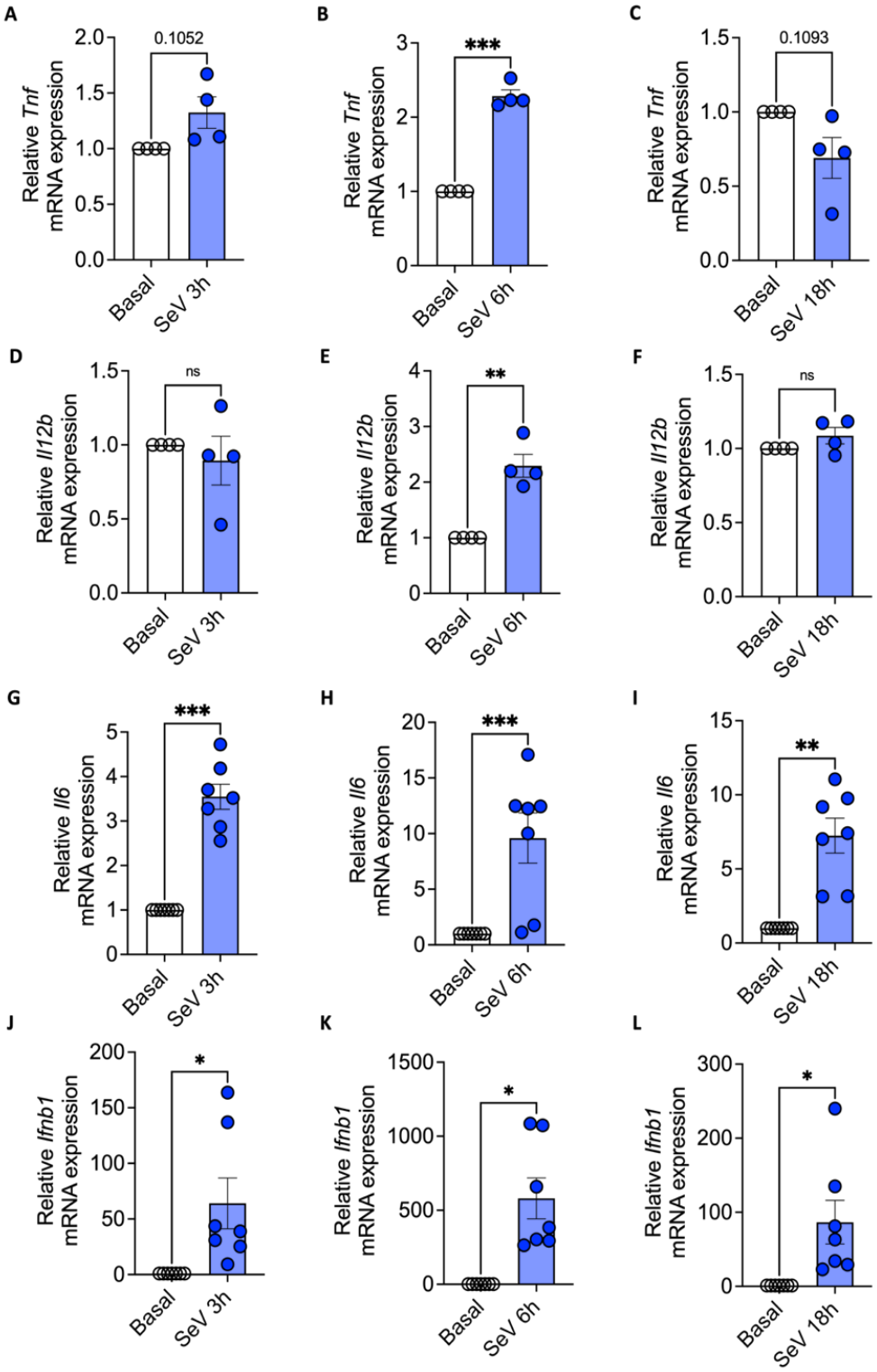


Figure 3.8 (Figure legend on the next page)

Figure 3.8 SeV stimulation induced GM-DC cytokine mRNA expression Scatter plot of (A-C) *Tnf* mRNA expression relative to *Actb* following SeV stimulation at different timepoints (n=4). (D-F) *Il12b* mRNA expression relative to *Actb* following SeV stimulation at different timepoints (n=4). (G-I) *Il6* mRNA expression relative to *Actb* following SeV stimulation at different timepoints (n=7). (J-L) *Ifnb1* mRNA expression relative to *Actb* following SeV stimulation at different timepoints (n=7). Statistical analysis performed using paired student's t-test or Wilcoxon test where appropriate, ns=p>0.05, *p ≤ 0.05, **p≤0.01, *** p≤ 0.001.

3.3.8 LPS stimulation of GM-DCs induced cytokine production

Having demonstrated the effect of LPS stimulation on mRNA expression, we next investigated the production of the cytokines encoded by the same genes by ELISA. We stimulated GM-DCs with LPS for 18 hours, followed by measuring cytokine levels in the supernatants (see section 2.9). *Tnf* encodes the TNFα cytokine. *Il12b* encodes the p40 subunit which heterodimerises with the p35 subunit to form the IL-12p70 cytokine. *Il6* encodes the IL6 cytokine. *Il10* encodes the IL10 cytokine. All of these cytokines were produced following LPS stimulation (Figures 3.9A-D), to varying levels. LPS stimulation resulted in the highest levels of TNFα and IL6, with a mean of approximately 1400 and 10,000pg/ml respectively (Figures 3.9A & C).

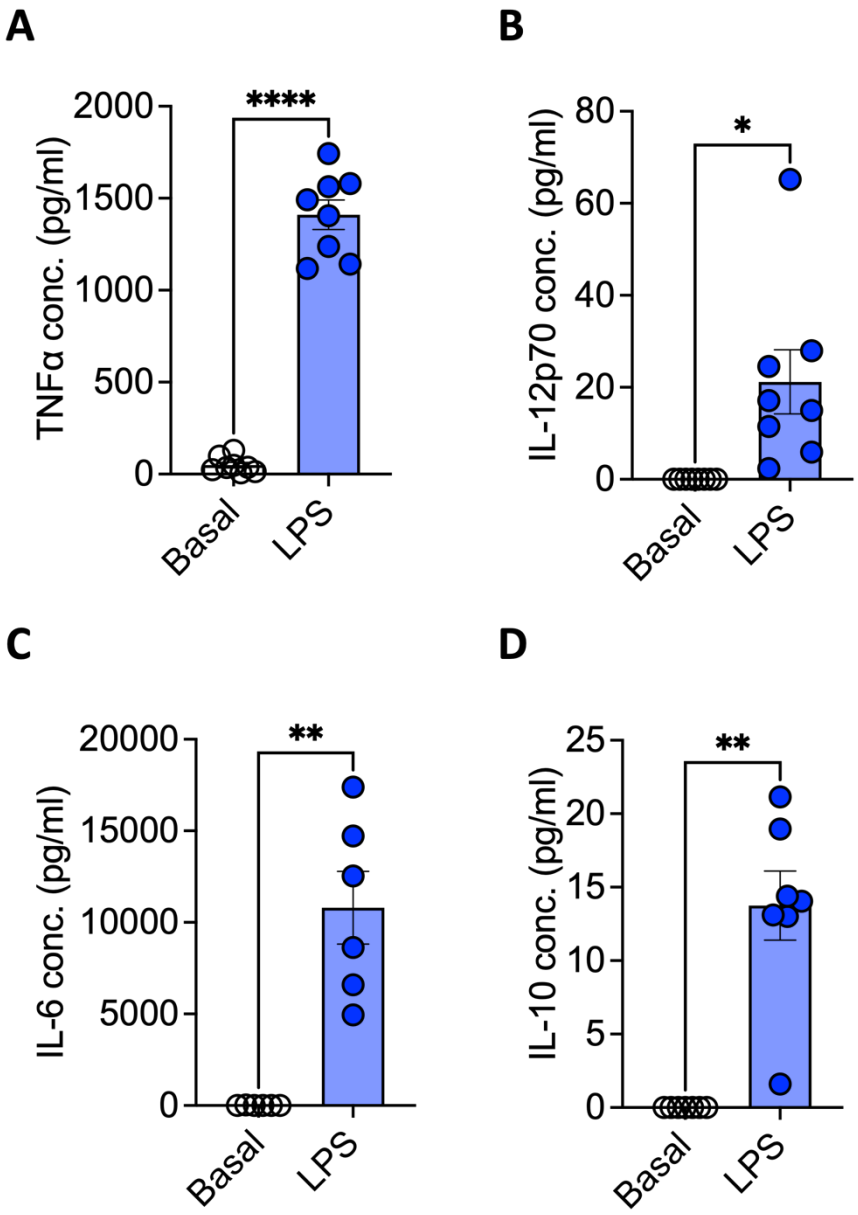


Figure 3.9 LPS stimulation induced GM-DC cytokine production Scatter plot of (A) TNFα production (pg/ml) following LPS 18-hour stimulation (n=8). (B) IL-12p70 production (pg/ml) following 18-hour LPS stimulation (n=8). (C) IL-6 production (pg/ml) following 18-hour LPS stimulation (n=6). (D) IL-10 production (pg/ml) following 18-hour LPS stimulation (n=7). Statistical analysis performed using paired student's t-test, *p ≤ 0.05, **p≤0.01, ****p<0.0001.

3.3.9 SeV stimulation of GM-DCs induced cytokine production

Next, we investigated SeV-stimulated GM-DC production of the cytokines using ELISA. Briefly, GM-DCs were stimulated with SeV for 18 hours, followed by the measuring of cytokine levels in the supernatants. SeV stimulation resulted in the production of TNFα and IL6 (Figures 3.10A & B). *Ifnb1* encodes the IFN-β antiviral cytokine. High levels of IFN-β were measured following SeV stimulation (Figure 3.10C), a mean of approximately 2500pg/ml.

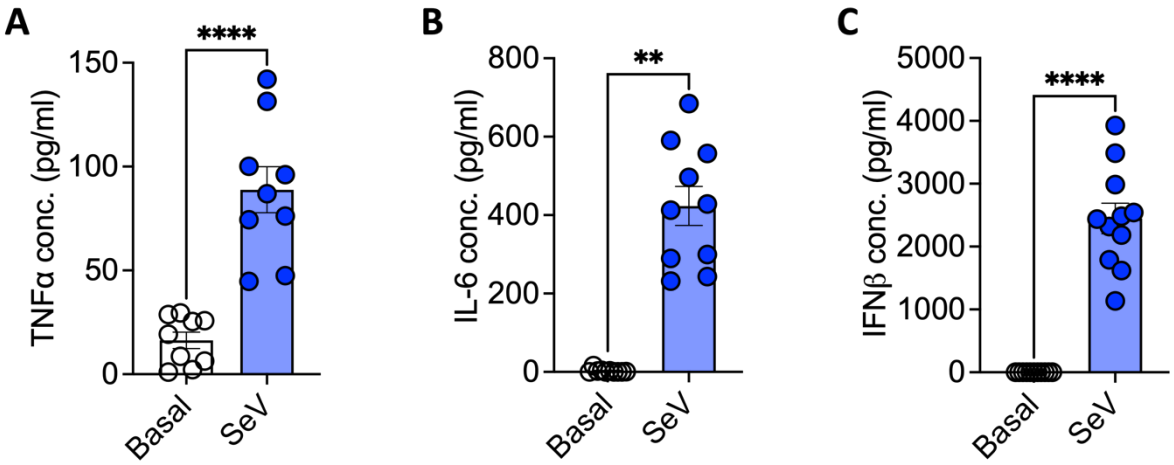


Figure 3.10 SeV stimulation induced GM-DC cytokine production Scatter plot of (A) TNFα production (pg/ml) following 18-hour SeV stimulation (n=9). (B) IL-6 production (pg/ml) following 18-hour SeV stimulation (n=10). (C) IFNβ production (pg/ml) following 18-hour SeV stimulation (n=11). Statistical analysis performed using paired student's t-test or Wilcoxon test where appropriate, **p≤0.01, *** p≤ 0.001, ****p<0.0001.

3.3.10 SeV stimulation activated the NF-κB signalling pathway

Having demonstrated that SeV stimulation induced mRNA expression (Figure 3.8), which subsequently was translated and resulted in cytokine production (Figure 3.10), we next investigated the signalling events following stimulation. SeV is a ssRNA virus. Upon entry into cells, the ssRNA activates intracellular signalling molecules. Immunoblotting was used to determine which signalling pathways are activated. These signalling pathways culminate in activation of transcription factors, which enter the nucleus and increase expression of target genes. Cells were stimulated with SeV for 6 hours and prepared for immunoblotting as per section 2.10. Samples were immunoblotted for phospho-IKBα (phosphorylated nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha). The transcription factor NF-κB is inhibited by IKBα, phosphorylation of the latter yields active NF-κB which can translocate to the nucleus for expression of its target genes (Beg *et al.*, 1993). Following 6-hour SeV stimulation, GM-DCs had more phospho-IKBα compared to untreated samples, depicted by the darker bands in the p-IKBα immunoblot (Figure 3.11A). β-Actin was used as the loading control, the bands are consistent between all samples. Densitometry analysis was performed on the generated blots, p-IKBα bands were normalised to β-Actin. Relative levels of p-IKBα were significantly higher in SeV stimulated GM-DCs compared to untreated (Figure 3.11B).

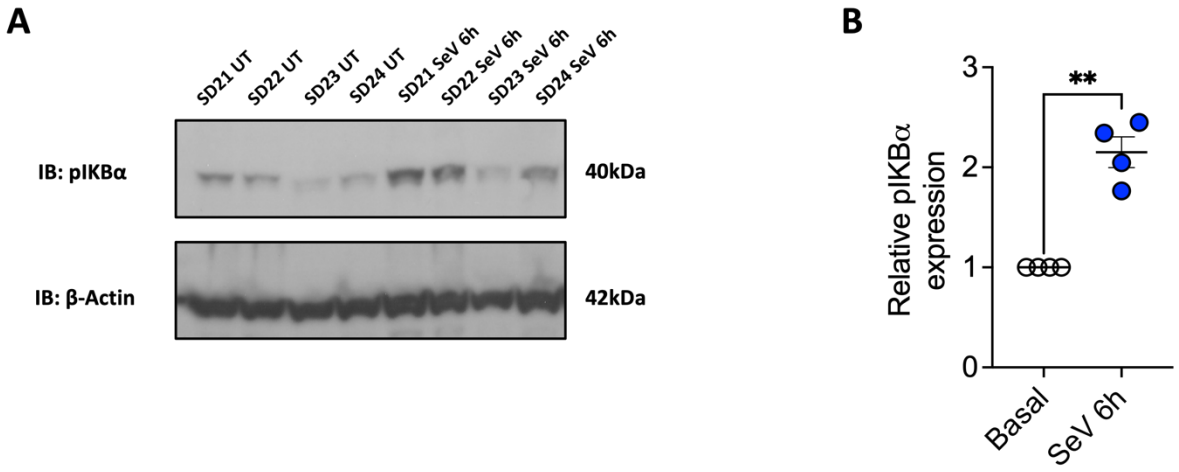


Figure 3.11 NF-κB pathway activated following SeV stimulation (A) Immunoblot of phospho-IκBα (40kDa) and β-Actin (42kDa) expression basally and following 6-hour SeV stimulation. (B) Densitometry displaying phospho-IκBα expression relative to β-Actin. Statistical analysis performed using paired student's t-test, **p≤0.01 (n=4).

3.4 Discussion

Dendritic cells (DCs) are critical mediators of the immune response, as they are professional antigen-presenting cells which circulate the body and upon antigen uptake, they migrate to T-cell rich zones to induce the adaptive immune response (Macatonia *et al.*, 1987; Crowley, Inaba and Steinman, 1990; Banchereau and Steinman, 1998). In this chapter, we aimed to characterize the functional responses of murine bone marrow derived DCs to two different stimuli. We first established and optimized a protocol for the generation of functional DCs from mice. Bone marrow precursors were cultured in the presence of the growth factor GM-CSF to yield bone marrow-derived dendritic cells, herein referred to as GM-DCs. GM-DCs were stimulated with either lipopolysaccharide (LPS) or Sendai virus (SeV), the former produces an inflammatory response (Granucci *et al.*, 1999), and the latter stimulates an antiviral response from the cells (Bedsaul, Zaritsky and Zoon, 2016). RNA sequencing (RNA-seq) provides a global view of a cell's transcriptional response to a stimuli or environment. The transcriptional response to LPS stimulation in GM-DCs has been widely studied (Efron *et al.*, 2005; Shalek *et al.*, 2013). However, the transcriptional response from SeV-stimulated GM-DCs has not been studied. To this end, we sought to characterise the transcriptional changes in SeV infected DCs by RNA sequencing.

GM-DCs were stimulated for 18 hours with SeV. As expected, SeV stimulation strongly affected gene expression within GM-DCs, with significant transcriptional remodeling noted, in processes ranging from signaling to metabolic processes. We show that SeV resulted in an increase in genes involved in the IFNA response, as expected as SeV induces a type I IFN response from cells, including GM-DCs (López *et al.*, 2003). Upon pathogen encounter, DCs undergo maturation to upregulate their antigen presenting machinery. The genomic material of SeV is encoded in their ssRNA. PRRs expressed on GM-DCs are required to recognise the ssRNA of SeV. SeV is recognised by RIG-I (Baum, Sachidanandam and García-Sastre, 2010) and MDA-5 (Yount *et al.*, 2008). We found that expression of these cytoplasmic viral genomic material sensors was upregulated, *Ddx58* and *Ifih1* respectively, following SeV infection. Additionally, their downstream signalling molecules, *IRF7,* expression was also upregulated. Therefore, we demonstrate that SeV infects GM-DCs and activates the viral sensors within the cell. This stimulates maturation of the cell, demonstrated by the increase in expression of

genes involved in antigen processing (*Tap1*), co-stimulatory molecules (*Cd40*) and cytokines which induce T cell polarisation (*Il6* & *Il27*) and differentiation (*Il15*). The chemokines, CXCL9, CXCL10 and CXCL11, interact with CXCR3 on T cells and promotes their differentiation to a Th1 phenotype and subsequently their migration to inflamed tissues (Loetscher *et al.*, 1996; Tannenbaum *et al.*, 1998). Increased expression of these chemokines along with the cytokines, including type I IFNs, and ISGs, portray activation and maturation of DCs, as they are primed to induce antiviral responses and activate adaptive immune cells. Upon SeV infection, inflammatory responses were upregulated, however, genes involved in metabolic pathways were decreased. *Myc* downregulation was observed in multiple myeloma cells infected with SeV (Jiang *et al.*, 2016). Therefore, potentially the same could occur here. *Myc* encodes the transcription factor, MYC, which targets many genes. A decrease in *Myc* can result in decreased protein and decreased expression of MYC target genes, which includes glycolytic enzymes, such as GAPDH.

After establishing that SeV results in the upregulation of genes involved in DC maturation, we next investigated one of the key effector functions of DCs, cytokine production. First, we investigated cytokine responses at an mRNA level, later confirming at a protein level in both LPS and SeV stimulated GM-DCs. LPS and SeV stimulation of their respective receptors on GM-DCs results in transcription factor activation. LPS stimulates TLR4 (Poltorak *et al.*, 1998), downstream signalling cascades results in the activation of the transcription factor NF-κB (Yao *et al.*, 1997). This has been extensively proven and activation of NF-κB results in the production of pro-inflammatory genes (Sharif *et al.*, 2007). SeV as previously mentioned is recognised by RIG-I and MDA-5, also TLR3 and TLR7 (Melchjorsen *et al.*, 2005; Peters, Chattopadhyay and Sen, 2008), these viral sensors can activate the transcription factor IRF3, SeV has been shown to induce apoptosis of infected cells through phosphorylation of IRF3 (pIRF3), its active form (Heylbroeck *et al.*, 2000). We show that SeV infection of GM-DCs induced the activation of the transcription factor NF-κB, demonstrated by the phosphorylation of its inhibitor IKBα. This process has been previously observed and subsequent DC maturation, including cytokine production, is dependent on NF-κB activation (López *et al.*, 2003). As LPS has been widely used to stimulate different immune cells, including GM-DCs, this was initially used to ascertain whether GM-DCs performed their appropriate functions.

Upon LPS stimulation, GM-DCs upregulated their expression of pro-inflammatory cytokines *(Tnf*, *Il12b* and *Il6*) and the anti-inflammatory cytokine (*Il10*), as previously described (Everts *et al.*, 2014). Increased mRNA expression correlated with increased protein production and secretion of all these cytokines following LPS stimulation of GM-DCs. TNF-α promotes the inflammatory state. *Il12b* encodes the p40 subunit of IL-12p70, and this cytokine promotes Th1 cell differentiation (Heufler *et al.*, 1996). IL-6 is a cytokine with ranging functions, one of which is the favouring of Th17 cell differentiation (Bettelli *et al.*, 2006). IL-10 is an anti-inflammatory cytokine, its release is important to ensure a controlled inflammatory response. IL-10 can impair DC maturation, pre-treatment with IL-10, followed by LPS stimulation, suppresses GM-DC antigen presenting capacity via downregulation of co-stimulatory molecules and reduced cytokine production (Bhattacharyya *et al.*, 2004). IL-10 presence during DC maturation can skew downstream T cell differentiation, favouring a Th2 phenotype over Th1 (De Smedt et al., 1997).

During RT-PCR, *Hprt* was used as the housekeeping gene for the LPS results. Analysis of the SeV results revealed changing *Hprt* Ct values between control and stimulated samples at different timepoints. Therefore, *Hprt* is not a suitable housekeeping gene in this instance and *Actb* was used instead. The unsuitability of *Hprt* has also been shown in other contexts (Townsend *et al.*, 2019). Expression of cytokines following SeV stimulation was measured at different timepoints, the 6-hour timepoint was when peak cytokine production was observed. Cytokines expressed following SeV stimulation were involved in inflammation (*Tnf*), T cell differentiation (*Il6* & *Il12b*) and antiviral responses (*Ifnb1*). Increased mRNA expression correlated with increased cytokine protein production and secretion. Production of these cytokines by SeV has previously been observed (Kurooka and Kaneda, 2007).

A limitation of our study is that RNA sequencing was performed at a single timepoint, 18-hours post-SeV stimulation. At this timepoint, specific genes would be altered, such as ISGs. As shown by RT-PCR, peak cytokine production between the three timepoints was at 6 hours, therefore, performing RNA sequencing at other timepoints would be ideal to study the impact of SeV on genes differentially expressed earlier, such as those involved in metabolic pathways.

As throughout stimulation, GM-DCs favour different metabolic pathways (GM-DC metabolism is discussed in the next chapter). However, due to the costs of RNA sequencing, only one stimulation timepoint was performed.

To ensure our GM-DCs were functioning appropriately, we stimulated them with LPS or SeV and we observed an increase in pro-inflammatory cytokines and antiviral cytokines, in the case of SeV (Figure 3.12). We also showed that NF-kB was activated upon SeV infection of GM-DCs. Additionally, we performed RNA sequencing of SeV-infected GM-DCs compared to untreated and determined various differentially expressed genes affected by SeV (Figure 3.12).

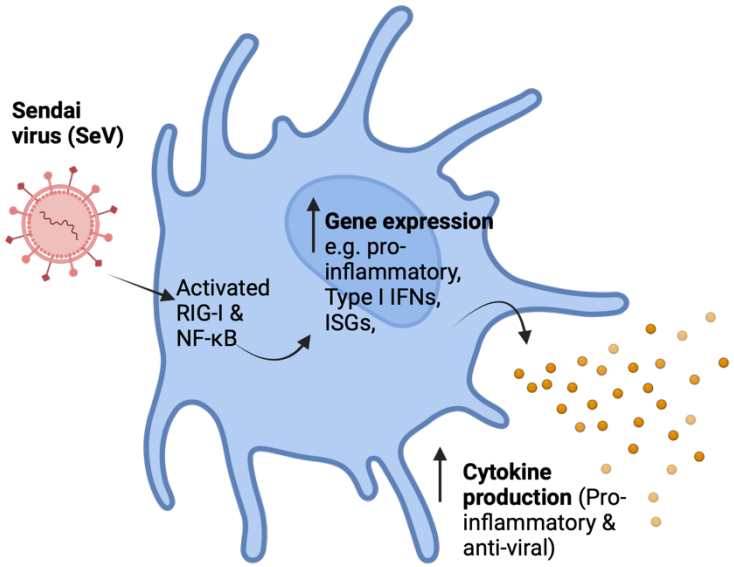


Figure 3.12 – Summary of Sendai virus (SeV) infection of GM-DCs from *wild type* C57BL/6J. SeV infection caused NF-κB activation in GM-DCs and induced transcriptional reprogramming within the cells. Genes from a wide range of processes were increased in expression, including pro-inflammatory cytokines and type I interferons. Increased cytokine mRNA expression correlated with increased protein production and secretion.

Chapter Four

Results

Characterisation of dendritic cell metabolism

4.1 Introduction

4.1.1 Immunometabolism overview

In the past decade, the field of immunometabolism has expanded exponentially. Immunometabolism applies to the interplay between system metabolism and the immune system, and also the intrinsic cellular metabolism of immune cells (O'Neill, Kishton and Rathmell, 2016). Different immune cells or immune cells during different stages of development utilise different metabolic pathways, to produce energy as ATP, metabolites and biointermediates (Michalek *et al.*, 2011; Berod *et al.*, 2014; Everts *et al.*, 2014; Thwe *et al.*, 2017). These metabolites can enter different pathways to generate other macromolecules, alter signalling pathways (He *et al.*, 2023) or alter transcriptional regulation (Langer *et al.*, 2002) or post-translational modifications (Bowman *et al.*, 2017). The consensus is that reprogramming of immune cell metabolism is vital for cell differentiation, proliferation, fate, and function.

Immune cells tend to utilise two major metabolic pathways to produce ATP: glycolysis and OXPHOS. Glycolysis is an inefficient process of producing ATP, as it only yields 2 net ATP per glucose molecule, however, this pathway produces metabolic intermediates which can offshoot into other metabolic pathways, for example, for the synthesis of nucleotides or amino acids (O'Neill, Kishton and Rathmell, 2016). The final reaction of glycolysis involves the production of pyruvate, a molecule which can be shuttled into either the mitochondria to enter the TCA cycle and subsequently OXPHOS or remains in the cytosol and is converted to lactate during anaerobic glycolysis (O'Neill, Kishton and Rathmell, 2016). A process known as aerobic glycolysis or the Warburg Effect, was discovered by Otto Warburg, where despite the presence of oxygen, glycolysis is highly upregulated (Warburg, 1925). Many immune cells undergo aerobic glycolysis, upon activation, and is required for proliferation (Krawczyk *et al.*, 2010; Michalek *et al.*, 2011). The TCA cycle is favoured by cells requiring more energy and longevity, therefore, non-proliferative or many quiescent cells upregulate the TCA cycle and OXPHOS (Yao *et al.*, 2019). The TCA cycle is also important as many other metabolic pathways intersect at different stages in the cycle. Lipids and amino acids can be broken down and can enter at various stages of the TCA cycle, allowing different macromolecules or stores to be utilised. TCA intermediates can also exit the cycle and the reverse can occur, where lipids and

amino acids are synthesised depending on cellular demand (O'Neill, Kishton and Rathmell, 2016).

Immune cells can sense nutrient or energy supply or detect hormones (e.g., growth factors, insulin) and these signals can regulate the intrinsic metabolism of the cell, in turn, their function. Two key molecules which detect nutrient availability are mTOR and AMPK (O'Neill, Kishton and Rathmell, 2016). mTOR and AMPK are both serine/threonine kinases (Laplante and Sabatini, 2009; Shaw, 2009), referred to as central metabolic regulators. mTOR detects growth factors and amino acids (Hay and Sonenberg, 2004; Sinclair *et al.*, 2013). mTOR signalling results in cell growth or proliferation and is highly activated during differentiation of immune cells, including T cells (Delgoffe *et al.*, 2009). mTOR increases anabolism (Hay and Sonenberg, 2004) whereas AMPK increases catabolism (Kemmerer *et al.*, 2015). AMPK detects nutrient deprivation, in turn, energy diminution (Carling, Zammit and Hardie, 1987; Munday *et al.*, 1988). AMPK detects the cell's energy status via cellular increases in AMP or ADP to ATP ratios, which results in the activation of the kinase (Xiao *et al*., 2011; Hardie, 2011). AMPK signalling re-establishes cell homeostasis by inhibiting mTOR, hence decreasing anabolic processes (e.g. protein synthesis), and consequently, represses activation of immune cells (Pandit *et al.*, 2022). Additionally, AMPK increases catabolic processes, e.g., lipid oxidation, to maintain the energy balance within the cell (O'Neill, Kishton and Rathmell, 2016). These master regulators function in concert with other signalling molecules or sensors which ultimately coordinate the appropriate metabolic responses within the cell. Dendritic cells utilise different metabolic pathways in their resting state and throughout their activation, these will be highlighted next.

4.1.2 Dendritic cell metabolism

Resting or immature DCs have a distinct metabolic phenotype compared to activated DCs (Krawczyk *et al.*, 2010). Bone marrow-derived dendritic cells have been extensively used to determine the metabolism of dendritic cells. These cells are either cultured with Flt3L or GM-CSF, termed FL-DCs or GM-DCs respectively (Zhang *et al.*, 2018). In a resting state many immune cells utilise OXPHOS to produce the ATP that they require (Brand and Hermfisse, 1997). Upon activation, these cells upregulate their rates of glycolysis to support the cells' metabolic requirements including the generation of biosynthetic precursors (Jacobs *et al.*,

2008). After LPS stimulation, an increase in OXPHOS is observed in DCs, but is subsequently downregulated to favour aerobic glycolysis (Everts *et al.*, 2012, 2014). Resting GM-DCs use fatty acid oxidation and glutaminolysis (the breakdown of the amino acid glutamine) to supply OXPHOS to meet the metabolic demands of this quiescent state. Following activation, notably, TLR stimulation, DCs upregulate their rates of glycolysis and lactate production (Everts *et al.*, 2012). However, the metabolic profile changes depending on the length of time after stimulation. DC activation involves antigen presentation, migration, cell-cell interactions and cytokine/chemokine secretion, the cell requires more nucleotides and amino acids for RNA and protein synthesis respectively. Glycolytic intermediates can contribute to both, along with fatty acid synthesis (Warburg, 1925; Everts *et al.*, 2014). Glycolytic intermediates can enter the pentose phosphate pathway to generate nucleotides and amino acids (Wood, 1985). The early increase in glycolysis also serves to provide pyruvate for TCA cycle function. Early LPS stimulation induces an increase in the TCA cycle and an increase in citrate (Everts *et al.*, 2014), which is shuttled into the cytosol. Fatty acid synthesis is a requisite for DC activation and is promoted by the citrate shuttle, providing the building blocks for Golgi apparatus and ER expansion, to accommodate heightened protein production upon DC activation (Everts *et al.*, 2014). GM-DCs express iNOS which mediates NO production (Lawless *et al.*, 2017). NO plays a role in inflammation, it can act as a signalling molecule and can trigger apoptosis of cells (Snyder *et al.*, 2009). NO can also inhibit the ETC in the mitochondria, and thus, inhibiting OXPHOS, which results in the commitment of GM-DCs to glycolysis and allow their survival (Everts *et al.*, 2012; Lawless *et al.*, 2017). This occurs between 18 to 48 hours after stimulation via a mTOR/HIF1α/iNOS axis (Lawless *et al.*, 2017). HIF1α protein increases following 24-hour LPS stimulation of GM-DCs (Lawless *et al.*, 2017). HIF1α is downstream of mTOR (Dodd *et al.*, 2015), and promotes glycolysis (Lum *et al.*, 2007) and is activated during low or lack of oxygen (Wang *et al.*, 1995). The initial glycolytic burst associated with DC activation requires a supply of glucose to sustain it. Glucose can be taken up from the surrounding microenvironment, however, glucose transporters (e.g. GLUT1) are upregulated later during stimulation (Thwe *et al.*, 2017). Thwe *et al.* (2017) demonstrated glycogen as an intracellular source of glucose, where glycogenolysis provides glucose monomers to fuel glycolysis and subsequently citrate and fatty acid synthesis. Later during stimulation, DCs can acquire glucose from the environment through their now upregulated glucose transporters on the cell surface.

Inhibition of glycogen breakdown hindered DC maturation and effector function (Thwe *et al.*, 2017).

Different metabolic pathways are favoured in different circumstances, of DC differentiation, activation, and maturation (Krawczyk *et al.*, 2010; Everts *et al.*, 2014; Thwe *et al.*, 2017). However, these changes in DC metabolism also influence their subsequent activation of the adaptive immune response, particularly the polarisation of T cells. TLR stimulation drives glycolysis, via mTORC1, which results in upregulation of MHC class II expression on murine DCs (Everts *et al.*, 2014; Lawless *et al.*, 2017). mTORC1 diminishes autophagy, the breakdown of cellular components (Hosokawa *et al.*, 2009; Dodd *et al.*, 2015). Therefore, Patente *et al.* (2019) suggested that increased mTORC1 activation, limits autophagy, promoting exogenous antigen presentation rather than endogenous cross-presentation, on MHC class II molecules (Schmid, Pypaert and Münz, 2007). Co-stimulatory molecule expression of CD40 and CD86 is dependent upon glycolysis (Everts *et al.*, 2014). Everts *et al.* (2014) demonstrated that glycolysis inhibition of GM-DCs resulted in reduced $CD4^+$ T cell priming. Expression of IL-12 was also dependent on glycolysis (Everts *et al.*, 2014), this enzyme induces the differentiation of Th1 cells (Heufler *et al.*, 1996). Therefore, GM-DC upregulation of glycolysis is required for Th1 polarisation. cDC1s which promote Th1 polarisation were also observed to be more glycolytic than cDC2s (Mashayekhi *et al.*, 2011; Du *et al.*, 2018). The signals which drive Th2 polarisation are less defined. House dust mite (HDM) extract can induce Th2 responses, stimulation of DCs with this allergen, displayed no reduction in OXPHOS and only a slight increase in glycolysis (Guak *et al.*, 2018). Greater AMPK phosphorylation was observed in murine lung cDC2s, which drove type 2 responses in the lung (Nieves *et al.*, 2016). PPAR-γ is a transcription factor involved in lipid metabolism (Dreyer *et al.*, 1992). Low PPAR-γ was postulated to be required for mediating Th2 responses by DCs (Legutko *et al.*, 2011; Khare *et al.*, 2015; Patente, Pelgrom and Everts, 2019). Patente *et al.* (2019) speculated the catabolic phenotype hinders MHC II and IL-12 expression, which instead promotes the Th2 polarisation, except there is no evidence yet. TGF-β and IL-6 induce Th17 polarisation (Bettelli *et al.*, 2006); IL-1β and IL-23 promote Th17 survival and expansion (Harrington *et al.*, 2005; Ghoreschi *et al.*, 2010). Glycolysis followed by fatty acid synthesis were required for IL-6 production by murine DCs (Everts *et al.*, 2014) and IL-1β and IL-23 production by human DCs (Hansen *et al.*, 2018). However, inhibition of glycolysis increased an ER stress sensor, Inositol-Requiring

Enzyme 1 alpha (IRE1α), and subsequently IL-23 expression during TLR stimulation of human DCs (Márquez *et al.*, 2017). Altogether, these papers demonstrate that DC polarisation of T cells depends on the stimulus, the subtype, or the setting, therefore, the metabolic pathway involved may also be dependent on these factors. Cholesterol accumulation in DCs was observed to stimulate Th17 polarising cytokine secretion (Westerterp *et al.*, 2017). DCs also induce regulatory T cells which is important in inducing immune tolerance (Darrasse-Jèze *et al.*, 2009), a process hijacked in cancer (Ramos *et al.*, 2012; Carrascal *et al.*, 2014). The mTORC1 inhibitor, rapamycin, promotes tolerogenic DCs during human monocyte differentiation towards DCs (Fischer *et al.*, 2009). Increased OXPHOS was observed to be required for the tolerogenic phenotype, however, so was an increase in glycolysis (Ferreira *et al.*, 2015). The increase in OXPHOS was dependent on FAO oxidation (Malinarich *et al.*, 2015), possibly through PPAR-γ signalling (Macdougall *et al.*, 2018). Ultimately, these results display the importance of metabolism in facilitating the appropriate DC effector function. Figure 4.1 displays the metabolic pathways important during the priming of different T cells and the importance of effector cytokine production by DCs. The ability of DCs to fuel these pathways depend on their environment and the importance of fuels will be highlighted next.

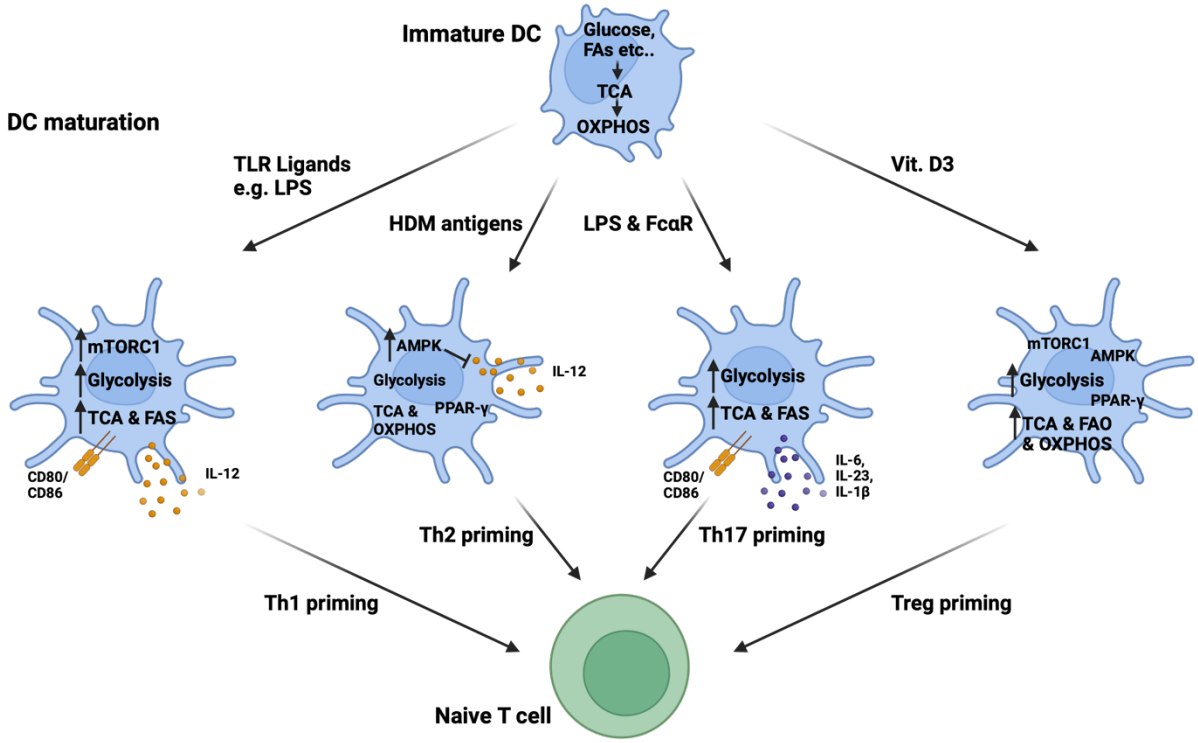


Figure 4.1 – DC metabolism and cytokine production influences T cell priming. Immature DCs utilise glucose, glutamine, and fatty acid oxidation to fuel the TCA cycle and oxidative phosphorylation to maintain their function. DCs stimulated with various ligands, leads to the favouring of different metabolic pathways, involving different metabolic regulators. These in turn affect DC effector function and influence T cell subtype differentiation (Adapted from Patente *et al.* (2019) & Wculek *et al.* (2019)).

4.1.3 Carbon flexibility

Immune cells circulate in their host in the blood or lymph and can localise to different tissues (Janeway *et al.,* 2001). These cells are exposed to a wide range of microenvironments which will comprise of fluctuating levels of nutrients or oxygen. In hypoxic environments such as in enlarged adipose tissue (which occurs during obesity) or in TMEs, low oxygen levels can impact cell function (Yang *et al.*, 2009). Additionally, in these environments, different nutrient levels can exist, e.g., in obesity, there can be an increase in circulating free fatty acids (Opie and Walfish, 2010) or in the TME, there can be reduced glucose or amino acids (e.g. glutamine) (Kamphorst *et al.*, 2015) and aberrant lipid levels (Yu *et al.*, 2021). In tumour microenvironments, tumour cells are glucose scavengers (Warburg, 1925) and activated T cells require glucose for their effector functions (Jacobs *et al.*, 2008). Therefore, DCs within the TME may be exposed to lower levels of glucose compared to normal conditions. Lawless *et al.*

(2017) demonstrated that GM-DC glucose utilisation at a later timepoint can impact their consequent T cell activation. Between 72 to 96 hours, GM-DC usage of glucose results in inefficient T cell stimulation. Glucose deprivation or inhibition of mTOR results in OXPHOS upregulation and greater T cell stimulation and increased GM-DC life span (Lawless *et al.*, 2017). Glucose deprivation experiments were performed by switching the normal glucose-containing media for media with galactose after 8 hours of LPS stimulation. Galactose can be metabolised through the Leloir pathway, a relatively slow process but its metabolism provides an alternative fuel source for glycolysis and OXPHOS (Caputto *et al.*, 1949). Glycolytic rates are lower in galactose-cultured GM-DCs and these cells maintained higher rates of OXPHOS (Lawless *et al.*, 2017).

Another carbon source that has been studied is fructose. Fructose is used as a sweetener and is naturally occurring in fruits (Walker, Dumke and Goran, 2014). The increased consumption of high fructose corn syrup and other sugar or sweet compounds can contribute to weight gain (Kasim-Karakas *et al.*, 1996) and increased risks of diabetes (Goran, Ulijaszek and Ventura, 2013; Chen *et al.*, 2020), heart disease (Stanhope *et al.*, 2015), obesity (Kasim-Karakas *et al.*, 1996) or NAFLD (Todoric *et al.*, 2020). Fructose is mostly metabolised in the liver, compared to glucose which exits and is utilised around the body (Topping and Mayes, 1971; Chandramouli *et al.*, 1993). However, low levels of fructose have been measured in circulation (Topping and Mayes, 1971). Higher fructose levels have been measured in some cases, e.g., in certain cancers (Chen *et al.*, 2016). Immune cells, especially those involved in immune surveillance (i.e., DCs) will be exposed to fluctuating nutrients in the different microenvironments of the host (Gottfried *et al.*, 2006; Yang *et al.*, 2009). Jaiswal *et al.* (2019) found increased pro-inflammatory cytokine in human Mo-DCs treated with high fructose compared to high glucose. High fructose treated Mo-DCs also induced greater T cell effector function, measured by their IFN-γ, compared to high glucose treated Mo-DCs. In 2021, Jones *et al.* sought to elaborate on the mechanistic actions of fructose in the metabolism of monocytes. Fructose promoted OXPHOS (demonstrated the same in galactose and no sugar conditions) compared to glucose-treated monocytes. Fructose treatment did not affect cell viability of LPS-stimulated monocytes compared to glucose treatment (Jones *et al.*, 2021). This demonstrated the flexibility of these immune cells in utilising a different carbon source to maintain its effector function. These fructose-mediated effects were also observed to increase

monocyte cytokine production, including pro-inflammatory IL-1β and TNFα, and anti-inflammatory IL-10. Cytokine mRNA expression was unchanged between the two conditions. Additionally, co-stimulatory or activation markers on monocytes were also unchanged. Jones *et al.* (2021) suggested a coupling between glycolysis and OXPHOS in fructose-treated cells as in the presence of 2-DG or oligomycin, which inhibits those pathways respectively, fructose-treated monocytes underwent drastic cell death compared to glucose-treatment (these cells were mostly unaffected by these inhibitors). Fructose reprogramming of the cells promoted glutaminolysis to support the TCA cycle and OXPHOS. These papers, Jaiswal *et al.* (2019) & Jones *et al.* (2021), formed the basis for investigating the impact of different carbon sources on GM-DC effector function.

In this chapter, we sought to investigate the metabolic pathways important for LPS and SeV stimulated GM-DCs. We also investigated the impact of different carbon sources on GM-DC function, whether the cells were flexible in their sugar usage.

4.2 Specific aims of Chapter 4

The specific aim of this chapter was to characterise GM-DC metabolism from mice on a standard diet, and therefore to:

1. Elucidate the metabolic pathways adopted by GM-DCs following LPS stimulation
2. Elucidate the metabolic pathways adopted by GM-DCs following SeV stimulation
3. Determine the carbon flexibility of GM-DCs in the presence of alternate fuel sources

4.3 Results

4.3.1 Stimulation of GM-DCs increased protein translation

First, GM-DCs were stimulated with either SeV or LPS and SCENITH analysis was performed, as per section 2.11.4. Argüello *et al.* (2020) published this technique where puromycin (a mimic of tyrosyl-tRNA) is incorporated into elongating peptide chains during cellular protein synthesis. The addition of puromycin leads to premature termination of the polypeptide chain. Incorporation of puromycin, at low concentrations, can be used to determine the degree of protein synthesis occurring within the cell (Starck *et al.*, 2004; Schmidt *et al.*, 2009). Approximately half of total cellular energy is utilised for protein synthesis. Therefore, this energy costly process (Buttgereit and Brand, 1995) can be used as a surrogate for cellular metabolic activity.

We demonstrated an increase in puromycin incorporation (geometric mean fluorescence intensity) following stimulation with either SeV or LPS (Figure 4.2), signifying an increase in protein synthesis. We observed a clear increase in puromycin following SeV stimulation (Figure 4.2A) compared to basal, correlating with a significant increase in translation (Figure 4.2C). The cells require proteins to increase their metabolic activity, to facilitate activated DC function. Following LPS stimulation, there is a slight increase in puromycin incorporation, in turn, translation (Figure 4.2B and D), however, not significant as with SeV.

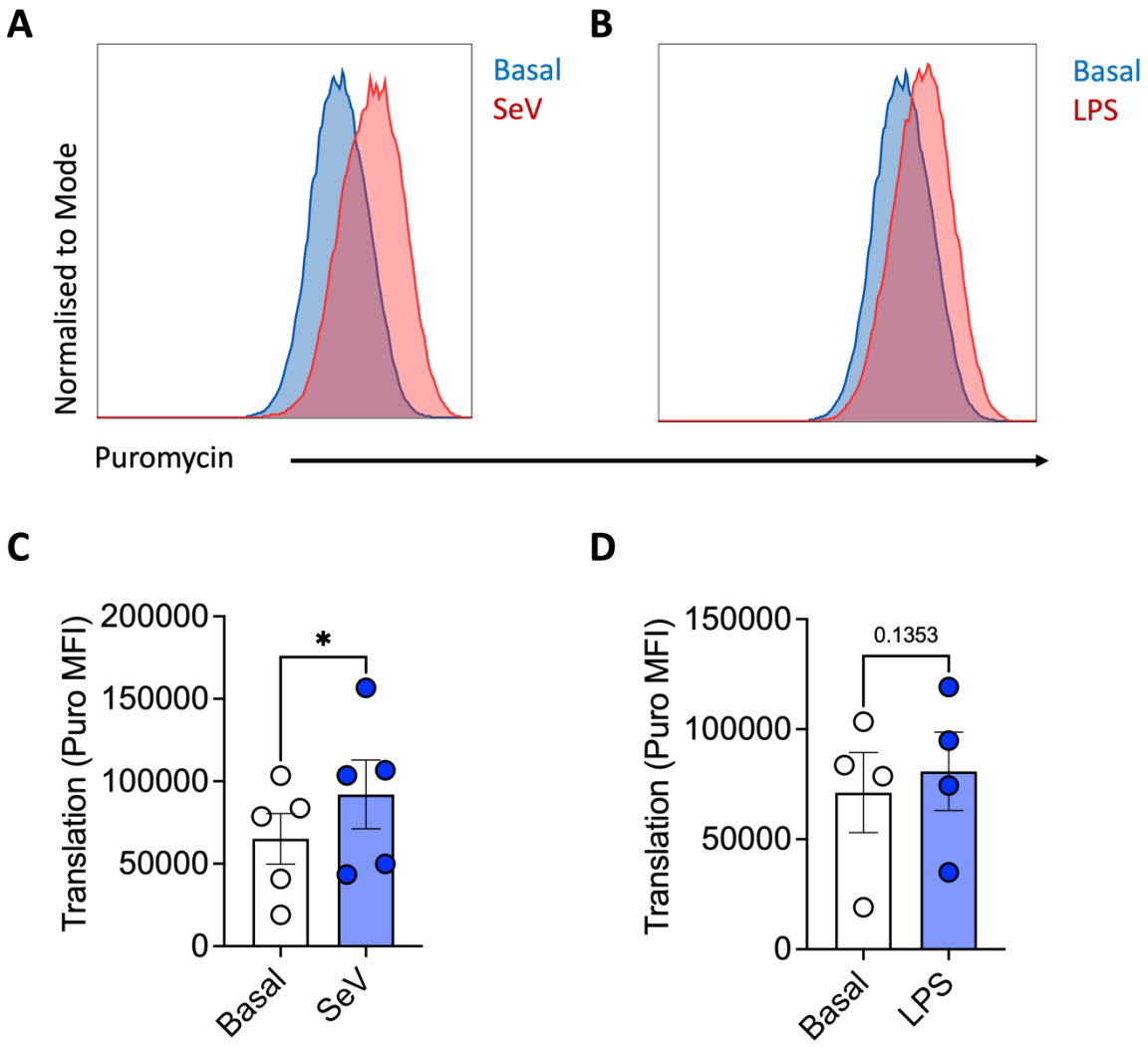


Figure 4.2 SeV and LPS stimulation increased protein translation (A & B) Representative histograms of puromycin incorporation in GM-DCs following 18-hour SeV stimulation (A) or LPS stimulation (B) respectively. (C & D) Scatter plot of puromycin incorporation (MFI) following 18-hour SeV stimulation (n=5) (C) or 18-hour LPS stimulation (n=4) (D). Statistical analysis performed using paired student's t-test, ns=$p>0.05$, *$p\leq0.05$.

4.3.2 Inhibition of key metabolic pathways resulted in a decrease in protein translation

Cells utilise various metabolic pathways to generate energy and metabolites for cell maintenance and function, including glycolysis and OXPHOS. 2-DG is a glucose mimic and acts as a competitive inhibitor (Crane and Solst, 1954). 2-DG competes with glucose for binding to hexokinase, in the first step of glycolysis (see Figure 4.3A), this results in 2-deoxy-D-glucose-6-phosphate (2-DG6P) formation (Tong *et al.*, 2000) and accumulation in cells which cannot be metabolised in the next step, hence, the inhibition of glycolysis. Oligomycin inhibits complex V, ATP synthase (Lardy, Johnson and McMurray, 1958), in OXPHOS (see Figure 4.3B), which prevents ATP synthesis, in turn, the inhibition of the ETC upstream. These two inhibitors have been extensively used to determine the metabolic pathways required for cell function.

We demonstrated the effects of 2-DG and/or oligomycin on protein translation of untreated or SeV-stimulated GM-DCs (Figure 4.3C). The addition of 2-DG and/or oligomycin to untreated and SeV-stimulated GM-DCs significantly decreased protein translation. Therefore, glycolysis and OXPHOS are required for protein synthesis. Next, we demonstrated the effects of 2-DG and/or oligomycin on protein translation of untreated or LPS-stimulated GM-DCs (Figure 4.3D). In untreated GM-DCs, 2-DG or oligomycin decreased protein translation to approximately the same extent. Following LPS stimulation, there was a slight difference between the effects of 2-DG or oligomycin, whereby 2-DG inhibited protein translation more than oligomycin. However, upon statistical analysis, no significant difference was observed between these two conditions. The addition of 2-DG and oligomycin completely abrogates protein translation for all conditions, untreated and SeV- or LPS-stimulated GM-DCs. Therefore, without either of these pathways, energy production is affected, hence, impacting GM-DC protein synthesis.

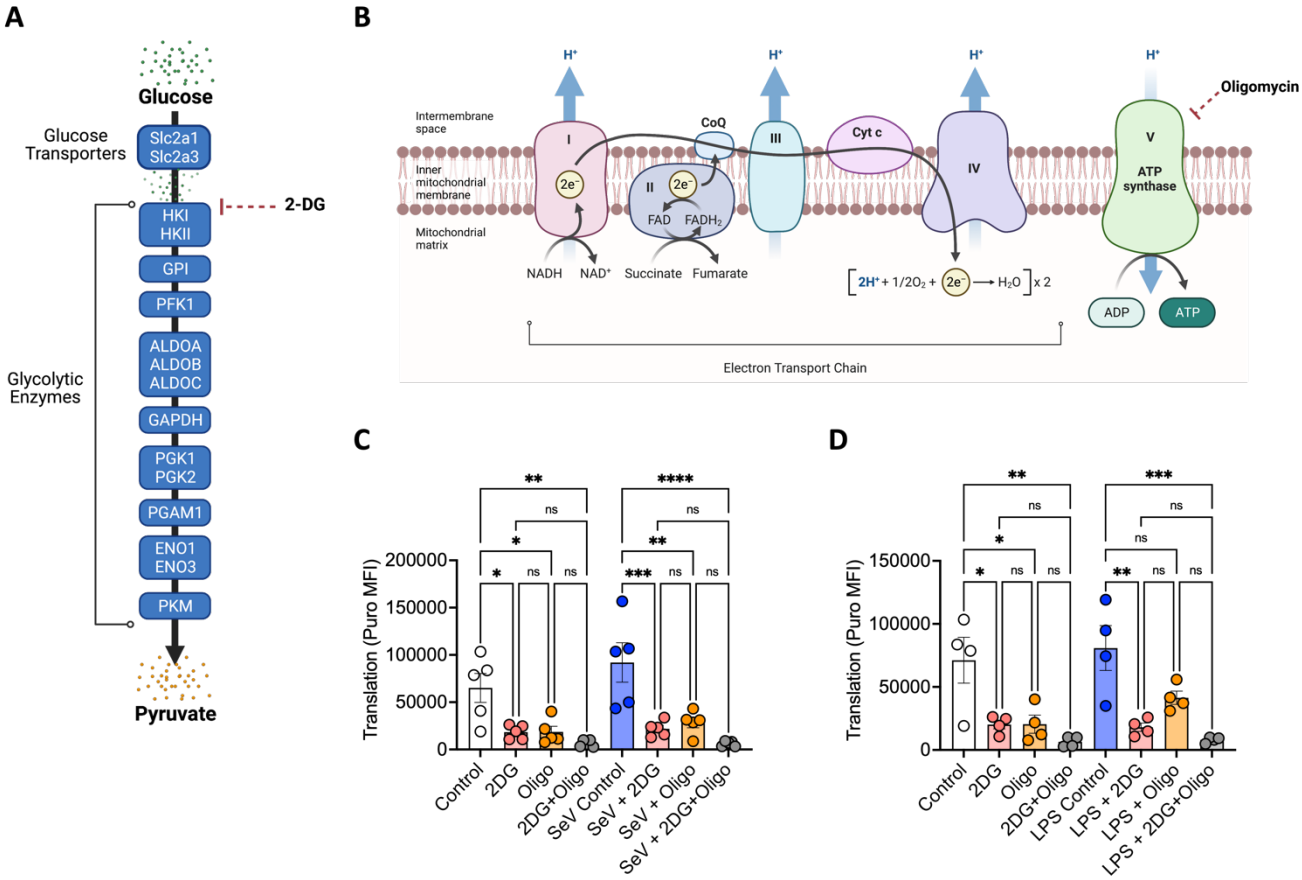


Figure 4.3 2-DG and Oligomycin inhibitors decreased protein translation (A) Simplified schematic of the glycolytic pathway displaying the inhibition of the first step of glucose metabolism by 2-DG. (B) Simplified schematic of OXPHOS displaying the inhibition of complex V, ATP synthase, by oligomycin (adapted from Biorender.com). (C & D) Scatter plot of puromycin incorporation (MFI) following 18-hour SeV (n=5) (C) or LPS (n=4) (D) stimulation in the presence or absence of 2-DG or oligomycin or both. Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction, ns=p>0.05, *p≤0.05, **p≤0.01, ***p≤0.001.

ALDOA= aldolase isozyme A, ALDOB= aldolase isozyme B, ALDOC= aldolase isozyme C, ENO1= enolase isozyme 1, ENO3= enolase isozyme 3, GAPDH= Glyceraldehyde 3-phosphate dehydrogenase, GPI= Glucose-6-phosphate isomerase, HKI= hexokinase isoenzyme 1, HKII= hexokinase isozyme 2, PFK1= phosphofructokinase isozyme 1, PGAM1= Phosphoglycerate Mutase isozyme 1, PGK1= phosphoglycerate kinase isozyme 1, PGK2= phosphoglycerate kinase isozyme 2, PKM= pyruvate kinase muscle isozyme, Slc2a1= solute carrier family 2 member 1, Slc2a3= solute carrier family 2 member 3. ADP= Adenosine diphosphate, ATP= Adenosine triphosphate, CoQ= coenzyme Q, Cyt c= cytochrome c, e^-= free electron, FAD= oxidized flavin adenine dinucleotide, $FADH_2$= reduced flavin adenine dinucleotide, H^+= hydrogen ion, H_2O= water molecule, I= complex I, II= complex II, III= complex III, IV= complex IV, NAD^+= oxidised nicotinamide adenine dinucleotide, NADH= reduced nicotinamide adenine dinucleotide, O_2= oxygen molecule, V= complex V.

4.3.3 SeV and LPS stimulation resulted in different metabolic dependencies and capacities

Based on puromycin MFI values obtained following addition of the different inhibitors, we next calculated the metabolic dependencies and capacities, as per Table 2.9 in materials & methods. Glucose dependence refers to the proportion of protein synthesis (and energy production) reliant on oxidation of glucose. Mitochondrial dependence refers to the proportion of protein synthesis (and energy production) reliant on OXPHOS. Glycolytic capacity is when OXPHOS is inhibited, the maximum capacity of the cell to support protein synthesis. Fatty acid and amino acid (AA) oxidation (FAO & AAO) capacity is when glucose oxidation is inhibited, the capacity of the cell to support protein synthesis/ATP production using fatty acids and amino acids (Argüello *et al.*, 2020). We calculated the aforementioned for untreated and SeV- or LPS-stimulated GM-DCs (Figure 4.4).

Glucose dependence of untreated GM-DCs was approximately 70% (Figures 4.4A & B), and upon stimulation, there was a slight increase for both stimulations, especially for LPS-stimulated GM-DCs (Figure 4.4B). These graphs show that GM-DCs are reliant upon glucose oxidation basally and upon stimulation to almost the same high extent.

GM-DCs were reliant upon OXPHOS basally (Figures 4.4C & D), to the same extent as glucose dependence, approximately 70%. Although upon stimulation, mitochondrial dependence was different depending on the stimulation. SeV stimulated GM-DCs were equally as reliant upon OXPHOS as untreated (Figure 4.4C), however, LPS stimulated GM-DCs had a significant decrease in mitochondrial dependence (Figures 4.4D). This correlated with the significant increase in glycolytic capacity observed in LPS-stimulated GM-DCs compared to untreated (Figure 4.4F). Therefore, GM-DCs following LPS stimulation are less reliant upon oxidative phosphorylation but rather other metabolic pathways to sustain the cell. SeV stimulated GM-DCs on the other hand have similar glycolytic capacity compared to its respective untreated (Figure 4.4E). GM-DCs following SeV stimulation are equally reliant on glucose oxidation and OXPHOS as untreated GM-DCs, unlike LPS-stimulated GM-DCs. As FAO & AAO capacity is calculated as the inverse of glucose dependence, a slight decrease is observed following stimulation compared to untreated (Figures 4.4G & H). The decrease is more noticeable following LPS stimulation of GM-DCs (Figure 4.4H). However, due to skewing of untreated samples, this may have affected statistical analyses, an increase in samples may have

demonstrated whether this trend is real. Basally there is slightly higher oxidation of fatty acids and amino acids than following stimulation, especially in LPS stimulated GM-DCs (Figure 4.4H).

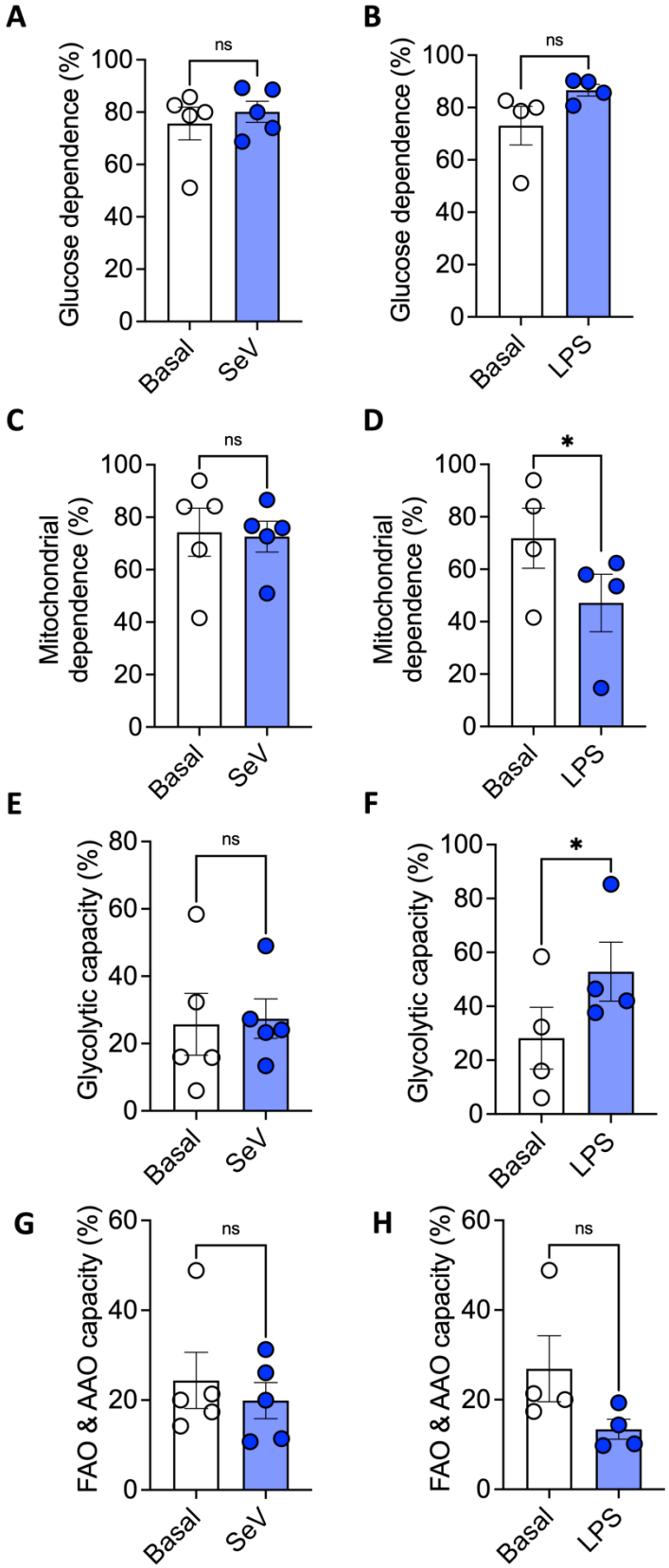


Figure 4.4 (Figure legend on the next page)

Figure 4.4 SeV and LPS stimulation favoured different metabolic pathways Scatter plot of (A & B) Glucose dependence of GM-DCs following 18-hour SeV (n=5) (A) or LPS stimulation (n=4) (B). (C & D) Mitochondrial dependence of GM-DCs following 18-hour SeV (n=5) (C) or LPS stimulation (n=4) (D). (E & F) Glycolytic capacity of GM-DCs following 18-hour SeV (n=5) (E) or LPS stimulation (n=4) (F). (G & H) Fatty acid oxidation (FAO) and amino acid oxidation (AAO) capacity of GM-DCs following 18-hour SeV (n=5) (G) or LPS stimulation (n=4) (H). Statistical analysis performed using paired student's t-test or Wilcoxon test, ns=$p>0.05$, *$p\leq0.05$.

4.3.4 2-DG reduced cytokine production following LPS stimulation

We previously showed that 2-DG inhibited protein synthesis following LPS stimulation (Figure 4.3D). Next, we wanted to investigate the effect of this glycolysis inhibitor on protein production, by measuring GM-DC cytokine production following LPS stimulation. We stimulated GM-DCs in the presence of 2-DG and measured cytokines in the supernatants via ELISAs (Figure 4.5). LPS stimulation increased cytokine production (Figure 3.9), the addition of 2-DG, resulted in a significant decrease in all cytokine production, TNFα (Figure 4.5A), IL-12p70 (Figure 4.5B), IL-6 (Figure 4.5C) and IL-10 (Figures 4.5D).

There was approximately a third of a decrease in TNFα production in the presence of 2-DG (Figure 4.5A). For IL-12p70, there was almost no IL-12p70 being produced in the presence of 2-DG, however, there were low levels of IL-12p70 being produced following LPS stimulation to begin with (Figure 4.5B). IL-6 production was halved in the presence of 2-DG (Figure 4.5C). There was approximately two-thirds of a decrease in IL-10 production in the presence of 2-DG (Figure 4.5D). Despite the addition of 2-DG, cytokines were still produced following LPS stimulation but not to the same extent as when it was absent, except in the case of IL-12p70 (Figures 4.5), where almost no IL-12p70 was produced in the presence of 2-DG. Therefore, glycolysis is required for optimal cytokine production.

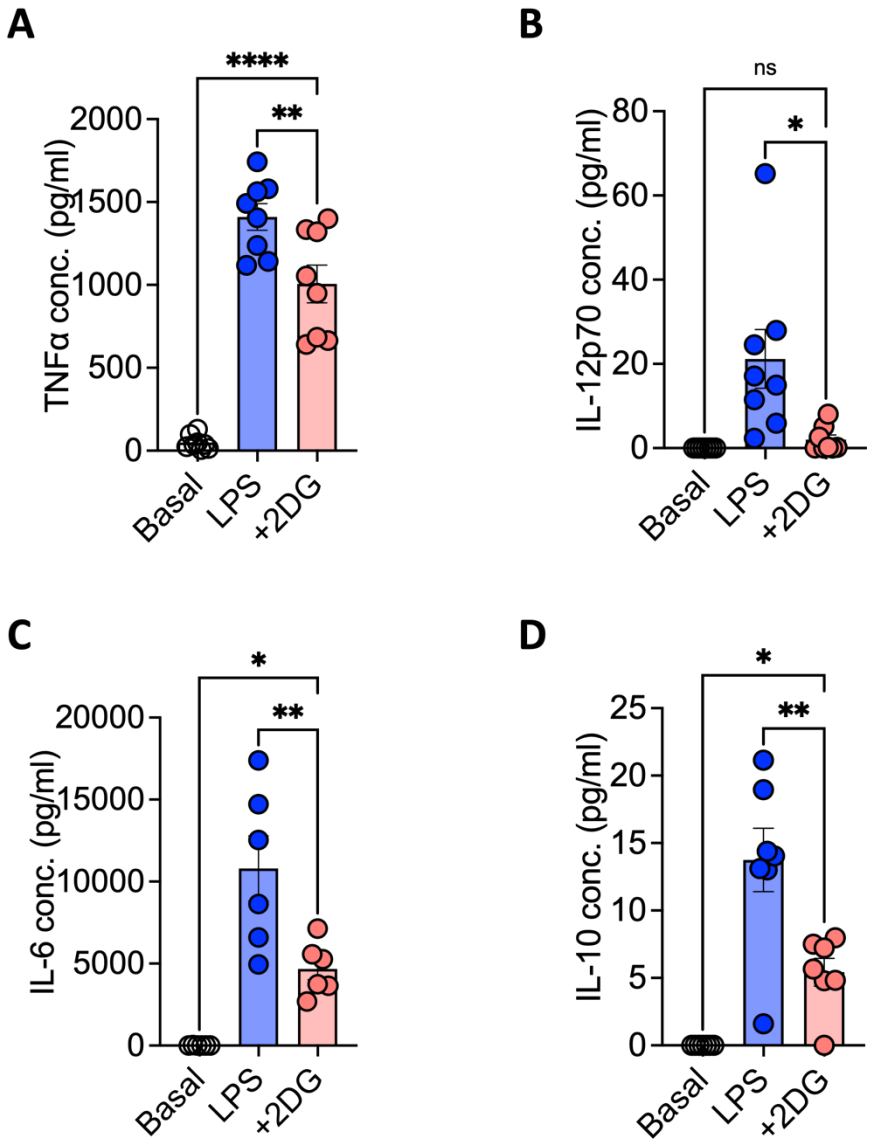


Figure 4.5 Hexokinase inhibitor 2-DG reduced GM-DC cytokine production following LPS stimulation (A-D) Scatter plot of TNFα (n=8) (A), IL-12p70 production (n=8) (B), IL-6 (n=6) (C) or IL-10 (n=7) production (pg/ml) following 18-hour LPS stimulation in the presence or absence of 2-DG. Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction or Kruskal-Wallis with Dunn's test where appropriate, ns=p>0.05, *p≤0.05, **p≤0.01, ****p<0.0001.

4.3.5 2-DG reduced cytokine mRNA expression following SeV stimulation

mRNA transcription precedes protein translation; hence, we studied the effects of the metabolic inhibitors at an earlier timepoint prior to protein quantification. We previously observed peak cytokine mRNA levels at 6 hours after stimulation (Figure 3.8). Therefore, we stimulated GM-DCs with SeV for 6 hours, in the presence of 2-DG, to determine the effect of glycolysis inhibition on mRNA expression. GM-DC expression of *Tnf* (Figure 3.8B)*, Il12b* (Figure 3.8E)*, Il6* (Figure 3.8H) and *Ifnb1* (Figure 3.8K) increased following 6-hour SeV stimulation. We now show the effect of 2-DG on GM-DC 6-hour SeV stimulation (Figure 4.6).

All cytokine mRNA expression significantly decreased in the presence of 2-DG (Figures 4.6A – C), except for *Ifnb1* where there is a trending decrease (Figure 4.6D). Depicting the need for glycolysis to generate appropriate functional responses, in this case, the transcription of genes normally activated upon SeV stimulation. In the case of *Tnf* and *Il12b*, the presence of 2-DG & SeV results in levels similar to basal GM-DCs of the respective cytokine, there is no significant difference between these two conditions (Figure 4.6A and B). For *Il6*, there is a slight increase in mRNA levels in the presence of 2-DG & SeV compared to basal, but not to the same extent as in SeV stimulation only (Figure 4.6C). For *Ifnb1*, there was an increase in mRNA levels in the presence of 2-DG & SeV compared to basal, except due to a range in the data, the difference is not as stark compared to SeV alone and untreated (Figure 4.6D).

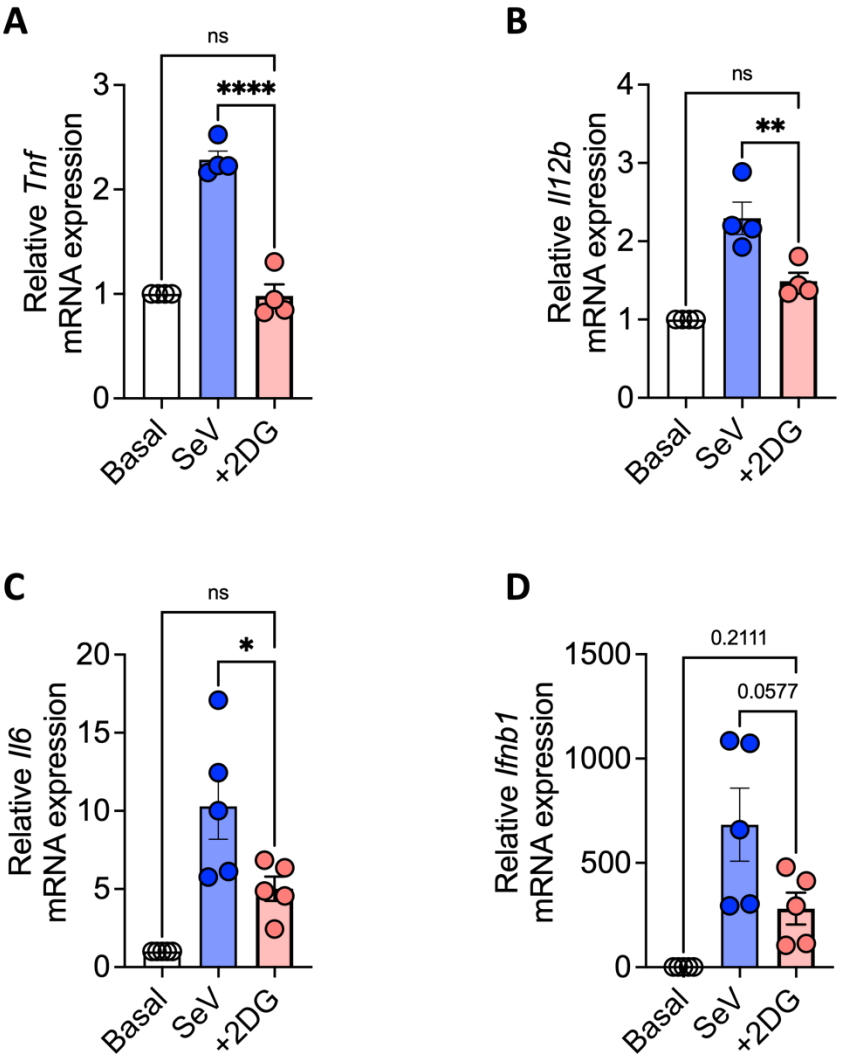


Figure 4.6 Hexokinase inhibitor 2-DG reduced GM-DC cytokine mRNA expression following 6-hour SeV stimulation (A-D) Scatter plot of *Tnf* (n=4) (A), *Il12b* (n=4) (B), *Il6* (n=5) (C) or *Ifnb1* (n=5) (D) mRNA expression relative to *Actb* following 6-hour SeV stimulation in the presence or absence of 2-DG. Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction, ns=p>0.05, *p≤0.05, **p≤0.01, ****p<0.0001.

4.3.6 2-DG reduced cytokine production following SeV stimulation

We demonstrated the reduction in mRNA expression by 2-DG (Figure 4.6). Next, we investigated the effect of 2-DG on subsequent protein production. We stimulated GM-DCs with SeV for 18 hours in the presence of 2-DG and cytokines were quantified in the supernatants. 2-DG resulted in the significant decrease of all cytokines (Figure 4.7). There was complete abrogation of IFNβ production by 2-DG (Figure 4.7C), as opposed to TNFα (Figure 4.7A) and IL-6 (Figure 4.7B) where there was still production of the respective cytokine in the presence of 2-DG, albeit lower. Therefore, expression (Figure 4.6) and production (Figure 4.7) of these cytokines are heavily reliant on glycolysis, particularly the production of IFNβ.

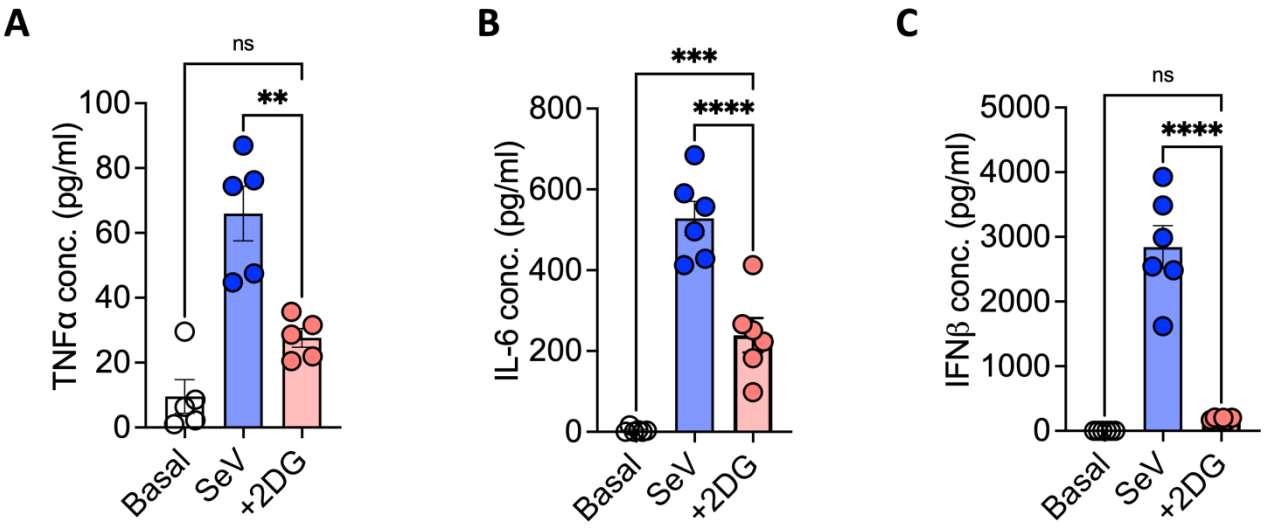


Figure 4.7 Hexokinase inhibitor 2-DG reduced GM-DC cytokine production following SeV stimulation (A-C) Scatter plot of TNFα (n=5) (A), IL-6 (n=6) (B) or IFNβ (n=6) (C) production (pg/ml) following 18-hour SeV stimulation in the presence or absence of 2-DG. Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction, ns=p>0.05, **p≤0.01, ****p<0.0001.

4.3.7 Heptelidic acid reduced cytokine production following SeV stimulation

Glycolysis is a pathway that yields substrates which can enter other metabolic pathways, such as the pentose phosphate pathway (PPP), fatty acid synthesis, amino acid synthesis and so on. 2-DG inhibits the first step of glycolysis which would affect all subsequent pathways where glucose oxidation is necessary. We investigated whether the effects observed in the presence of 2-DG, was due to the reliance on glycolysis or inhibitor-specific, to do so we utilised other glycolysis inhibitors. Heptelidic acid inhibits GAPDH (Endo *et al.*, 1985), the enzyme in the sixth step of glycolysis which converts glyceraldehyde 3-phosphate (GA3P) to 1,3-bisphosphoglycerate (1,3-BPG) (Figure 4.8A). We stimulated GM-DCs with SeV, in the presence of heptelidic acid.

We demonstrated significant reduction in cytokine production following 18-hour SeV stimulation, in the presence of heptelidic acid (Figures 4.8B – D). The inhibitor concentration used was 5µM, based off previous studies (Salabei *et al.*, 2015; Galván-Peña *et al.*, 2019). For TNFα and IL-6, their respective production was inhibited by heptelidic acid (Figures 4.8B and C) to a similar extent as 2-DG (Figures 4.7A and B). IFNβ production was inhibited by heptelidic acid (Figure 4.8D) but not to the same extent as 2-DG (Figure 4.7C). Using multiple inhibitors, we show the requirement of glycolysis for functional GM-DC responses. In the presence of heptelidic acid and SeV, there was still higher cytokine production compared to basal GM-DCs, except in the case of TNFα, where production was somewhat comparable to untreated GM-DCs.

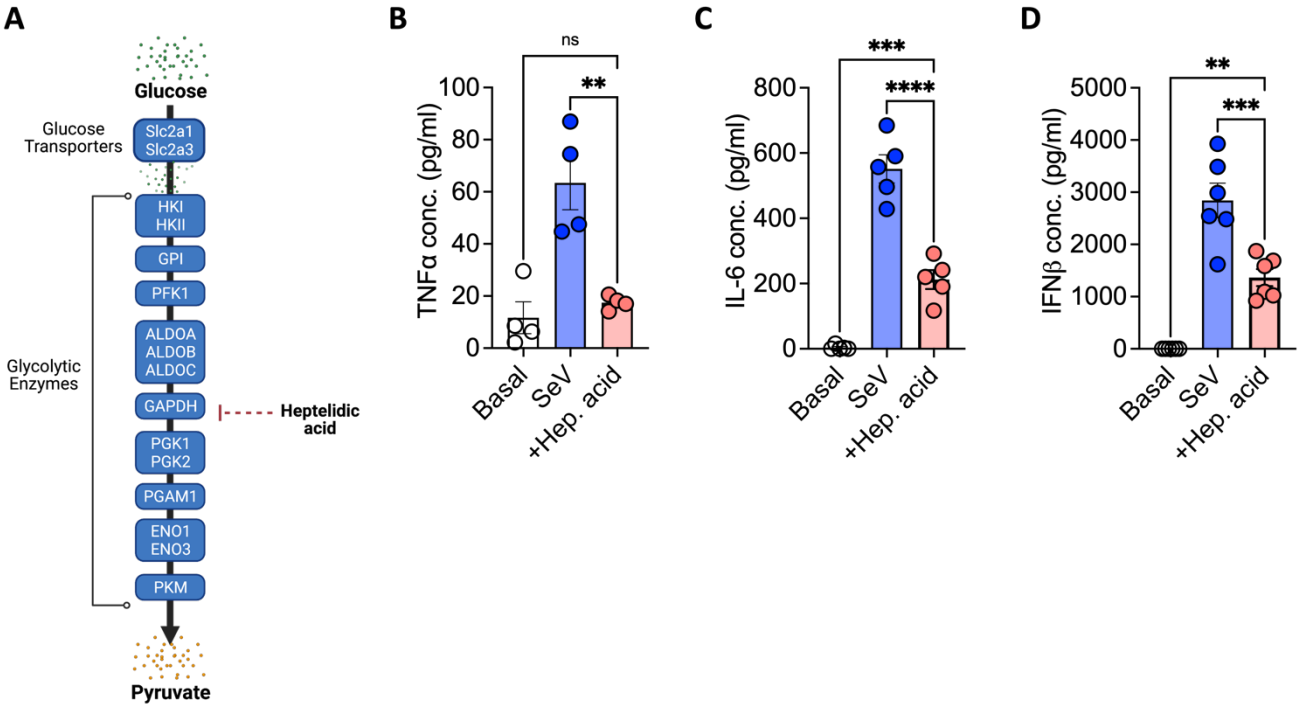


Figure 4.8 GAPDH inhibitor Heptelidic acid reduced GM-DC cytokine production (A) Schematic of the glycolytic pathway displaying the inhibition of the sixth step of glucose metabolism by heptelidic acid. (B-D) Scatter plot of TNFα (n=4) (B), IL-6 (n=5) (C) or IFNβ (n=6) (D) production (pg/ml) following 18-hour SeV stimulation in the presence or absence of heptelidic acid. Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction, ns=p>0.05, **p≤0.01,*** p≤ 0.001, ****p<0.0001.

ALDOA= aldolase isozyme A, ALDOB= aldolase isozyme B, ALDOC= aldolase isozyme C, ENO1= enolase isozyme 1, ENO3= enolase isozyme 3, GAPDH= Glyceraldehyde 3-phosphate dehydrogenase, GPI= Glucose-6-phosphate isomerase, HKI= hexokinase isoenzyme 1, HKII= hexokinase isozyme 2, PFK1= phosphofructokinase isozyme 1, PGAM1= Phosphoglycerate Mutase isozyme 1, PGK1= phosphoglycerate kinase isozyme 1, PGK2= phosphoglycerate kinase isozyme 2, PKM= pyruvate kinase muscle isozyme, Slc2a1= solute carrier family 2 member 1, Slc2a3= solute carrier family 2 member 3.

4.3.8 AZ67 had no effect on cytokine production following SeV stimulation

PFKFB3 converts fructose-6-phosphate (F6P) to fructose-2,6-bisphosphate (F-2,6-BP), and vice versa (Bartrons *et al.*, 1983). F-2,6-BP is a potent activator of phosphofructo-1-kinase (PFK1) (Van Schaftingen *et al.*, 1981) which is required for the third step of glycolysis, the phosphorylation of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F-1,6-BP). This step is referred to as the first committed glycolytic step. 3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) has been used as an inhibitor of PFKFB3 in previous studies (Clem *et al.*, 2008; Kotowski *et al.*, 2020), however, it was demonstrated that it led to side effects, such as lactic acid accumulation, and did not bind to PFKFB3 (Emini Veseli *et al.*, 2020). Other PFKFB3 inhibitors have been developed, including a molecule referred to as AZ PFKFB3 67 (AZ67) which was demonstrated to be a selective inhibitor (Emini Veseli *et al.*, 2020).

First, we optimised the concentration of AZ67 to use for experiments. We exposed GM-DCs to different AZ67 concentrations. Cell viability and their effect on cytokine production was determined. We utilised the highest concentration of AZ67 which did not significantly kill cells for subsequent experiments, which was 10µM. AZ67 targets the PFKFB3 enzyme in the glycolytic pathway (Figure 4.9A). We stimulated GM-DCs with SeV, in the presence of AZ67. We demonstrated that AZ67 did not alter cytokine production of TNFα (Figure 4.9B), IL-6 (Figure 4.9C) and IFNβ (Figure 4.9D). Cytokine production was comparable to SeV stimulation only. Compared to the previous two glycolytic inhibitors, AZ67 did not reduce cytokine production. However, AZ67 indirectly inhibited a step of glycolysis, unlike 2-DG and heptelidic acid which inhibited the enzymes directly involved in their respective steps.

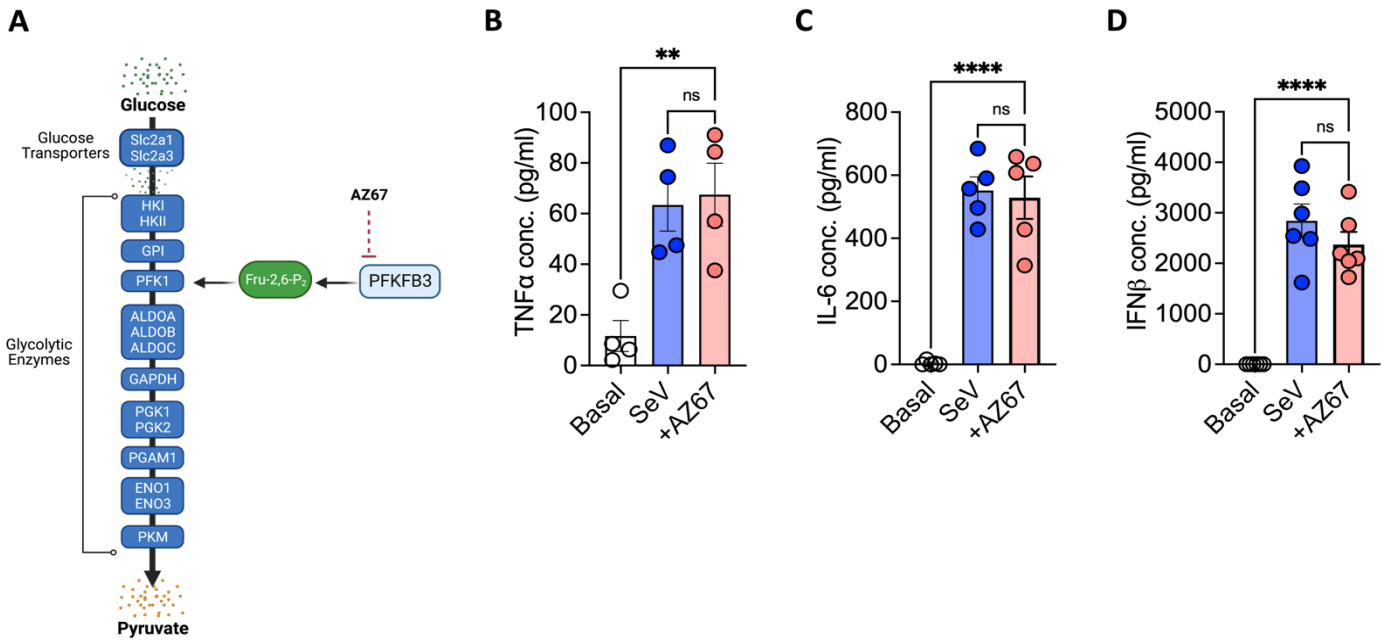


Figure 4.9 PFKFB3 inhibitor AZ67 had no effect on GM-DC cytokine production (A) Schematic of the glycolytic pathway displaying the indirect inhibition of the third step of glucose metabolism by AZ67. (B-D) Scatter plot of TNFα (n=4) (B), IL-6 (n=5) (C) or IFNβ (n=6) (D) production (pg/ml) following 18-hour SeV stimulation in the presence or absence of AZ67. Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction, ns=p>0.05, **p≤0.01, ****p<0.0001.

ALDOA= aldolase isozyme A, ALDOB= aldolase isozyme B, ALDOC= aldolase isozyme C, ENO1= enolase isozyme 1, ENO3= enolase isozyme 3, Fru-2,6-P_2= fructose-2,6-bisphosphate, GAPDH= Glyceraldehyde 3-phosphate dehydrogenase, GPI= Glucose-6-phosphate isomerase, HKI= hexokinase isoenzyme 1, HKII= hexokinase isozyme 2, PFKFB3= phosphofructokinase-2/fructose-2,6-bisphosphatase isoform 3, PFK1= phosphofructokinase isozyme 1, PGAM1= Phosphoglycerate Mutase isozyme 1, PGK1= phosphoglycerate kinase isozyme 1, PGK2= phosphoglycerate kinase isozyme 2, PKM= pyruvate kinase muscle isozyme, Slc2a1= solute carrier family 2 member 1, Slc2a3= solute carrier family 2 member 3.

4.3.9 Oligomycin caused an increasing trend in GM-DC cytokine mRNA expression following 3-hour LPS stimulation, with a significant increase in *Il12b* and *Il10*

As previously mentioned, oligomycin serves as an inhibitor of OXPHOS. We previously demonstrated that oligomycin reduced GM-DC protein synthesis following 18-hour LPS stimulation (Figure 4.3D). mRNA transcription precedes protein translation, hence, the effect of inhibitors was studied at an earlier timepoint prior to protein quantification. We stimulated GM-DCs with LPS for 3 hours, in the presence of oligomycin, to determine the effect of OXPHOS inhibition on mRNA expression.

We observed an increasing trend in cytokine mRNA expression, in the presence of oligomycin (Figure 4.10). The increase was much less noticeable for *Tnf* and *Il6* (Figures 4.10A and C). However, *Il12b* (Figure 4.10B) and *Il10* (Figure 4.10D) expression following stimulation more than doubled in the presence of oligomycin. Therefore, inhibiting OXPHOS within the first 3 hours of LPS stimulation, increases gene transcription, especially of *Il12b* and *Il10*. In all cases (Figures 4.10A - D), LPS-stimulated GM-DCs, in the presence of oligomycin, have greater cytokine mRNA expression compared to untreated GM-DCs.

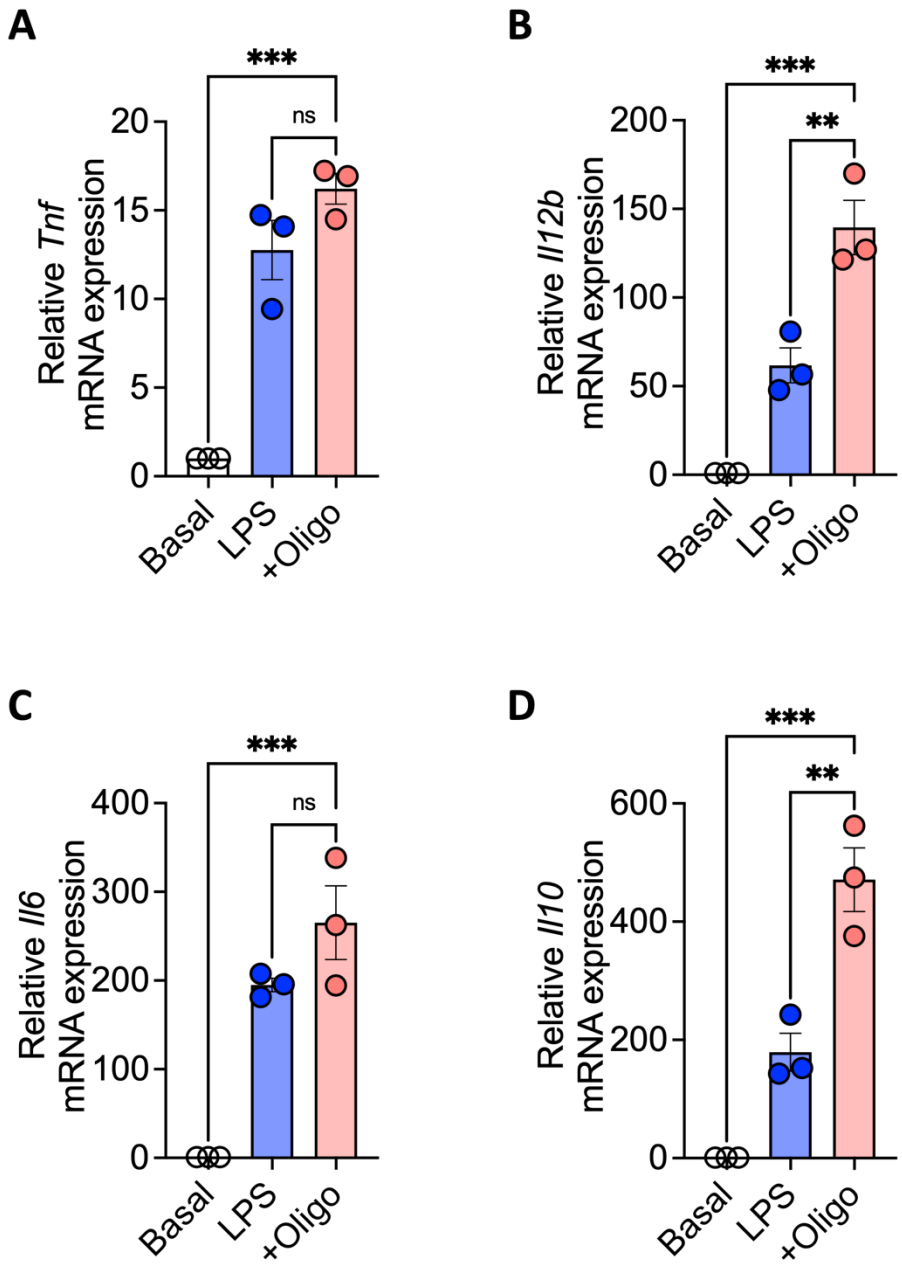


Figure 4.10 ATP synthase inhibitor oligomycin increased GM-DC *Il12b* and *Il10* cytokine mRNA expression following 3-hour LPS stimulation (A-D) Scatter plot of *Tnf* (A), *Il12b* (B), *Il6* (C) or *Il10* (D) mRNA expression relative to *Hprt* following 3-hour LPS stimulation in the presence or absence of oligomycin (n=3). Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction, ns=p>0.05, ** p≤0.01, ***p≤0.001.

4.3.10 Oligomycin reduced GM-DC TNFα cytokine production, but not other functional cytokines, following 18-hour LPS stimulation

Oligomycin addition during LPS stimulation increased mRNA expression of certain cytokines (Figure 4.10). Next, we sought to investigate its effect on cytokine protein production. We stimulated GM-DCs for 18 hours with LPS, in the presence of oligomycin to determine the effect of this OXPHOS inhibitor on protein production. We measured cytokine levels in the resulting supernatants (Figure 4.11).

LPS stimulation increased GM-DC cytokine production (Figure 3.9). The addition of oligomycin resulted in a significant reduction in TNFα production (Figure 4.11A). However, for all other cytokines, IL-12p70 (Figure 4.11B), IL-6 (Figure 4.11C) and IL-10 (Figure 4.11D), addition of oligomycin resulted in cytokine production comparable to LPS stimulation only. Therefore, despite the trending increase in mRNA expression in the following 3-hour LPS stimulation, in the presence of oligomycin, after 18-hour LPS stimulation, this did not translate to the protein level. LPS stimulation and the presence of oligomycin still resulted in a significant increase in cytokines compared to untreated GM-DCs.

Inhibition of OXPHOS did not affect protein production (Figure 4.11), except in the case of TNFα where there was a decrease but still quite a large amount was produced. This correlated with the decrease in mitochondrial dependence and increase in glycolytic capacity after LPS stimulation (Figures 4.4D and F respectively). The decrease in all cytokine production in the presence of 2-DG portrays the importance of glucose oxidation through other pathways, rather than OXPHOS.

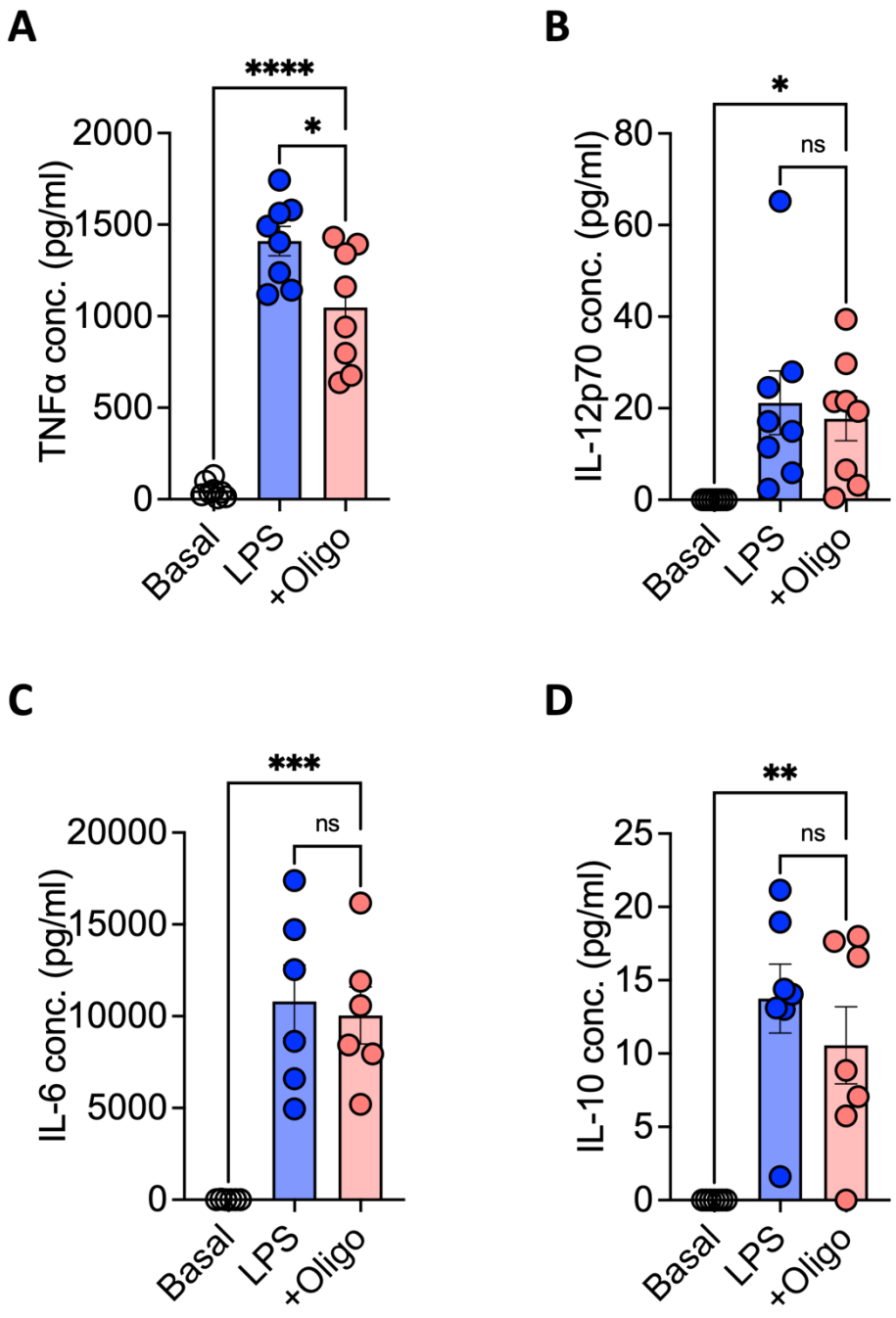


Figure 4.11 ATP synthase inhibitor oligomycin reduced GM-DC TNFα cytokine production, but not other functional cytokines, following 18-hour LPS stimulation (A-D) Scatter plot of TNFα (n=8) (A), IL-12p70 (n=8) (B), IL-6 (n=6) (C) or IL-10 (n=7) (D) production (pg/ml) following 18-hour LPS stimulation in the presence or absence of oligomycin. Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction, ns=p>0.05, *p≤0.05, **p≤0.01, *** p≤ 0.001, ****p<0.0001.

4.3.11 Oligomycin caused an increasing trend in GM-DC cytokine mRNA expression, following 6-hour SeV stimulation, with a significant increase in *Il12b*

Oligomycin reduced GM-DC protein synthesis following 18-hour SeV stimulation (Figure 4.3C). We previously showed with various SeV timepoints that peak mRNA level occurred at 6 hours (Figure 3.8). Hence, we stimulated GM-DCs with SeV for 6 hours, in the presence of oligomycin, to determine the effect of OXPHOS inhibition on mRNA expression.

An increasing trend in cytokine mRNA expression was observed (Figure 4.12). *Il12b* mRNA expression was significantly increased in the presence of oligomycin compared to SeV stimulation only (Figure 4.12B). The increase was much less noticeable for *Tnf* and *Il6* (Figures 4.12A and C), this could possibly be ameliorated with an increase in sample numbers. Based on Figure 4.12D, we observed that mean *Ifnb1* levels, in oligomycin-treated GM-DCs, almost doubled compared to SeV-stimulated GM-DCs only (Figure 4.12D). However, due to the data spread, this may have affected statistical analysis. Increasing sample numbers would possibly prove whether the trend was real.

Inhibiting OXPHOS during 6-hour SeV stimulation increased gene transcription of *Il12b,* with a trending increase in *Tnf* and *Ifnb1*. The inhibition of glycolysis with 2-DG decreased mRNA expression of *Il12b* (Figure 4.6B), signifying the importance of glucose oxidation, but not OXPHOS during the first 6 hours for mRNA expression of certain cytokines. In all cases (Figures 4.12A – D), SeV-stimulated GM-DCs, in the presence of oligomycin, have greater cytokine mRNA expression compared to untreated GM-DCs.

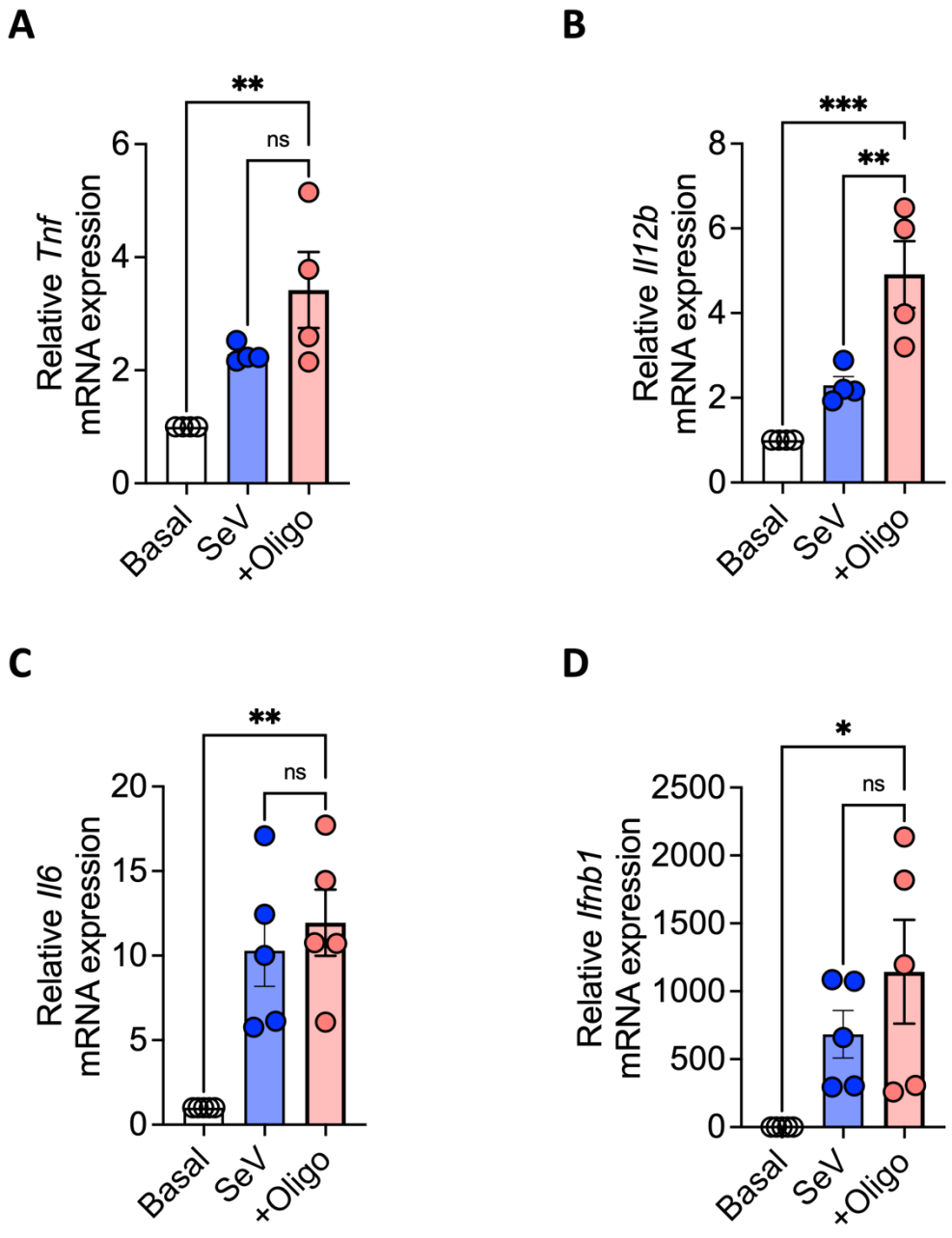


Figure 4.12 ATP synthase inhibitor oligomycin significantly increased GM-DC *Il12b* cytokine mRNA expression, but not other functional cytokines, following 6-hour SeV stimulation
(A-D) Scatter plot of *Tnf* (n=4) (A*), Il12b* (n=4) (B*), Il6* (n=5) (C) or *Ifnb1* (n=5) (D) mRNA expression relative to *Actb* following 6-hour SeV stimulation in the presence or absence of oligomycin. Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction, ns=p>0.05, *p≤0.05, **p≤0.01, ***p≤0.001.

4.3.12 Oligomycin had no effect on cytokine production following SeV stimulation

Oligomycin addition during SeV stimulation increased mRNA expression of *Il12b,* with a trending increase of *Tnf* and *Ifnb1* (Figure 4.12). Next, we sought to investigate its effect on cytokine protein production. We stimulated GM-DCs for 18 hours with SeV, in the presence of oligomycin, to determine the effect of this OXPHOS inhibitor on protein production. We measured cytokine levels in the resulting supernatants (Figure 4.13).

SeV stimulation increased GM-DC cytokine production (Figure 3.10). Here, we show that the addition of oligomycin during 18-hour SeV stimulation, had no effect on GM-DC cytokine production of TNFα, IL-6 and IFNβ, compared to SeV stimulation only (Figures 4.13A – C respectively). Therefore, despite the trending increase in mRNA expression at 6 hours (Figure 4.12), after 18-hour SeV stimulation and oligomycin, this was not translated to the protein level. SeV stimulation and the presence of oligomycin still resulted in a significant increase in cytokines compared to untreated GM-DCs.

In the presence of 2-DG, there was decreased cytokine production (Figure 4.7). This portrayed a requirement for glycolysis for cytokine production, especially IFNβ production, but as OXPHOS inhibition did not affect cytokine protein production (Figure 4.13), glucose oxidation can occur through other pathways downstream of glycolysis circumventing the blocking of OXPHOS.

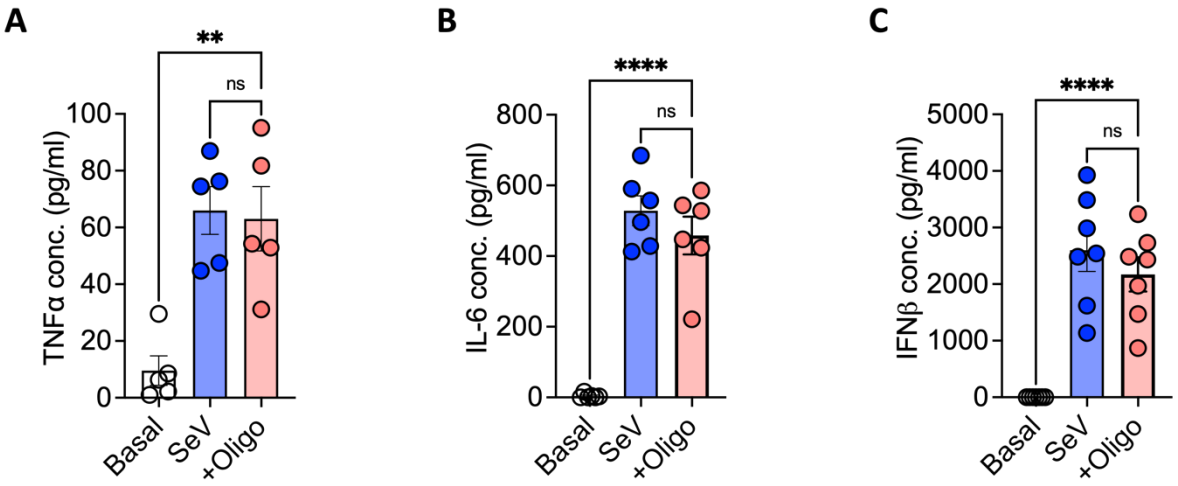


Figure 4.13 ATP synthase inhibitor oligomycin had no effect on GM-DC cytokine production following 18-hour SeV stimulation (A-C) Scatter plot of TNFα (n=5) (A), IL-6 (n=6) (B) or IFNβ (n=7) (C) production (pg/ml) following 18-hour SeV stimulation in the presence or absence of oligomycin. Statistical analysis performed using paired Ordinary one-way ANOVA with Tukey's correction, ns=p>0.05, **p≤0.01, ***p<0.0001.

4.3.13 CP91149 significantly decreased IL-10 production following LPS stimulation, with minimal effects on other functional cytokines

Whilst initially investigating the impact of metabolic inhibitors on GM-DC function, the effect of the glycogen phosphorylase inhibitor, CP91149, on GM-DC cytokine production was investigated. Previous work by Thwe *et al.* (2017) demonstrated the importance of glycogen for early DC responses. Glycogen phosphorylase releases glucose-1-phosphate monomers from glycogen stores, which is subsequently converted to glucose-6-phosphate (G6P) by phosphoglucomutase (Burke *et al.*, 1987; Adeva-Andany *et al.*, 2016). G6P can enter glycolysis for further oxidation. CP91149 targets glycogen phosphorylase, inhibiting glycogenolysis (Figure 4.14A).

We stimulated GM-DCs with LPS for 18 hours, in the presence of CP91149. We demonstrated that CP91149 had no effect on TNFα (Figure 4.14B) and IL-12p70 (Figure 4.14C) production. However, a decrease in IL-10 was observed in the presence of CP91149 (Figure 4.14D). LPS stimulation and the presence of CP91149 still resulted in a significant increase in all cytokines compared to untreated GM-DCs.

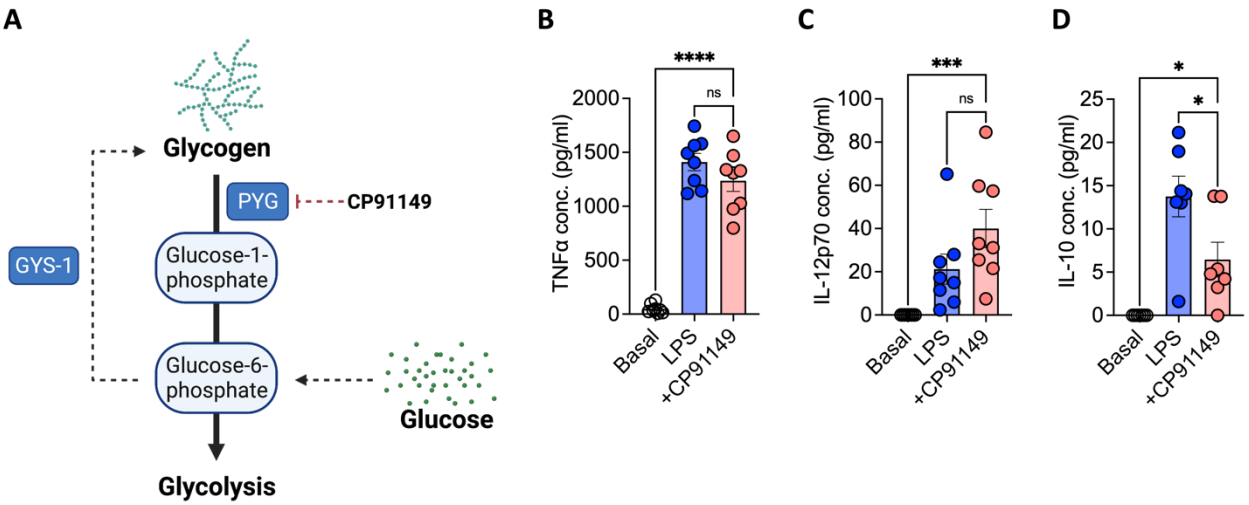


Figure 4.14 Glycogen phosphorylase inhibitor CP91149 significantly decreased IL-10 production following LPS stimulation, with minimal effects on other functional cytokines

(A) Simplified schematic of glycogenolysis displaying the inhibition of glycogen breakdown by CP91149. (B-D) Scatter plot of TNFα (n=8) (B), IL-12p70 (n=8) (C) or IL-10 (n=7) (D) production (pg/ml) following 18-hour LPS stimulation in the presence or absence of CP91149. Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction, ns=p>0.05, *p≤0.05, ***p≤0.001, ****p<0.0001.

GYS-1=glycogen synthase 1, PYG= glycogen phosphorylase.

4.3.14 Low glucose had no impact on GM-DC cytokine production

So far, we have shown that metabolic inhibitors have impacted GM-DCs differently, in terms of cytokine mRNA expression and protein production. Previous work from Lawless *et al.* (2017) in GM-DCs demonstrated the varied effects of galactose on their function. Previous work from Jones *et al.* (2021) in bone marrow-derived macrophages (BM-DMs) and monocytes, demonstrated the pro-inflammatory effects of other carbon sources on their function.

We sought to investigate whether altering the carbon source impacted GM-DC function. GM-DCs were cultured in varied carbon sources, as per section 2.5. On day 10 of growth, we harvested GM-DCs, re-suspended the cells in the respective specified media and the cells were allowed to rest for a few hours. Here, GM-DCs were stimulated in low glucose (1mM) or normal media (11mM). We subsequently stimulated the cells with LPS for 18 hours. Cytokine production was quantified in the resulting supernatants (Figure 4.15).

We observed no difference in cytokine production between GM-DCs in low or high glucose, both basally and during LPS stimulation (Figure 4.15). LPS stimulation of GM-DCs in low glucose media resulted in a significant increase of cytokines compared to basal, TNFα (Figure 4.15A) and IL-10 (Figure 4.15C). IL-12p70 concentrations in low glucose cultured GM-DCs ranged and could possibly be impacting statistical analysis, but we did observe an increase in its production (Figure 4.15B). Comparing low glucose cultured and normal glucose cultured conditions, we demonstrated no difference in GM-DC cytokine production.

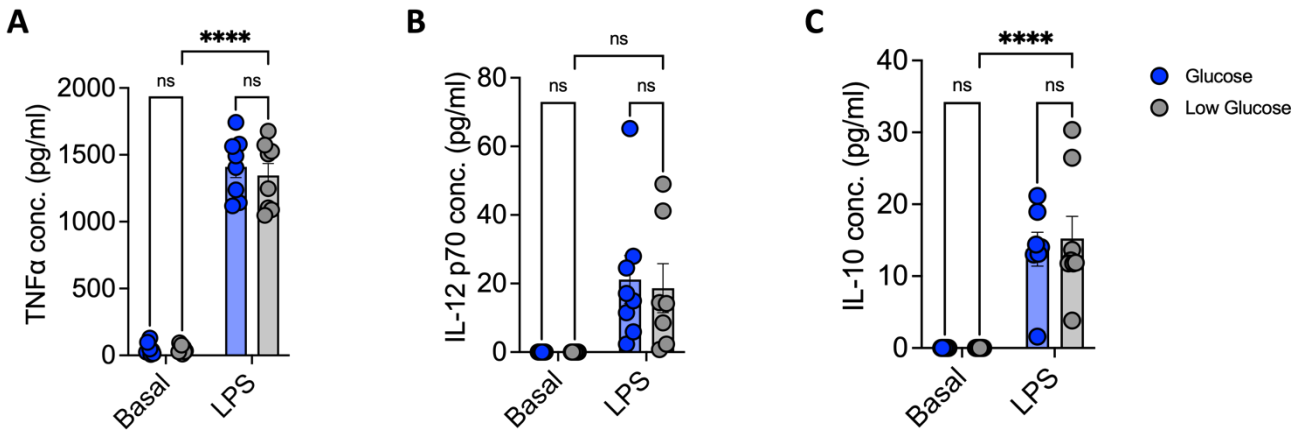


Figure 4.15 Low Glucose resulted in similar GM-DC cytokine production to normal glucose media basally & following LPS stimulation (A-C) Scatter plot of TNFα (n=8) (A), IL-12p70 (Glucose n=8, Low Glucose n=7) (B) or IL-10 (Glucose n=7, Low Glucose n=8) (C) production (pg/ml) in glucose (11mM) or low glucose (1mM) following 18-hour LPS stimulation. Statistical analysis performed using two-way ANOVA with Tukey's correction, ns=p>0.05, ****p<0.0001.

4.3.15 GM-DC cytokine production was largely unaffected in the presence of alternate carbon sources

Following GM-DC harvest, we re-suspended the cells in their respective medias and allowed them to rest. Cells were cultured in either their normal culture media with glucose (11mM), or fructose (10mM) or galactose (10mM). We subsequently stimulated the GM-DCs with LPS for 18 hours. Cytokines were quantified in the resulting supernatants (Figure 4.16).

Jaiswal *et al.* (2019) found that Mo-DCs treated with high fructose produced more pro-inflammatory cytokines compared to high glucose. In 2021, Jones and colleagues demonstrated an increase in cytokines produced by fructose-cultured monocytes compared to glucose-cultured, hence a more inflammatory phenotype. To this end, we investigated the effects of fructose on GM-DCs compared to those stimulated in normal glucose-containing media (Figures 4.16A – C). However, we found that fructose-cultured GM-DCs did not display altered cytokine production compared to glucose-cultured. TNFα (Figure 4.16A), IL-12p70 (Figure 4.16B) and IL-10 (Figure 4.16C) concentrations were comparable between glucose- and fructose- cultured GM-DCs following LPS stimulation. LPS stimulation of GM-DCs in fructose media resulted in a significant increase of cytokines compared to basal fructose condition, TNFα (Figure 4.16A) and IL-10 (Figure 4.16C). Except for IL-12p70 (Figure 4.16B), where low concentrations were measured in fructose media, which could possibly affect subsequent analysis, but we did observe an increase in its production.

We investigated the effects of galactose on GM-DCs compared to those stimulated in normal glucose-containing media (Figures 4.16D – F). Galactose is metabolised through a pathway known as the Leloir pathway (Caputto *et al.*, 1949), which induces low rates of glycolysis (Chang *et al.*, 2013). However, we demonstrate that after 18-hour LPS stimulation, galactose-cultured GM-DCs were able to produce cytokines to comparable levels as glucose-cultured cells, TNFα (Figure 4.16D) and IL-10 (Figure 4.16F). Except for IL-12p70 (Figure 4.16E), where its production is significantly reduced in galactose-cultured cells compared to glucose. 18-hour LPS stimulation of GM-DCs in galactose media also resulted in a significant increase of cytokines compared to basal galactose condition, TNFα (Figure 4.16D) and IL-10 (Figure 4.16F), except IL-12p70 where production was impaired in galactose-cultured GM-DCs (Figure 4.16E).

We demonstrate that GM-DCs display carbon flexibility and are able to utilise different carbon sources to replace glucose for cytokine production, one of their key functions. Except for IL-12p70 production in galactose media.

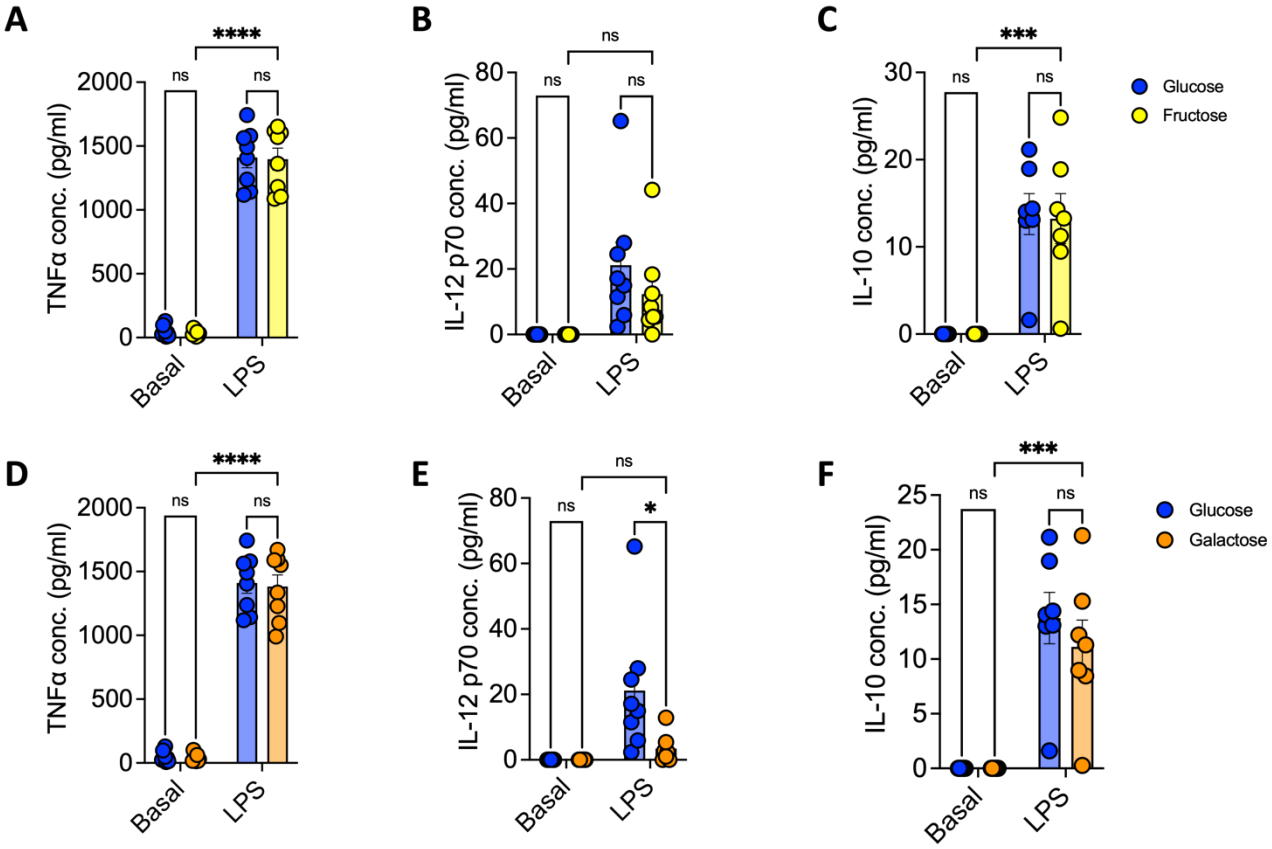


Figure 4.16 GM-DCs exhibited carbon source flexibility based on similar cytokine production when cultured in media with alternative carbon sources (A-C) Scatter plot of TNFα (n=8) (A), IL-12p70 (n=8) (B) or IL-10 (n=7) (C) production (pg/ml) in glucose (11mM) or fructose (10mM) following 18-hour LPS stimulation. (D-F) Scatter plot of TNFα (n=8) (D), IL-12p70 (Glucose n=8, Galactose n=7) (E) or IL-10 (n=7) (F) production (pg/ml) in glucose (11mM) or galactose (10mM) following 18-hour LPS stimulation. Statistical analysis performed using two-way ANOVA with Tukey's correction, ns=p>0.05, *p≤0.05, **p≤0.01, *** p≤ 0.001, ****p<0.0001.

4.3.16 Oligomycin impacted cell viability and cytokine production of fructose-cultured GM-DCs

Jones *et al.* (2021) demonstrated a favouring of oxidative metabolism in monocytes cultured in fructose. Most monocytes cultured in fructose underwent apoptosis following the addition of oligomycin compared to no significant decrease in monocyte viability cultured in glucose. Therefore, we wanted to investigate whether GM-DCs utilised the same pathways for fructose metabolism. We cultured GM-DCs in fructose media, in the presence of oligomycin, and determined cytokine production and cell viability (Figure 4.17).

Firstly, we observed a drastic decline in TNFα production in fructose-cultured and LPS-stimulated GM-DCs, in the presence of oligomycin, compared to LPS stimulation only (Figure 4.17A). This data was compared to glucose-cultured GM-DCs. We normalised TNFα levels from glucose- and fructose-cultured GM-DCs (Figure 4.17B). We previously showed that oligomycin reduced TNFα production from glucose-cultured GM-DCs (Figure 4.11A). However, in fructose-cultured GM-DCs, we show that oligomycin abrogated TNFα production, compared to LPS stimulation only and compared to glucose-cultured, LPS- and oligomycin-stimulated cells (Figure 4.17B).

Next, we investigated whether this abrogation of cytokine production was due to cell death. Fructose did not impact cell viability, as the percentage of live cells was similar between glucose- and fructose-cultured GM-DCs, basally and following LPS stimulation (Figure 4.17C). Oligomycin resulted in some cell death for glucose-treated GM-DCs, whereas oligomycin resulted in complete cell death for fructose-treated GM-DCs (Figure 4.17C). This data resembles findings in monocytes (Jones *et al.,* 2021), which suggested OXPHOS is required for cell survival and function when cultured in fructose.

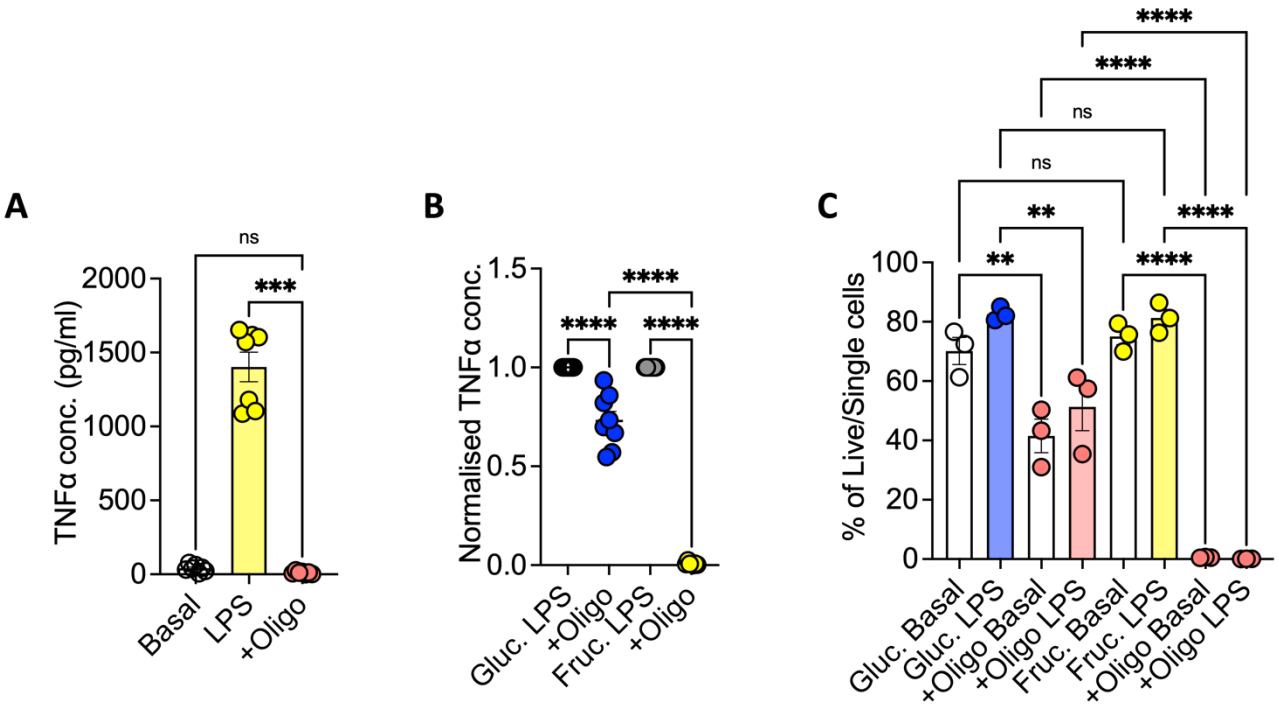


Figure 4.17 ATP synthase inhibitor oligomycin and fructose drastically reduced GM-DC cytokine production Scatter plot of (A) TNFα production (pg/ml) from GM-DCs in fructose-containing media (10mM) following 18-hour LPS stimulation in the presence or absence of oligomycin (n=7). (B) Normalised TNFα production from GM-DCs in glucose- (11mM) or fructose-containing media (10mM) following 18-hour LPS stimulation in the presence or absence of oligomycin (Glucose n=8, Fructose n=7). (C) Percentage of live GM-DCs per single cell population in glucose- and fructose-containing media following 18-hour LPS stimulation in the presence or absence of oligomycin (n=3). Statistical analysis performed using Kruskal-Wallis with Dunn's test and Ordinary one-way ANOVA with Tukey's correction where appropriate, ns=p>0.05, **p≤0.01, ***p≤0.001, ****p<0.0001.

4.3.17 Oligomycin had no impact on cell viability and cytokine production of GM-DCs cultured in equimolar glucose & fructose

In physiological conditions, cells would not be exposed to only one carbon source. As fructose altered the metabolic programme of the cell. We next wanted to investigate whether the presence of glucose, alongside fructose, impacted the function of the cells. We cultured GM-DCs in equimolar solutions of glucose and fructose (glucose:fructose) (5mM each) and stimulated the cells with LPS for 18 hours and determined cytokine production and cell viability (Figure 4.18).

We observed that glucose:fructose-cultured GM-DCs produced similar levels of TNFα following LPS stimulation as glucose only-cultured GM-DCs (Figure 4.18A). The addition of oligomycin to glucose:fructose-cultured GM-DCs did not affect TNFα production compared to LPS stimulation only. Interestingly, TNFα levels were also not different to glucose-cultured GM-DCs in the presence of oligomycin. Thereby demonstrating the ability of GM-DCs to utilise the available glucose present in the media. Viability staining showed a decrease in live cells in the presence of oligomycin, basally and following LPS stimulation, in both types of media (Figure 4.18B). There was no difference in cell viability between these culture medias in the presence of oligomycin, basally and following LPS stimulation (Figure 4.18B), compared to the stark decrease in viability observed for fructose-cultured GM-DCs, in the presence of oligomycin (Figure 4.17C). The glucose in the media, at the concentration of 5mM - physiological concentration (Matschinsky and Wilson, 2019) - compensated and allowed GM-DC survival and cytokine production.

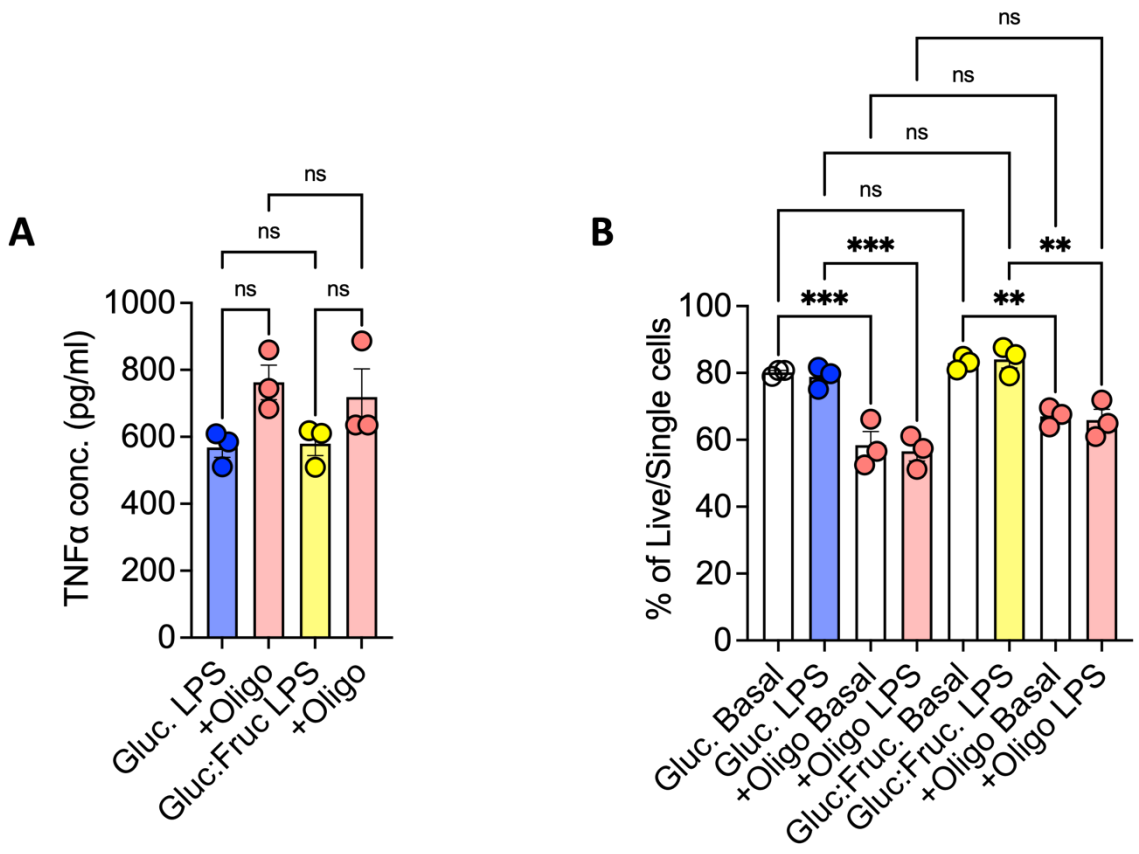


Figure 4.18 Equimolar concentrations of glucose and fructose in the presence of oligomycin affected GM-DC survival to a similar extent as normal glucose concentration Scatter plot of (A) TNFα production (pg/ml) from GM-DCs in glucose- (11mM) or glucose (5mM) and fructose (5mM)-containing media following 18-hour LPS stimulation in the presence or absence of oligomycin (n=3). (B) Percentage of live GM-DCs per single cell population in glucose- or glucose and fructose-containing media following 18-hour LPS stimulation in the presence or absence of oligomycin (n=3). Statistical analysis performed using ordinary one-way ANOVA with Tukey's correction or Kruskal-Wallis with Dunn's test where appropriate, ns=$p>0.05$, **$p\leq0.01$, ***$p\leq0.001$.

4.3.18 Oligomycin impacted cell viability and cytokine production of GM-DCs cultured in no glucose

Dziurla *et al.* (2010) demonstrated reduced cytokine production by human $CD4^+$ T cells in the absence of glucose. We previously showed that GM-DC cytokine production in low glucose was not impacted compared to normal media (Figure 4.15). Next, we determined whether no glucose impacted cytokine production and cell viability.

We found that GM-DCs cultured in no glucose, produced the same levels of TNFα as those cultured in normal glucose-containing media (Figure 4.19A). However, the addition of oligomycin abrogated TNFα production completely (Figure 4.19A), similar to findings observed in fructose-cultured GM-DCs (Figure 4.17A). We did observe that no glucose resulted in a decrease in viable cells, basally and following LPS stimulation (Figure 4.19B), this decrease in viability was also observed in monocytes (Jones *et al.*, 2021). The presence of oligomycin and no glucose-media resulted in complete cell death, basally and in LPS-stimulated GM-DCs, compared to no glucose-media only. A similar observation to fructose-cultured GM-DCs (Figure 4.17C).

We show that no glucose-cultured GM-DCs, treated with oligomycin, led to significant reduction in viable cells compared to normal glucose-cultured GM-DCs treated with oligomycin. This was associated with a dramatic decrease in TNFα production. Another metabolic pathway which may possibly link to OXPHOS may be sustaining the cells, in the absence of oligomycin. However, the addition of oligomycin leads to OXPHOS inhibition and metabolism is abrogated, leading to cell death and no functional responses.

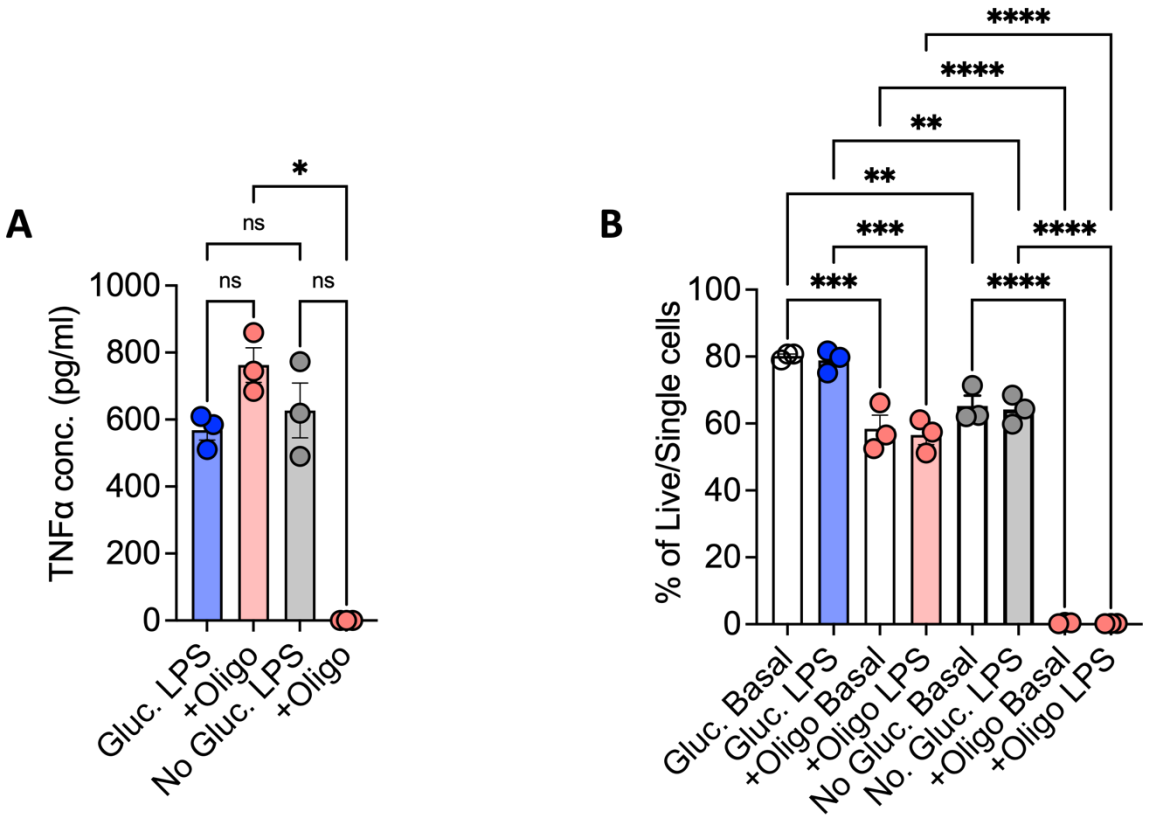


Figure 4.19 Oligomycin and no glucose-containing media dramatically affected GM-DC survival and cytokine production Scatter plot of (A) TNFα production (pg/ml) from GM-DCs in glucose- (11mM) or no glucose-containing media following 18-hour LPS stimulation in the presence or absence of oligomycin (n=3). (B) Percentage of live GM-DCs per single cell population in glucose- and no glucose-containing media following 18-hour LPS stimulation in the presence or absence of oligomycin (n=3). Statistical analysis performed using ordinary one-way ANOVA with Tukey's correction or Kruskal-Wallis with Dunn's test where appropriate, ns=p>0.05, *p≤0.05, **p≤0.01, ***p≤0.001, ****p<0.0001.

4.4 Discussion

Immunometabolism is a relatively novel field of immunology. During the last decade, DC metabolism has been deeply investigated, yet there remain many gaps in our knowledge. DCs are important for immune surveillance. DCs patrol the body where they can be exposed to different microenvironments. These microenvironments differ in physiological composition (e.g., nutrients or oxygen saturation) and can impact DCs differently (Gottfried *et al.*, 2006; Yang *et al.*, 2009). DCs sample the environment to detect any foreign invaders or damage signals which are deleterious to the host, ultimately, with the aim of initiating and informing the adaptive immune response. The field of immunometabolism studies the metabolic pathways utilised by immune cells to promote their survival or differentiation and support their effector functions (Mathis and Shoelson, 2011). GM-DCs have been extensively used to investigate DC metabolism (Everts *et al.*, 2014; Thwe *et al.*, 2017). In this chapter, we aimed to characterize the metabolic pathways required for bone murine DC activation upon two different stimuli, a TLR agonist (LPS) and infection with SeV. We also investigated their metabolic flexibility.

DC activation increases transcription and translation of molecules required for DC maturation, such as co-stimulatory molecules and pro-inflammatory cytokines (Krawczyk *et al.*, 2010; Everts *et al.*, 2014). These processes are energetic, with the cell having to synthesise new proteins. This is supported by our data, where SeV infection of GM-DCs increased the levels of protein synthesis within the cell. Increased protein synthesis would permit the increased levels of cytokine production observed following SeV infection. Surprisingly, LPS-stimulated GM-DCs did not result in a significant increase in protein synthesis, based on puromycin incorporation, despite actual increased cytokine production observed. The increase in protein synthesis in SeV-stimulated cells could also be a result of hijacking of the translation machinery of the cell for viral protein synthesis, as displayed by different viruses (Aragón *et al.*, 2000; Connor and Lyles, 2002).

The use of metabolic inhibitors is widely used to determine which metabolic pathways are important for cellular function. LPS-treated GM-DCs have previously been shown to upregulate glycolysis swiftly following activation (Krawczyk *et al.*, 2010), along with an initial

burst in mitochondrial activity, but a long-term commitment to glycolysis (Everts *et al.*, 2012). Viruses, especially RNA viruses, have been shown to hijack cell metabolism and increase glycolysis (Fontaine *et al.*, 2015; Ren *et al.*, 2021). The effect of SeV on cell metabolism, in general, has not been extensively studied and its effect on GM-DC metabolism has not been investigated. We noted a decrease in protein translation and cytokine production following glycolysis inhibition, through the hexokinase inhibitor, 2-DG. This was expected for LPS-stimulated GM-DCs. A decrease in cytokine-producing (IL-6, IL-12 & TNF) GM-DCs has been observed in the presence of 2-DG and 6-hour LPS stimulation (Everts *et al.*, 2014). They did not observe any changes in mRNA expression at 3-hour LPS stimulation with 2-DG. Glycolysis is important in the beginning and is more favoured with time, therefore, this could explain why 2-DG has more of an effect at a later stage (6 hours). Certain other findings were also unexpected. LPS and SeV appear to favour different metabolic pathways. Following 18-hour stimulation of both, SCENITH was performed and the metabolic dependencies and capacities of the GM-DCs from both conditions were calculated. Both LPS- and SeV-stimulated GM-DCs had similar glucose dependencies, which is expected as both rely on glycolysis. However, these different stimulations displayed differential mitochondrial dependencies. GM-DCs had no difference in mitochondrial dependence following SeV stimulation, whereas LPS stimulation resulted in lower mitochondrial dependence. Previously, Argüello *et al. (*2020) demonstrated a decrease in the mitochondrial dependency and an increase in the glycolytic capacity of LPS-treated cDC1 and cDC2 populations derived from FL-DCs. This correlated with what we observed, presumably due to the increase in glycolysis, 18 hours after stimulation, these GM-DCs rely less on OXPHOS and have greater capacity to support the cell via glycolysis. SeV infection did not alter the mitochondrial dependence or the glycolytic capacity, demonstrating a role for both glycolysis and OXPHOS for GM-DC responses. Rezinciuc *et al.* (2020) also demonstrated differences in the metabolism of GM-DCs following influenza A virus (another RNA virus) stimulation compared to other TLR ligands. Many studies are conducted using TLR ligands (including those mimicking viral ligands) and it is important to note that they may not simulate the same responses as an active viral infection.

Another interesting observation was that 2-DG inhibition of SeV stimulated GM-DCs resulted in decreased cytokine production, as expected, however there was a dramatic decrease in IFNβ production. The addition of an alternative glycolysis inhibitor, heptelidic acid (GAPDH

inhibitor), also reduced IFNβ production, but to a lesser extent. Based on the literature, I suggest three possible reasons for this observation. Firstly, a recently published paper from He *et al.* (2023), highlighted the importance of the adaptor protein MAVS in coordinating RLR signalling and glucose metabolism. Upon RLR detection of viral pathogens, MAVS activation and aggregation results in a signalling cascade which results in transcription factor (e.g., IRF3 and NF-κB) activation and ultimately expression of type I interferons and pro-inflammatory molecules (Seth *et al.*, 2005; Hou *et al.*, 2011). MAVS can localize to the mitochondria, mitochondria-associated ER membranes (MAMS) and peroxisomes (Seth *et al.*, 2005; Dixit *et al.*, 2010; Horner *et al.*, 2011). MAVS located on MAMS were demonstrated to cause a shift of glycolysis into the hexosamine biosynthesis pathway (HBP) and resulted in the expression of type I IFNs (He *et al.*, 2023). This pathway branches off glycolysis after the production of F6P (the second step of glycolysis) (Marshall, Bacote and Traxinger, 1991), this step proceeds the hexokinase reaction and precedes the GAPDH reaction. Possibly inhibiting hexokinase, would prevent both glycolysis and its diversion into HBP, halting IFNβ production drastically, whereas inhibiting GAPDH, would not prevent F6P diversion into HBP, so IFNβ inhibition is not as extreme. Another possibility is that 2-DG has other side effects, where it affects protein glycosylation, required by both the host and the virus (Datema and Schwarz, 1979; Kurtoglu *et al.*, 2007; Berthe *et al.*, 2018). IFNβ requires glycosylation (Sommereyns and Michiels, 2006), therefore, this could be a possible avenue of how 2-DG off target effects can impact IFNβ production. Lastly, the inhibitor-specific effects of heptelidic acid could be due to the concentration used and was not as potent as the effects of 2-DG. Experiments using higher heptelidic acid concentrations could elucidate this observation. The AZ67 inhibitor had no effect on cytokine production. Its selective target is PFKFB3 (a PFK2 isoform) which allosterically activates the glycolytic enzyme PFK1 (Van Schaftingen *et al.*, 1981). In LPS-activated GM-DCs, PFK2 knockdown had no effect on the increased glycolytic rates or DC activation (Everts *et al.*, 2014). Based on that observation, AZ67 inhibition of PFKFB3 may not affect SeV infection. The effect of glycolysis inhibition on GM-DCs during LPS or SeV stimulation is summarised in Figure 4.20.

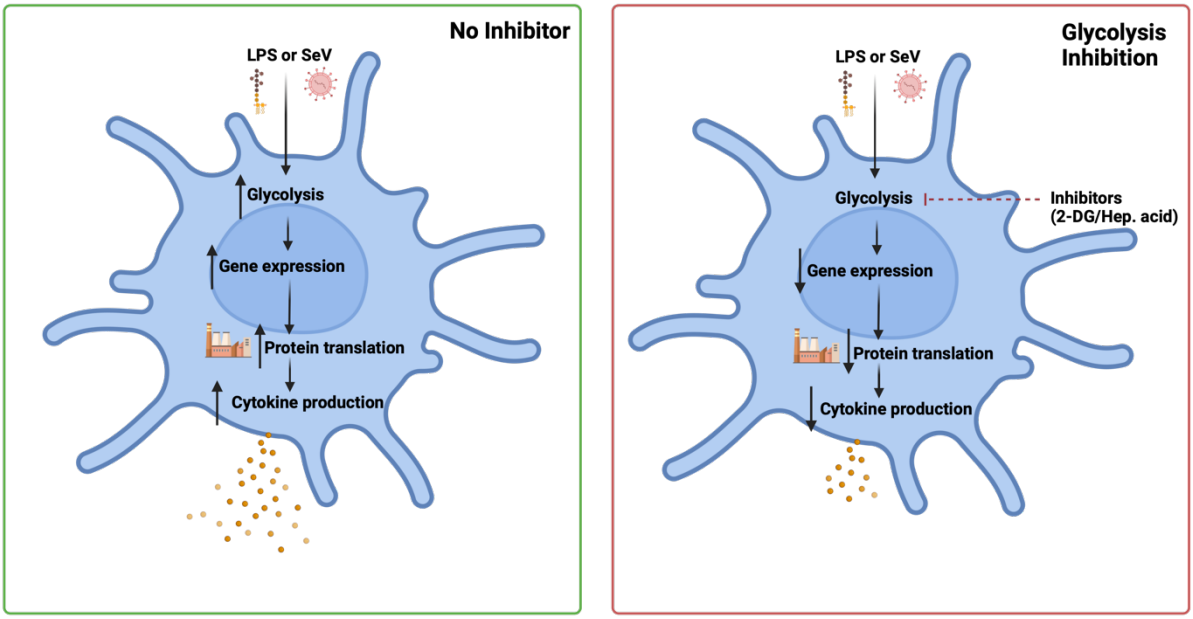


Figure 4.20 – Inhibition of glycolysis reduced GM-DC functionality. Upon LPS or SeV stimulation, GM-DCs upregulate glycolysis and increase mRNA expression of pro-inflammatory or antiviral genes. This was associated with an increase in protein translation and subsequent cytokine production. Inhibition of glycolysis reduced gene expression, along with a decrease in protein translation and cytokine production.

Early inhibition of OXPHOS caused mRNA expression of GM-DC cytokines to trend upwards or significantly increase (particularly *Il12b* for both LPS and SeV). However, this mRNA expression must not be maintained as it does not correlate with protein production in the presence of oligomycin after 18 hours. Cytokine production is either unaffected by oligomycin or a slight decrease observed in only TNFα in LPS-stimulated GM-DCs. At 18 hours, LPS-stimulated GM-DCs have downregulated OXPHOS due to NO production inhibiting the ETC (Everts *et al.*, 2012). Therefore, as this pathway is not favoured at later timepoints, this could explain why cytokine production is unaffected by oligomycin inhibition. CP91149, the glycogen phosphorylase inhibitor, also did not affect cytokine production 18 hours after LPS stimulation. Thwe *et al.* (2017) demonstrated the importance of glycogen in GM-DC function during early LPS activation. At later timepoints, glucose is obtained extracellularly and sustains cell functions, therefore, no differences in cytokine production was expected. Despite, glycogen affecting DC effector function earlier during stimulation, after 18 hours, the same levels of cytokines (TNFα and IL-12p70) are observed. *In vivo*, DCs reach the draining lymph nodes or

organs to activate T cells around this time (Kissenpfennig *et al.*, 2005), therefore, the levels of cytokines being unchanged, would maintain the appropriate responses.

Glycolysis appears to be the most important pathway for initial and sustained GM-DC effector function. However, the cells can compensate with other pathways, but it is noted that cytokine production is not to the same extent, except in the case of IFNβ, where there is complete abrogation following inhibition of hexokinase. Glycolysis requires glucose or other derivatives which can enter the pathway, therefore, we wanted to investigate whether changes in the fuel source available affected subsequent GM-DC effector function.

Long-term commitment to glycolysis in GM-DCs involves the activation of mTORC1, which activates HIF1α and the enzyme iNOS (Jantsch *et al.*, 2008; Lawless *et al.*, 2017). iNOS produces NO which serves to inactivate the ETC, in turn, OXPHOS (Everts *et al.*, 2012). GM-DCs were cultured in glucose for 24 hours then switched to galactose containing media, this resulted in a dramatic decrease in ATP production (after 15 minutes) and cell death (after an additional 24 hours) (Everts *et al.*, 2012). In $iNOS^{-/-}$ mice, these effects in the galactose media were not observed (Everts *et al.*, 2012). Lawless *et al.* (2017) stimulated GM-DCs with LPS for 8 hours in normal glucose-containing media then switched the media to either glucose- or galactose-containing media. GM-DCs in the galactose media favoured OXPHOS, importantly, there was no effect on cell viability. An increase in *Il12a* mRNA was also observed. This coincided with decreased HIF1α activation and nitrite production (Lawless *et al.*, 2017). We stimulated GM-DCs with LPS in the presence of galactose and found no differences in cytokine production of TNFα and IL-10, except IL-12p70. Similar was observed in the presence of fructose, where LPS-stimulated GM-DC cytokine production was unaffected. These different studies depict the importance of when the cells are exposed to the fuel source. At a later stage, as in the case of Everts *et al.* (2012), the cells have already activated iNOS which blocks OXPHOS with NO. Therefore, switching to fuel sources which rely on OXPHOS, would impact GM-DC survival and function. Whereas possibly if the commitment to glycolysis does not occur, i.e., no nitric oxide production, then the cells remain functional through their use of OXPHOS. This displays a metabolic flexibility within GM-DCs, where in the absence of glucose, the cells can still be activated and generate the appropriate effector cytokine responses. In conditions with equimolar glucose and fructose, glucose was still present at 5mM (the same

as physiological conditions) and the inhibition of OXPHOS had no impact, therefore, the cells utilize the glucose in solution.

Similar to galactose, Lawless *et al.* (2017) demonstrated inactivation of HIF1α in glucose-deprived GM-DCs. These glucose-deprived GM-DCs rather displayed activation of AMPK and inhibition of mTORC1. These master regulators sense glucose deprivation (Inoki, Zhu and Guan, 2003). Low glucose was also observed to increase *Il12a* expression, after 8-hour LPS stimulation in normal glucose-containing media (Lawless *et al.*, 2017). We demonstrated no changes in cytokine production after 18 hours of LPS stimulation in low (1mM) glucose and no glucose media. Jones *et al.* (2021) demonstrated an increase in oxygen consumption rate (OCR), similar to fructose and galactose media. Inhibition of OXPHOS in GM-DCs in no glucose media resulted in cell death. The absence of glucose would favour mitochondrial respiration and the activation of AMPK would facilitate the oxidation of other substrates (e.g., glutamine, fatty acids). The effect of oligomycin during the use of different fuel sources is summarized in Figure 4.21.

Collectively, we observed that LPS and SeV favour different metabolic pathways within GM-DCs, both require glucose to sustain glycolysis, however, LPS-treated GM-DCs rely less on the mitochondria after 18 hours unlike SeV-treated GM-DCs which appear still reliant on mitochondrial oxidative processes. GM-DCs also displayed carbon fuel flexibility, through their ability to use fructose, galactose, low or no glucose to fuel their cytokine production. However, these carbon sources require functional mitochondria to perform oxidative phosphorylation, compared to normal glucose-containing media where GM-DCs are largely unaffected in the absence of OXPHOS.

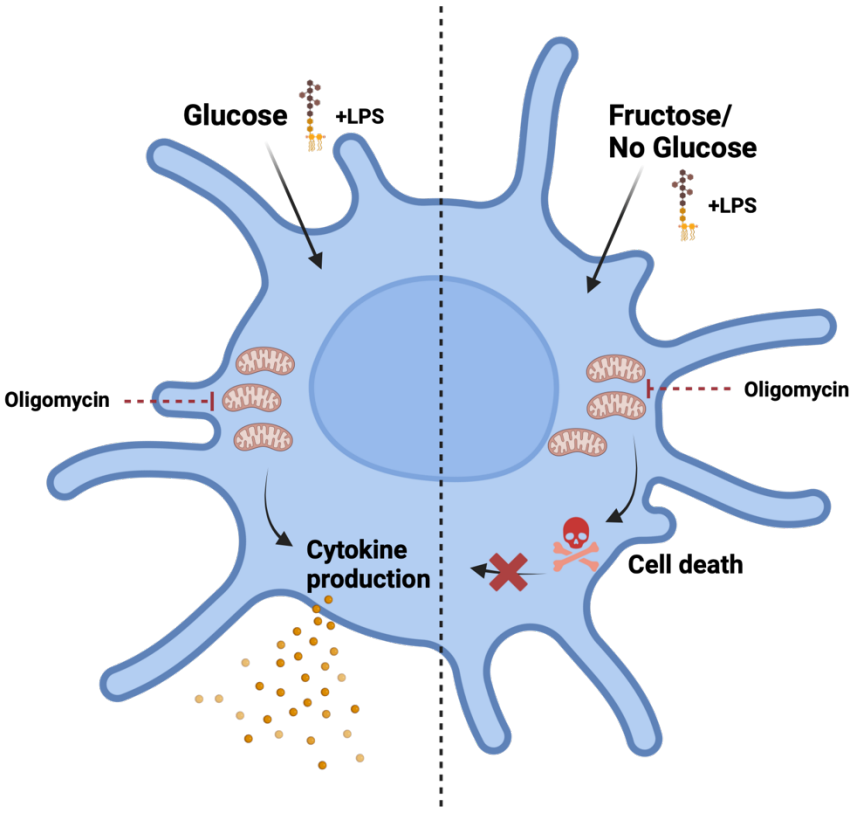


Figure 4.21 – Summary of the effect of OXPHOS inhibition during the use of alternative carbon sources. GM-DCs produced cytokine following LPS stimulation in glucose- or fructose-containing media or no glucose media. Inhibition of oxidative phosphorylation by oligomycin resulted in cell death in fructose or no glucose media, hence no cytokine production. In glucose-containing media, oligomycin addition did not hinder cytokine production.

Chapter Five

Results

Impact of diet-induced obesity on dendritic cell functional responses

5.1 Introduction

5.1.1 Obesity Overview

The World Health Organisation (WHO) defines obesity as an excessive or abnormal accumulation of fat, which can impair an individuals' health (WHO, 2021). Obesity is a multifactorial disease (Bray, Kim and Wilding, 2017) and is the leading cause of disability in the WHO European region ('WHO EUROPEAN REGIONAL OBESITY REPORT 2022', 2022).

Simplistically, an increase in adipose tissue, causes changes in the individual's hormones, metabolism and various cell types, and eventually results in a chronic, low-grade inflammatory state (Fantuzzi, 2005). A dysregulation of adipocytes and immune cells disrupts adipose tissue homeostasis (Fantuzzi, 2005). This interplay results in the inflammatory state and eventually may lead to various co-morbidities, which includes an increased susceptibility, morbidity and mortality to infections (Haslam and James, 2005). Most recently, this was observed during the coronavirus disease 2019 (COVID-19) pandemic, where PWO had increased morbidity and mortality rates (Popkin *et al.*, 2020). Dysregulation of adipose tissue homeostasis occurs via increased adipocyte stress, increased concentrations of FFAs, increased ROS and altered metabolic processes (Haslam and James, 2005; Wolowczuk *et al.*, 2008; Minihane *et al.*, 2015). FFA can activate toll-like receptors, TLR4 in particular, and downstream signalling results in the production of cytokines by e.g., macrophages (Shi *et al.*, 2006). 'Stressed' macrophages were observed in AT, where during a state of obesity, a pro-inflammatory M1-like polarisation of AT-resident macrophages is favoured as opposed to the anti-inflammatory M2-like state (Lumeng, Bodzin and Saltiel, 2007). Other pro-inflammatory cells such as $CD8^+$ T and $CD4^+$ Th1 cells are favoured and their accumulation have been observed, in contrast to the reduction in anti-inflammatory $CD4^+$ Th2 and regulatory T cells (Nishimura *et al.*, 2009; Chan *et al.*, 2017). A systemic inflammatory state may eventually be generated due to the production of pro-inflammatory cytokines and increased levels of pro-inflammatory cells (Fantuzzi, 2005). Obesity is associated with major alterations to immune cell populations responsible for host-pathogen defences. Both innate and adaptive cell populations can be affected. Favouring of Th1 or Th17 effector T cell differentiation also promotes the inflammatory state (Endo *et al.*, 2015; Surendar *et al.*, 2019), alongside a decline in inflammation resolution (Luo *et al.*, 2019). Obesity can lead to the impairment of immune responses either directly or indirectly and this

has significant implications for infection response or vaccination. PWO may generate suboptimal or inadequate responses following vaccination against a specific pathogen (Mckee *et al.*, 1990; Banga *et al.*, 2014). Hence rendering vaccinated PWO more susceptible to infection by these vaccinated pathogens.

Obesity is associated with an increased risk of over 200 chronic co-morbidities, such as cancer, T2DM, obstructive sleep apnoea, polycystic ovary syndrome (PCOS) (Haslam and James, 2005; Horn, Almandoz and Look, 2022). These factors can contribute to a decreased quality of life and reduced life expectancy for PWO (Peeters *et al.*, 2003; Kolotkin and Andersen, 2017). In addition to these chronic illnesses, a subpar immune system can result in PWO succumbing to infection more frequently or not clearing infections as rapidly as metabolically healthy individuals (Huttunen and Syrjänen, 2012). The increased risk of infection will be discussed further next.

5.1.2 Obesity results in viral susceptibility in host

Obesity has previously been identified as a major risk factor for viral infections. The strongest evidence for obesity susceptibility to viral infection comes from the influenza A H1N1 pandemic (H1N1pdm) which occurred in 2009-2010 (van Kerkhove *et al.*, 2011). Van Kerkhove *et al.* (2011) analysed data from approximately 70,000 pH1N1 positive patients who were hospitalised in 19 countries or administrative regions, to determine the risk factors associated with this viral influenza pandemic. Risk factors were compared based on the severity level of the infection, which were: hospitalisation, ICU admission and fatality. Severity of infection positively correlated with the percentage of H1N1 positive patients with at least one underlying chronic condition. Severe H1N1pdm infection risk factors were observed to be similar to those of seasonal influenza, including immunodeficiency and chronic conditions such as CVD, pulmonary disease or diabetes (Fiore *et al.*, 2010; van Kerkhove *et al.*, 2011). However, morbid obesity was then recognised as a risk factor for severe influenza infection (van Kerkhove *et al.*, 2011). As severity of disease increased, so did the percentage of patients with a BMI ≥ 30kg/m^2, the same was observed for patients with morbid obesity (BMI > 40kg/m^2), however, there were disparities between countries (van Kerkhove *et al.*, 2011). This may be due to the differing extents of obesity within the populations of different countries. As previously mentioned, patients with obesity or morbid obesity have a greater risk of ICU

admission (Fezeu *et al.*, 2011; Simonnet *et al.*, 2020), but there are associated complications, where these same individuals have been reported to have an increased susceptibility to nosocomial or secondary infections (Choban *et al.*, 1995). Therefore, if PWO are hospitalised, they have additional risks of being infected with other microbial infections, causing e.g., ventilator-associated pneumonia or catheter-related infections. These significantly increase their risk of mortality.

Obesity has also been observed to alter lung physiology. Alterations include: reducing the total lung capacity; affecting the breathing of the individual by increasing their respiratory rate; increasing the work of breathing; reducing the lung compliance; and affecting the strength of the respiratory muscles, such as reducing the maximum voluntary ventilation (Sharp *et al.*, 1964; Zerah *et al.*, 1993; Pelosi *et al.*, 1998; Kress *et al.*, 1999; Murugan and Sharma, 2008; Hodgson, Murphy and Hart, 2015). Such changes to the respiratory system of PWO can drastically affect the progression of a respiratory illness, which is why obesity has been listed as a risk factor for several respiratory illnesses (Guerra *et al.*, 2002; Murugan and Sharma, 2008; Young, Peppard and Gottlieb, 2012; Bhatt, Guleria and Kabra, 2021). Including susceptibility to infections of the upper and lower respiratory tracts (Maccioni *et al.*, 2018). A Polish study of 1,129 children of 9 years of age observed a higher BMI correlated with acute respiratory infection susceptibility (Jedrychowski *et al.*, 1998). Children with a BMI greater than or equal to 20kg/m^2, classified as overweight, had doubled the risk of infection compared to those of a lower BMI (Jedrychowski *et al.*, 1998). These are the overall impacts of obesity on the host. On a cellular level, obesity results in dysfunctional cells, ranging from haematopoietic stem cells to adipocytes (McLaughlin *et al.*, 2016; Lee *et al.*, 2018). Susceptibility to infection or obesity-related comorbidities can be due to their suboptimal immune responses or an over-active inflammatory state and eventual exhaustion of immune cells.

Lipid accumulation, due to obesity, can occur in immune cells, impeding their function, such as NK cells (Michelet *et al.*, 2018). NK cells are important cells of the innate immune response against viral infections. A lipid-rich environment has been shown to cause a metabolic paralysis of NK cells impeding their function (Michelet *et al.*, 2018). Additionally, a decrease in IFN-γ production by NK cells was observed (Michelet *et al.*, 2018), interferons are essential

anti-viral cytokines (Liu *et al.*, 2012). NK cells can detect virally infected cells and function by releasing cytotoxic granules capable of killing these infected cells (Brandstadter and Yang, 2011). The inability of these key innate cells to function within PWO may be correlated with their increased susceptibility to viral infections (van Kerkhove *et al.*, 2011; Popkin *et al.*, 2020). DIO was observed to increase the mortality rate of mice following influenza infection compared to control. These mice with DIO had more inflammation and damage to their lungs (Smith *et al.*, 2007), along with increased decreased NK cell cytotoxicity (Smith *et al.*, 2007) and decreased effector $CD8^+$ T cells (Green *et al.*, 2022) following influenza infection, compared to control mice. Diet-induced obesity was also associated with decreased $CD8^+$ effector memory T cells in the lungs over time following primary influenza infection in mice (Karlsson, Sheridan and Beck, 2010b). Upon a secondary infection, these memory T cells also had diminished effector function, depicted by reduced IFN-γ production (Karlsson, Sheridan and Beck, 2010a). APCs are required to activate the adaptive arm of the immune response (Medzhitov and Janeway, 1997). A professional APC with the ability of stimulating naïve T cells to differentiate into memory T cells is a DC (Quigley, Huang and Yang, 2007). A brief overview of the impact of obesity on DCs will be discussed next.

5.1.3 Impact of obesity on dendritic cells

Dendritic cells are phagocytic, innate immune cells which act as professional antigen presenting cells to activate the adaptive immune response (Banchereau *et al.*, 2003; Steinman, 2003). DCs circulate the body in an immature state, upon antigen encounter, DCs undergo maturation (Banchereau *et al.*, 2003). Immature DCs have a high phagocytic capacity, sampling antigen in their surroundings (Albert *et al.*, 1998; Ackerman and Cresswell, 2003; Kim and Kim, 2019). Upon maturation, their phagocytic machinery is downregulated and DCs upregulate the expression of molecules required for antigen presentation (Kim and Kim, 2019). DCs express MHCs to present antigen and co-stimulatory molecules which provide additional signals for efficient T cell activation (Kim and Kim, 2019). DCs also produce cytokines which can polarise T cell differentiation and enhance the activation of other immune cells (Sallusto and Lanzavecchia, 2002; Nagorsen, Marincola and Panelli, 2004). DCs have been impacted in the context of obesity, in both humans and mice (O'Shea *et al.*, 2013). Obesity can alter DC effector function. Deletion of *Flt3l* in mice ($Flt3l^{-/-}$), caused DC depletion and resulted in obesity resistance or reduced body weight compared to their control respectively

(Stefanovic-Racic *et al.*, 2012). This was linked to their role in recruiting immune cells, DCs increased in the adipose tissue in mice on a HFD compared to control and subsequently correlated with an increase in macrophage infiltration in the adipose tissue (Stefanovic-Racic *et al.*, 2012). An increase in macrophage infiltration would promote the inflammatory state within the adipose tissue, observed in obesity (Fujisaka *et al.*, 2013). Obesity also promoted DC activation and maturation, demonstrated by increased MHC molecules and co-stimulatory molecules (e.g., CD40 or CD86) in adipose tissue and liver (vanderZande *et al.*, 2023). Similarly in humans, increasing BMI correlated with increased DC2 ($CD1c^+$ $CD11c^+$ DCs) and $CD83^+$ cells, a DC maturation marker, in human adipose tissue (Bertola *et al.*, 2012). In both studies, these DC populations affected T helper cell populations. vanderZande et al. (2023) demonstrated an increase in Th1 cells and a decrease in Th2 and Treg cells in the adipose tissue. Along with an increase in Th1, Th17 and Treg cells in the liver (vanderZande *et al.*, 2023). Bertola *et al.* (2012) demonstrated in mice and humans that the $CD11c^+$ DCs correlated with increased Th17. Th17 cells produce IL-17, a cytokine linked with exacerbating inflammation in obesity and can promote autoimmune diseases (Jhun *et al.*, 2012). In humans, reduced numbers of DCs were observed in the circulation of PWO compared to controls (O'Shea *et al.*, 2013). A decrease in the DC maturation marker, CD83, was observed in PWO compared to controls (O'Shea *et al.*, 2013). Obesity impacts cells differently, in this context, depending on the type of DC or their localisation, different effects were observed.

Chen *et al.* (2022) demonstrated an increase in DCs in the spleen of mice on a HFD compared to mice on a LFD. The spleen is a major hub for DC-T cell interactions, where these interactions result in T cell activation (Bronte and Pittet, 2013). These same splenic HFD DCs displayed reduced capacity to present antigen to $CD4^+$ T cells compared to LFD splenic DCs (Chen *et al.*, 2022). No differences were observed in co-stimulatory molecules; therefore, this did not account for the defect observed. Genes involved in mitochondrial homeostasis, including lipid catabolism and fatty acid oxidation, were upregulated in HFD splenic DCs compared to LFD (Chen *et al.*, 2022). This correlated with an increase in mitochondrial respiration observed in the HFD splenic DCs. The increase in mitochondrial respiration was not related to increased mitochondria or lipid content, but rather, increased fatty acid oxidation fuelling the HFD splenic DCs (Chen *et al.*, 2022). Altogether, Chen *et al.* (2022) found that this increase in FAO observed in HFD splenic DCs, resulted in increasing mitochondrial ROS which consequently

hindered their ability to present antigen to T cells. Obesity results in oxidative stress, including an increase in ROS, which can be detrimental to cells. This is not the only example of how obesity impacts metabolism. Metabolism is closely linked to cellular function (O'Neill, Kishton and Rathmell, 2016), and next the impact of obesity on the metabolism will be explored.

5.1.4 Obesity impacts host metabolism

The immune system is one of the systems which utilises the most energy within the body, especially during infection (Almajwal *et al.*, 2018; Ganeshan *et al.*, 2019). During a resting state, immune cells are not highly metabolically active, but upon activation, immune cells become highly metabolically active to fuel the demands of the cell, from energy to the building blocks required for protein synthesis (Krawczyk *et al.*, 2010; O'Neill, Kishton and Rathmell, 2016). Nutrient availability for the cell is closely associated with their function. In obesity, where the microenvironment consists of nutritional imbalances, cellular metabolism is impacted. The increase of FFAs in obesity can impact cell function (Michelet *et al.*, 2018). FFA can induce ROS production, in turn, promoting ER stress and increased AT expression of pro-inflammatory genes (Erbay *et al.*, 2009; Hotamisligil, 2010; Savva *et al.*, 2023). Additionally, the enlargement of the adipose tissue, impedes blood supply to the tissue, consequently promoting a hypoxic environment due to the lack of oxygen to the area (Chan *et al.*, 2017). Low oxygen levels activate HIF-1α, a subunit of the transcription factor HIF-1 (Wang *et al.*, 1995). This upregulates pro-inflammatory gene expression, which recruits immune cells into the adipose tissue and promotes the local inflammatory state, which eventually worsens in obesity to the systemic inflammatory state (Chan *et al.*, 2017). The adipose tissue is a metabolically active organ, in addition to its function as an energy store (Coelho, Oliveira and Fernandes, 2013). Other metabolic organs are also affected: skeletal muscle, liver, pancreas (Marchesini *et al.*, 2008; Ye *et al.*, 2019; Ahuja *et al.*, 2022).

Metabolism can be considered in terms of whole-body metabolism, the reactions which occur in the tissues and organs to ensure correct host function, or cellular metabolism (Mathis and Shoelson, 2011; O'Neill, Kishton and Rathmell, 2016). Immunometabolism is the study of the metabolism within the immune cells and its importance to the cell's function. Obesity can have varied effects on the metabolism of different immune cells, hence affecting their function. Obesity can alter the metabolic pathway favoured by a cell, e.g., increasing

mitochondrial respiration in HFD splenic DCs and ultimately increasing ROS and impeding their effector function (Chen *et al.*, 2022). The addition of antioxidants has previously been shown to improve T cell proliferation and reduce oxidative stress (Norell *et al.*, 2009). ROS accumulation causes immune dysfunction, e.g., preventing the proliferation of T cells and inducing apoptosis instead (Case *et al.*, 2011). Saturated fatty acids can be detected by TLR4 on the surface of innate cells, which contributes to the pro-inflammatory state due to the production of pro-inflammatory cytokines downstream (Weatherill *et al.*, 2005; Milanski *et al.*, 2009). The production and secretion of the cytokine TNF has been well-documented in its ability to impair insulin sensitivity and glucose metabolism and contribute to insulin resistance within the host (Nieto-Vazquez *et al.*, 2008). The saturated fatty acid, palmitate, is found at high levels in HFD mice and activates the inflammasome within macrophages, resulting in the production and secretion of IL-1β and IL-18 (more pro-inflammatory cytokines) (Wen *et al.*, 2011). mTORC1 is a master regulator of cellular metabolism (O'Neill, Kishton and Rathmell, 2016). Its key role is to sense nutrients, growth factors and the energy status of the cell and functions accordingly (Laplante and Sabatini, 2009; Shaw, 2009; O'Neill, Kishton and Rathmell, 2016). Obesity has led to dysfunctional mTORC1 signalling which can affect the activation of subsequent metabolic pathways. An innate lymphocyte subset important for host immune defences is the MAIT cell (Treiner *et al.*, 2003). MAIT cells upon activation increase their rates of glycolysis to support their effector functions (O'Brien *et al.*, 2019). However, in PWO, MAIT cells were shown to have reduced levels of glycolysis, following activation, compared to healthy controls (O'Brien *et al.*, 2019). Glycolysis is intrinsically linked to their production of IFN-γ, therefore, the reduced rates of glycolysis correlated with decreased IFN-γ producing MAIT cells in PWO compared to controls (O'Brien *et al.*, 2019). The reduction in glycolysis was due to defective mTORC1, demonstrated by a reduction in phosphorylation of ribosomal protein S6 (a target of mTORC1) in PWO compared to controls (O'Brien *et al.*, 2019), and defects in MYC, another metabolic regulator (Kedia-Mehta *et al.*, 2023). Therefore, obesity can have varied impacts on cells, especially immune cells, which can then impact their ability to efficiently protect their host from pathogens.

Due to the interplay between metabolism and immune cell function, we sought to investigate whether the metabolism of GM-DCs was impacted by obesity. A murine model of diet-induced obesity was used to study the impact of obesity.

5.1.5 DIO-model of obesity

Obesity is a worldwide epidemic (WHO, 2021) and murine models have been used for decades to investigate the impact of obesity, as some experiments are more invasive or terminal, and these experiments are impossible in humans. Obesity is a multifactorial disease, but the diet is one of the dominant contributors to the obesogenic phenotype (George *et al.*, 1990; Hariri and Thibault, 2010). Weight gain in mice is induced through supplementation with a HFD (Hariri and Thibault, 2010). The HFD has a higher composition of fat, widely used diets consist of either 45% or 60% fat, compared to the standard diet which tends to consist of 10% fat (Speakman, 2019). The diets can also differ based on their carbohydrate composition and type of fats in the diet itself (Speakman, 2019). HFD induces weight gain and adipose tissue increase, which can result in metabolic effects observed in obesity, such as glucose intolerance and insulin resistance (Takahashi, Ikemoto and Ezaki, 1999). Animal models are favoured due to the ease of weight gain when fed a HFD. The type of mouse used is also an important factor, as not all mice have increased adiposity on a HFD (West *et al.*, 1992). C57BL/6J mice on a HFD are more prone to developing obesity and tend to be widely used in obesity studies (Hu *et al.*, 2018). Due to ease of standardisation, diet-induced models of obesity are used, and the impact of anti-obesity drugs can also be investigated (Lu *et al.*, 2022).

SeV was used to stimulate GM-CSF cultured bone marrow-derived dendritic cells (GM-DCs) from both C57BL/6J mice on a SD or HFD for 16 weeks, to determine the impact of obesity on antiviral responses.

5.2 Specific aims of Chapter 5

The specific aim of this chapter was to investigate the impact of obesity on GM-DC function and metabolism, and therefore to:

1. Establish a murine model of diet-induced obesity
2. Characterise transcriptional changes in SeV-infected DCs from SD or HFD mice by RNA sequencing
3. Define the functional characteristics of GM-DCs following SeV infection from mice on the SD or HFD
4. Define the metabolic characteristics of GM-DCs following SeV infection from mice on the SD or HFD

5.3 Results

5.3.1 Mice on the HFD had greater body weight and epididymal adipose tissue

Murine models of obesity have been used extensively in research to deduce the effects of obesity. For our experiments, male mice of approximately 6-8 weeks of age, were allowed to acclimatise then placed on either the SD or HFD for 16 weeks. The SD contained 18% kcal from fat, compared to the HFD which contained 60% kcal from fat. We weighed the mice weekly and the mice on the HFD had a greater average weight than the mice on the SD, from week 1 until the end of the 16 weeks (Figure 5.1A). At the end of the 16 weeks, the HFD mice on average had gained approximately 15 grams compared to their starting weight, compared to SD mice which had on average gained approximately 5 grams compared to their starting weight (Figure 5.1B).

At the end of the 16 weeks, the mice were sacrificed and HFD mice had a greater body weight compared to SD mice (Figure 5.1C). On the day of sacrifice, we extracted and weighed epididymal AT from mice on each diet. HFD mice had more adipose tissue compared to SD mice (Figure 5.1D). As expected, more adipose tissue meant greater body weight. A 16-week mouse model of obesity generated larger mice with more adipose tissue compared to mice on the SD, these mice were used for subsequent experiments, to determine the impact of obesity on GM-DCs.

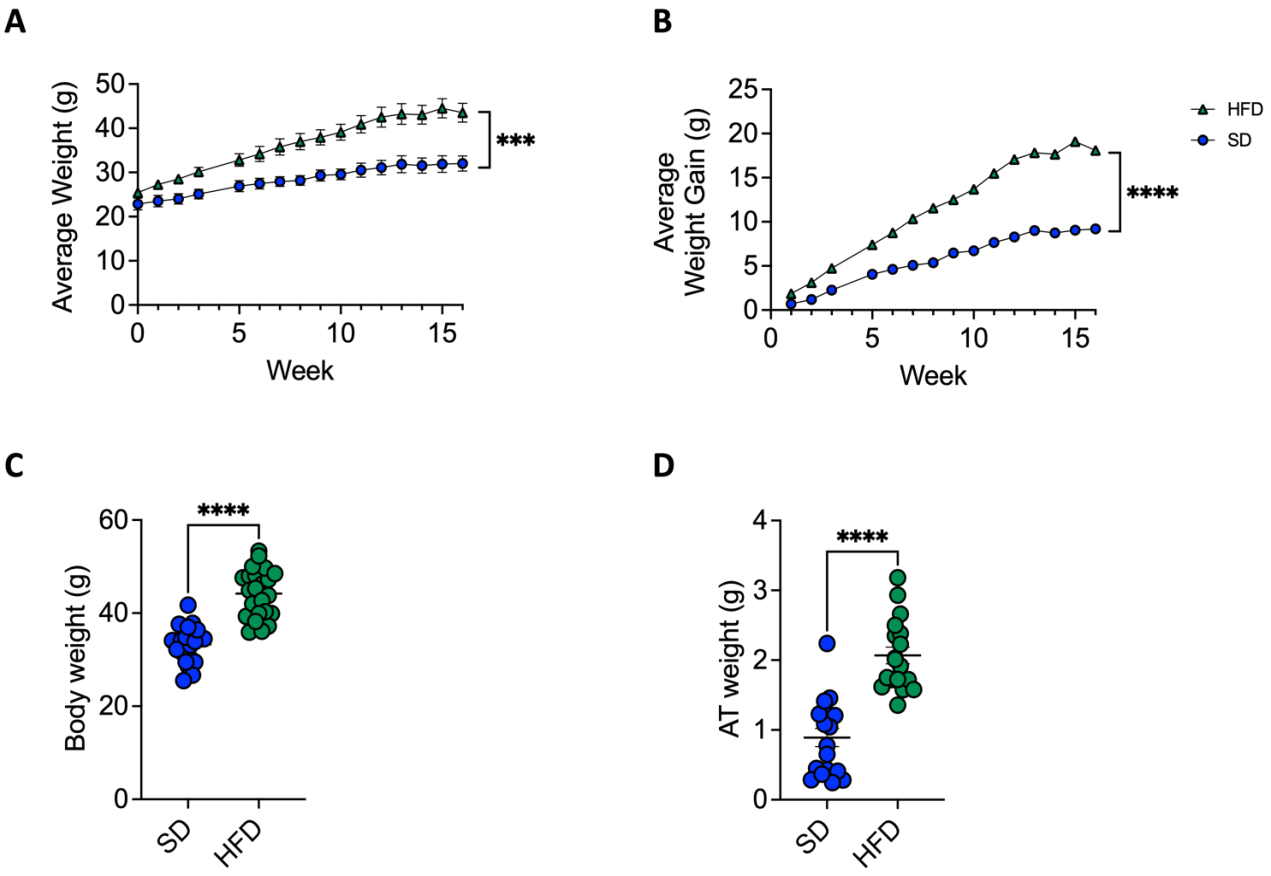


Figure 5.1 16-week murine model of obesity (A) Average weight of mice on either SD or HFD weighed weekly for 16 weeks (n=5). (B) Average weight gain of SD or HFD mice relative to starting average weight at week 0 (n=5). (C) Scatter plot of mice weights on the day of sacrifice at the end of the 16-week diet (SD n=21, HFD n=23) (D) Scatter plot of epididymal adipose tissue extracted and weighed on the day of sacrifice at the end of the 16-week diet (n=18). Statistical analysis performed using area under curve (AUC) for (A), linear regression for (B) and unpaired student's t-test for (C) & (D), ***p≤0.001, ****p<0.0001.

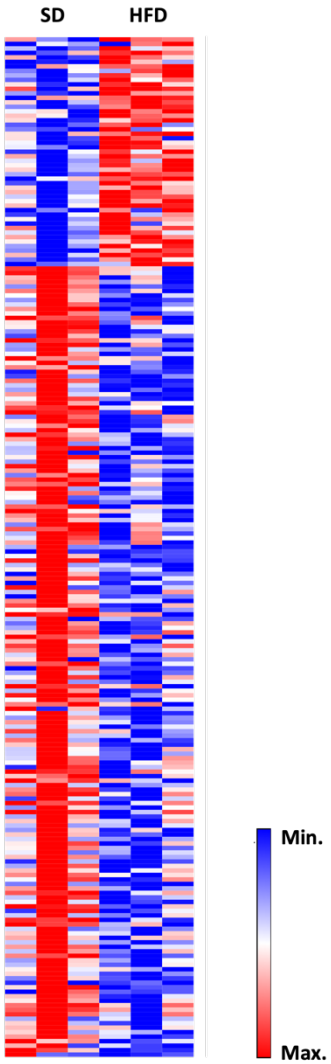
5.3.2 HFD yielded differential gene expression in untreated GM-DCs compared to SD

First, we extracted bone marrow precursors from mice on the SD or HFD and cultured bone marrow-derived dendritic cells in the presence of recombinant GM-CSF. RNA was isolated and RNA sequencing was performed (see section 2.8). The total number of genes differentially expressed was calculated, we observed 227 differentially expressed genes between SD and HFD untreated GM-DCs (Figure 5.2A). 51 genes were increased in expression and 176 genes were decreased in expression in HFD GM-DCs compared to SD GM-DCs (Figure 5.2A). All the 227 differentially expressed genes were displayed as a heatmap (Figure 5.2B). Next, we performed pathway analysis of all differentially expressed genes, and highlight the key processes the genes are involved in (Figure 5.2C). In summary, the major pathways altered by the high fat diet include cellular processes (25.8%), regulation (15.8%) and metabolic processes (13.3%) (Figure 5.2C). We demonstrated that obesity induced changes on an mRNA level in untreated GM-DCs (after 10 days of culture) whether this affected their functional properties warranted further investigation.

A

	SD vs HFD Untreated
Total differentially expressed genes	227
Increased	51
Decreased	176

B Untreated



C

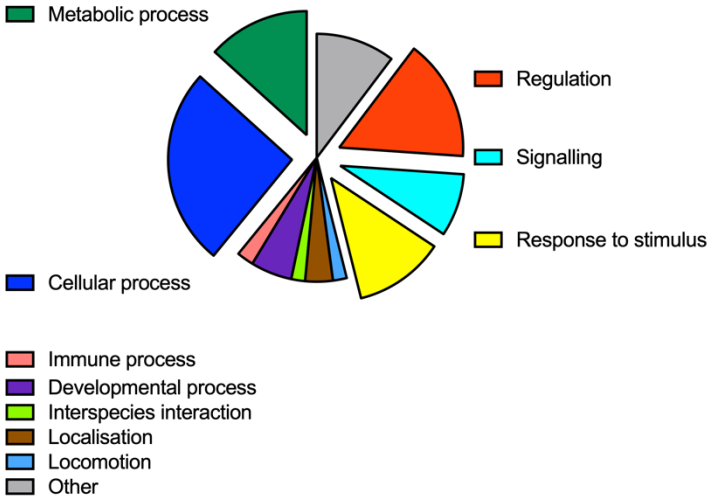


Figure 5.2 RNA sequencing revealed differential gene expression between untreated SD and HFD GM-DCs (A) Total number of genes differentially expressed between untreated SD and HFD GM-DCs. (B) Normalised differentially expressed genes between untreated SD and HFD GM-DCs (n=3) ($p \leq 0.05$). (C) Pathway analysis of biological processes affected between untreated SD and HFD GM-DCs.

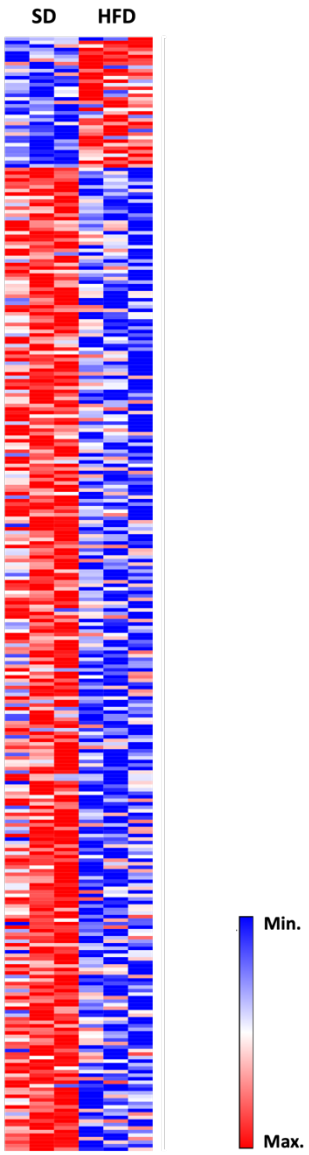
5.3.3 HFD yielded differential gene expression in SeV-stimulated GM-DCs compared to SD

First, GM-DCs from mice on the SD or HFD were stimulated with SeV for 18 hours. RNA was isolated and RNA sequencing was performed (see section 2.8). The total number of genes differentially expressed was calculated, we observed 316 differentially expressed genes between SD and HFD SeV-stimulated GM-DCs (Figure 5.3A). 37 genes were increased in expression and 279 genes were decreased in expression in HFD GM-DCs compared to SD GM-DCs (Figure 5.3A). All the 316 differentially expressed genes were displayed as a heatmap (Figure 5.3B). Next, we performed pathway analysis of all differentially expressed genes, and highlight the key processes the genes are involved (Figure 5.3C). In summary, the major pathways altered by the high fat diet, during SeV infection of GM-DCs, include cellular processes (26.4%), regulation (17.5%), metabolic processes (14.2%) and an evident pathway, response to stimulus (11.3%) (Figure 5.3C). We demonstrated that obesity induced changes on an mRNA level in both untreated (Figure 5.2) and SeV-stimulated (Figure 5.3) GM-DCs, compared to controls.

A

	SD vs HFD SeV
Total differentially expressed genes	316
Increased	37
Decreased	279

B SeV



C

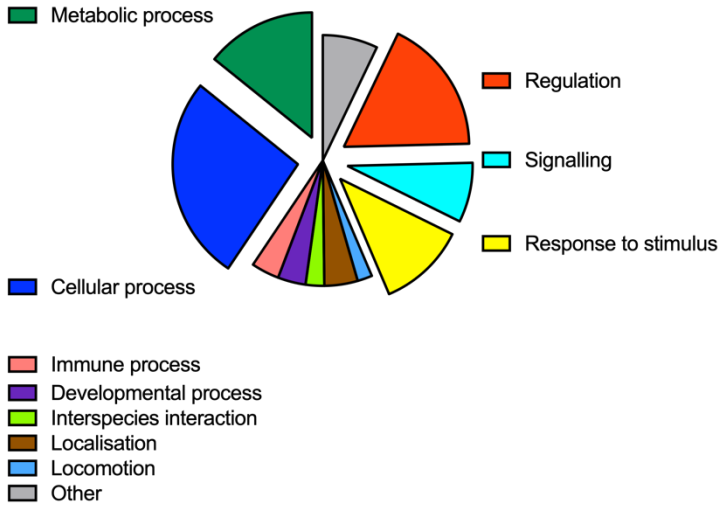


Figure 5.3 RNA sequencing revealed differential gene expression between SeV-stimulated SD and HFD GM-DCs (A) Total number of genes differentially expressed between 18-hour SeV-stimulated SD and HFD GM-DCs. (B) Normalised differentially expressed genes between SD and HFD GM-DCs following 18-hour SeV stimulation (n=3) ($p \leq 0.05$). (C) Pathway analysis of biological processes affected between SeV-stimulated SD and HFD GM-DCs.

5.3.4 HFD reduced gene expression of genes in GM-DCs ranging from antiviral responses to signalling

Our RNA sequencing data provided an array of differentially expressed genes, we investigated certain genes further based on their respective protein's functions in different pathways: antiviral responses, signalling or metabolism. Schlafen 4 (Slfn4) is part of a family of proteins, many of which their functions have not yet been elucidated. Slfns were found to be activated by Type I IFNs (Katsoulidis *et al.*, 2010), similar to ISGs (Kim and Weitzman, 2022). Different members of this family have demonstrated roles in cell differentiation, proliferation, and inhibition of viral replication (Schwarz, Katayama and Hedrick, 1998; Geserick *et al.*, 2004; Brady *et al.*, 2005; Ding *et al.*, 2022; Jitobaom *et al.*, 2023). In untreated GM-DCs, we observed lower *Slfn4* expression in HFD mice compared to SD (Figure 5.4A). Following SeV stimulation, *Sfln4* expression gradually increased for both groups, however, expression remained lower for HFD GM-DCs compared to SD, especially at the 18-hour timepoint where we observed peak expression (Figure 5.4B).

Il27 encodes the IL-27 cytokine which leads to the differentiation of specific T cell subsets, and inhibition of others, and IL-10 upregulation (Owaki *et al.*, 2005; Murugaiyan *et al.*, 2009). Therefore, this protein can have both pro-inflammatory or anti-inflammatory roles depending on the context. *Il27* expression trended downwards in untreated HFD GM-DCs compared to SD (Figure 5.4C). Following SeV stimulation, we found increased *Il27* expression which peaked at the 6-hour timepoint. At this timepoint we observed quite reduced *Il27* expression in HFD GM-DCs compared to SD (Figure 5.4D). *Il27* expression in both SD and HFD GM-DCs was lower at the 18-hour timepoint.

Oas2 encodes 2'-5'-Oligoadenylate Synthetase 2 (OAS2), a protein induced by interferons which subsequently activates ribonuclease L (RNase L) (Zhou, Hassel and Silverman, 1993; Kakuta, Shibata and Iwakura, 2004). RNase L inhibits protein synthesis, in turn, viral replication by degrading cellular and viral RNA (Zhou, Hassel and Silverman, 1993). *Oas2* expression trended downwards in untreated HFD GM-DCs compared to SD (Figure 5.4E). Following SeV stimulation, we demonstrated increased *Oas2* expression which peaked at the 18-hour timepoint. At 18 hours, expression was approximately halved in HFD GM-DCs compared to SD (Figure 5.4F).

Ptgs2 encodes PTGS2, also known as COX-2. PTGS produces prostaglandin and PTGS2 is the inducible isozyme of PTGS, induced during inflammation (Hla and Neilson, 1992). Prostaglandins function to control inflammation. *Ptgs2* expression was similar in untreated GM-DCs from both SD and HFD mice (Figure 5.4G). We observed increased *Ptgs2* expression at later SeV timepoints, the peak seen at 6 hours and a decline at 18-hours in SD GM-DCs. HFD GM-DC *Ptgs2* expression did not increase to the same extent as SD GM-DCs (Figure 5.4H).

Therefore, we demonstrate different genes involved in processes necessary for successful viral clearance are altered in obesity. Even basally, *Slfn4* expression was affected. Additionally, a key observation is the importance of time-points, the duration of the stimulation and when gene expression was measured. As at different stages of stimulation, certain genes were more upregulated compared to others (Figures 5.4B, D, F & H).

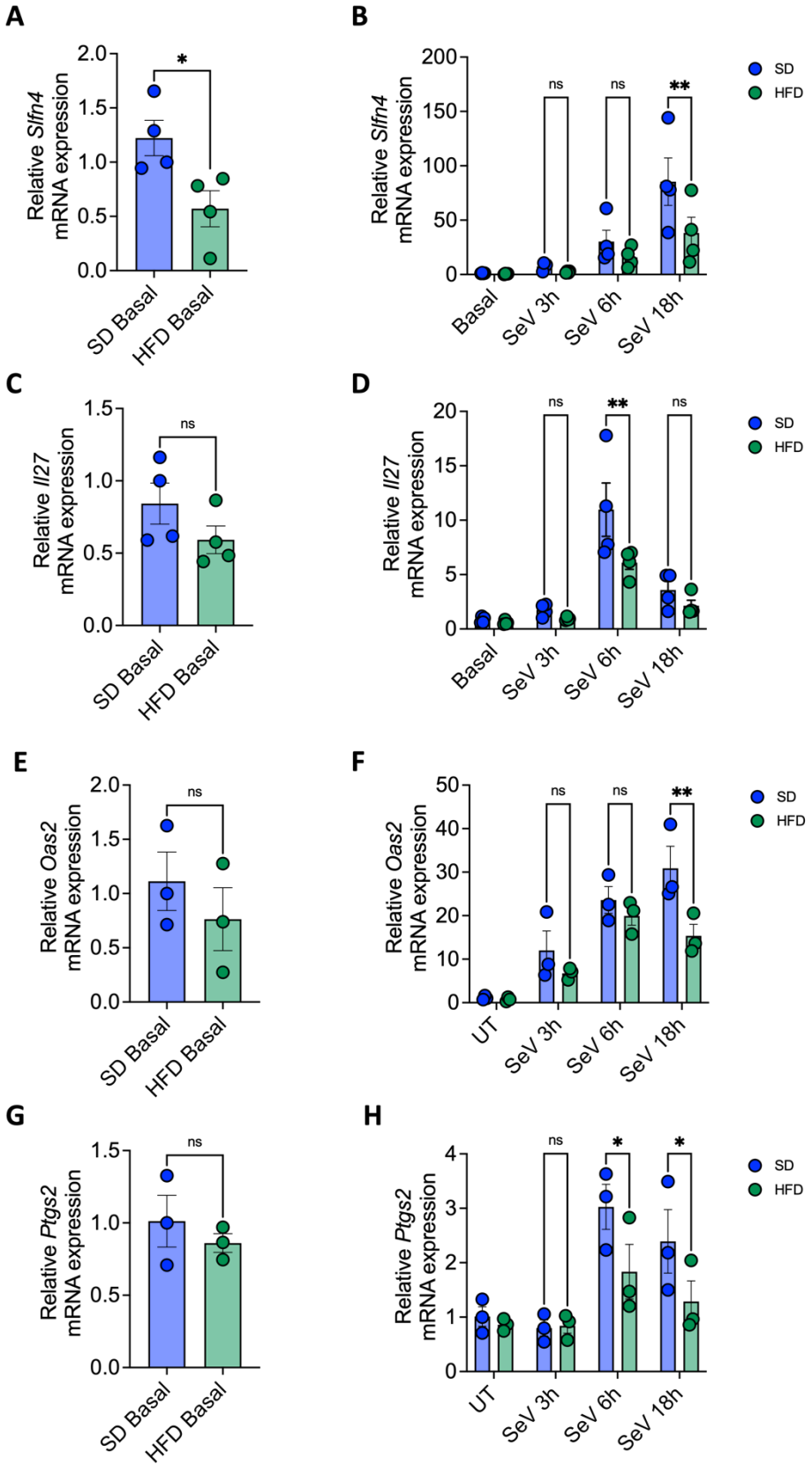


Figure 5.4 (Figure Legend on the next page)

Figure 5.4 Genes important for anti-viral responses were differentially expressed between SD and HFD GM-DCs Scatter plot of (A-B) *Slfn4* mRNA expression relative to *Actb* basally (A) and following SeV stimulation at different timepoints (B) between SD and HFD (n=4). (C-D) *Il27* mRNA expression relative to *Actb* basally (C) and following SeV stimulation at different timepoints (D) between SD and HFD (n=4). (E-F) *Oas2* mRNA expression relative to *Actb* basally (E) and following SeV stimulation at different timepoints (F) between SD and HFD (n=3). (G-H) *Ptgs2* mRNA expression relative to *Actb* basally (G) and following SeV stimulation at different timepoints (H) between SD and HFD (n=3). Statistical analysis performed using unpaired student's t-test or Two-way ANOVA with Tukey's correction, ns= $p>0.05$, *$p\leq0.05$, **$p\leq0.01$.

5.3.5 HFD decreased *Ifnb1* mRNA expression, with minimal effects on other cytokines, in GM-DCs following SeV stimulation

Cytokine production is a key function of dendritic cells. Upon activation, signalling events occur which culminate in increased target gene expression. Expression of pro-inflammatory genes and, in this case, anti-viral genes occur. *Tnf* and *Il6* encode TNFα and IL-6 respectively, these cytokines are key inducers of inflammation. *Ifnb1* encodes IFNβ, an important antiviral cytokine. *Il12b* encodes the IL-12 p40 subunit which heterodimerises with IL-12 p35 subunit to form IL-12p70 or with IL-23 p19 subunit to form IL-23 (Oppmann *et al.*, 2000), both resulting cytokines are important for inducing or maintaining specific T cell polarisation (Heufler *et al.*, 1996; Khayrullina *et al.*, 2008). Therefore, we sought to investigate the effect of obesity on mRNA expression of these vital GM-DC cytokines at different SeV stimulation timepoints.

Tnf expression trended downwards in untreated HFD GM-DCs compared to SD (Figure 5.5A). Following SeV stimulation, we demonstrated peak *Tnf* expression at 6 hours, however, no significant difference was seen between SD and HFD GM-DCs for *Tnf* expression (Figure 5.5B). Again, no significant difference in *Il12b* expression was noted in untreated HFD GM-DCs compared to SD (Figure 5.5C). Following SeV stimulation, we demonstrated *Il12b* expression doubled at 6 hours, but no differences were observed between SD and HFD GM-DCs (Figure 5.5D). *Il6* expression trended downwards in untreated HFD GM-DCs compared to SD (Figure 5.5E). Following SeV stimulation, we found that *Il6* expression increased for both SD and HFD GM-DCs, with a peak at 6 hours, but no differences between the two groups was observed (Figure 5.5F). The HFD had no significant effect on gene expression of these inflammatory cytokines.

Ifnb1 expression ranged for both SD and HFD untreated GM-DCs, where levels were quite low, and mean expression for HFD GM-DCs was slightly higher than SD (Figure 5.5G). Following SeV stimulation, we demonstrated *Ifnb1* expression was highest at 6 hours, with a clear difference between SD and HFD GM-DCs. Interestingly, *Ifnb1* expression was significantly lower in the HFD GM-DCs (Figure 5.5H). By 18 hours, *Ifnb1* expression had declined once again.

Overall, cytokine mRNA expression was highest following 6-hour SeV stimulation. At 18 hours, mRNA levels either returned to baseline (Figure 5.5D), was lower than basal conditions (Figure

5.5B) or was merely lower than 6-hour stimulation (Figure 5.5F & H). Importantly, we demonstrated obesity affected *Ifnb1* at 6-hour SeV stimulation, but no large impact on any other cytokine mRNA expression was observed.

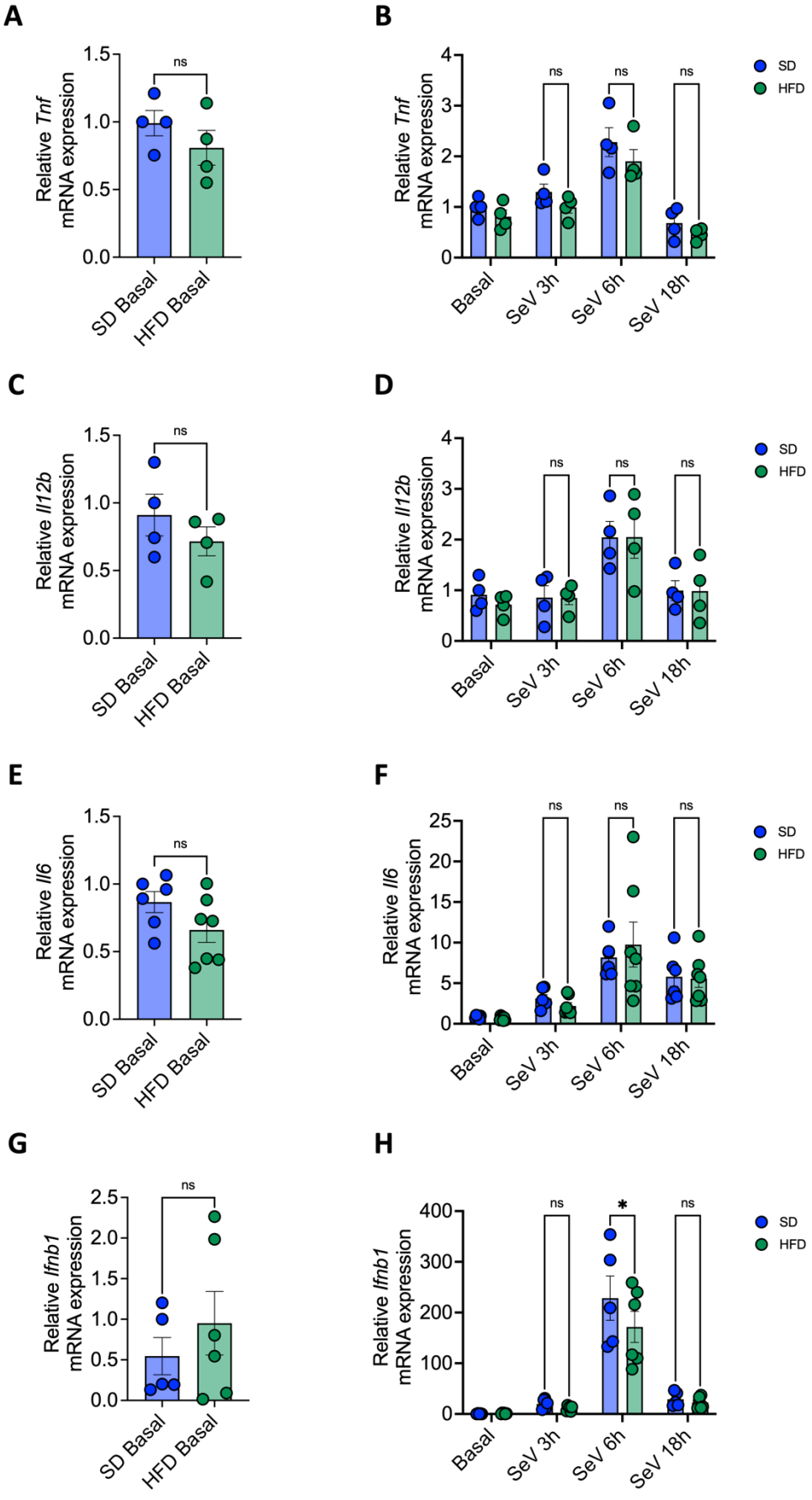


Figure 5.5 (Figure legend on the next page)

Figure 5.5 HFD decreased *Ifnb1* mRNA expression, with minimal effects on other cytokines, in GM-DCs following SeV stimulation Scatter plot of (A-B) *Tnf* mRNA expression relative to *Actb* basally (A) and following SeV stimulation at different timepoints (B) between SD and HFD (n=4). (C-D) *Il12b* mRNA expression relative to *Actb* basally (C) and following SeV stimulation at different timepoints (D) between SD and HFD (n=4). (E-F) *Il6* mRNA expression relative to *Actb* basally (E) and following SeV stimulation at different timepoints (F) between SD and HFD (SD n=6, HFD n=7). (G-H) *Ifnb1* mRNA expression relative to *Actb* basally (G) and following SeV stimulation at different timepoints (H) between SD and HFD (SD n=5, HFD n=6). Statistical analysis performed using unpaired student's t-test or Two-way ANOVA with Tukey's correction, ns=$p>0.05$, *$p\leq0.05$.

5.3.6 HFD decreased GM-DC cytokine production

To further elucidate the impact of obesity on functional properties of DCs, we stimulated GM-DCs from mice on SD or HFD with SeV for 18 hours. We harvested the supernatants and cytokine production was measured via ELISAs. Following stimulation, both SD and HFD GM-DCs were able to produce all cytokines, TNFα, IL-6 and IFNβ (Figure 5.6). We noted no significant difference in TNFα levels between HFD GM-DCs and SD controls. However, we demonstrated that IL-6 (Figure 5.6B) and IFNβ (Figure 5.6C) production were significantly lower in HFD GM-DCs when compared to SD controls.

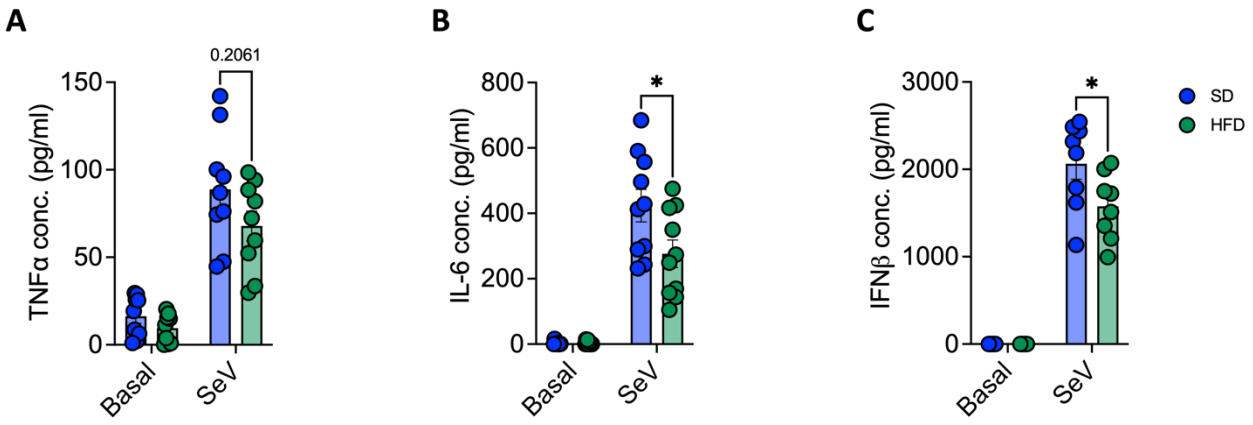


Figure 5.6 Obesity reduced GM-DC cytokine production upon SeV stimulation Scatter plot of (A) TNFα production (pg/ml) following 18-hour SeV stimulation (n=9). (B) IL-6 production (pg/ml) following 18-hour SeV stimulation (n=10). (C) IFNβ production (pg/ml) following 18-hour SeV stimulation (n=8). Statistical analysis performed using Two-way ANOVA with Tukey's correction, ns=p>0.05, *p≤0.05.

5.3.7 2-DG reduced *Tnf* and *Il12b* cytokine mRNA expression in SD GM-DCs, but only *Tnf* in HFD GM-DCs, following SeV stimulation

2-DG inhibits the first reaction of glycolysis, its target is depicted in Figure 4.3A. We previously demonstrated peak cytokine mRNA expression at 6-hour SeV stimulation, therefore, we investigated the impact of inhibiting glycolysis at this timepoint. 2-DG approximately halved the expression of *Tnf* in both SD and HFD GM-DCs, affecting both groups to the same extent (Figure 5.7A). Interestingly, inhibition of glycolysis reduced *Il12b* expression in only SD GM-DCs, and its expression was unaffected in HFD GM-DCs (Figure 5.7B). Like *Il12b*, *Il6* expression trended downwards for SD GM-DCs in the presence of 2-DG, but *Il6* expression in 2-DG presence is comparable to SeV only for HFD GM-DCs (Figure 5.7C). *Ifnb1* expression in the presence of 2-DG trended downwards for both SD and HFD GM-DCs, the difference is starker for SD GM-DCs possibly since *Ifnb1* expression is higher in SD SeV only compared to HFD (Figure 5.7D).

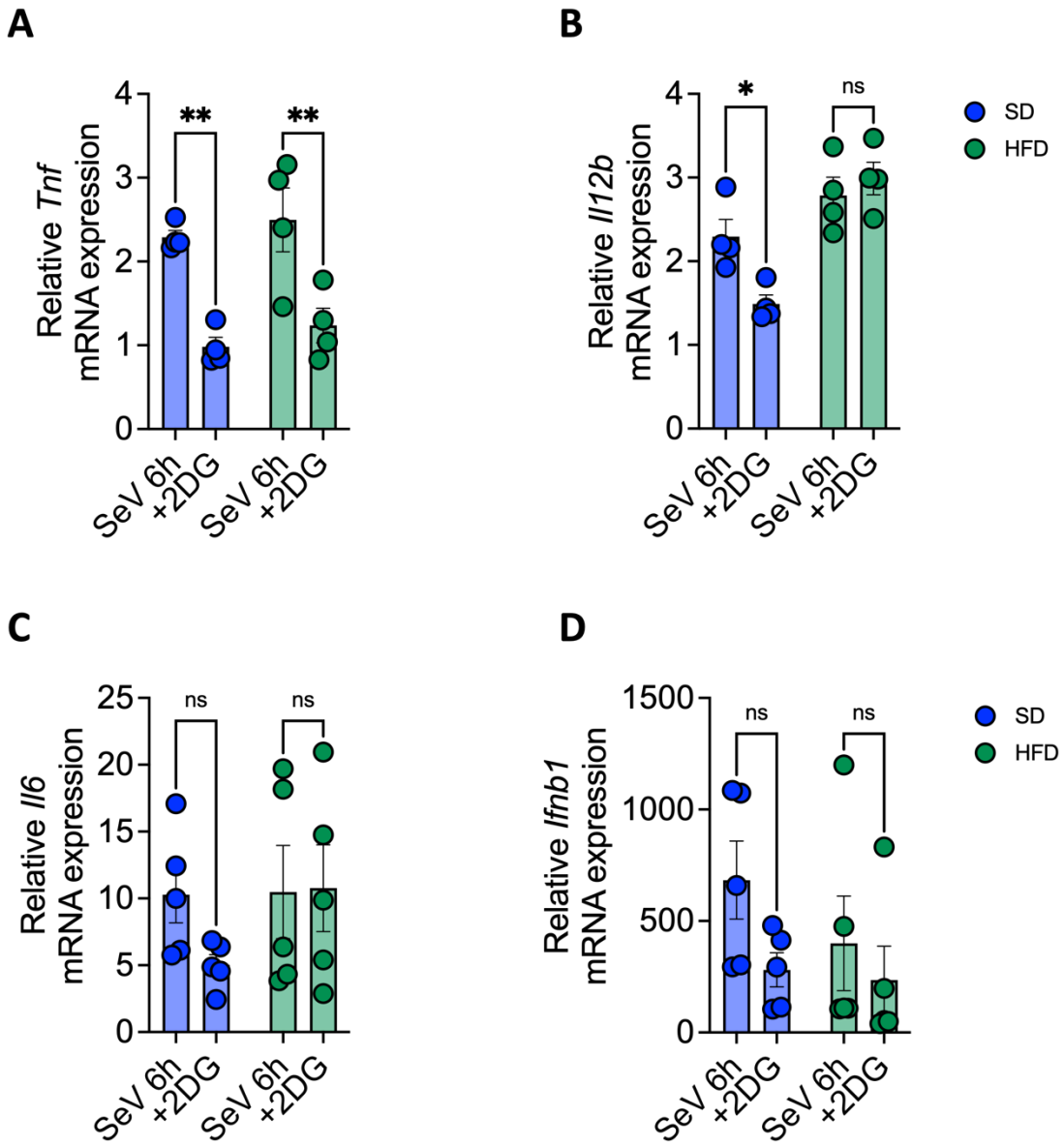


Figure 5.7 Hexokinase inhibitor 2-DG reduced *Tnf* and *Il12b* cytokine mRNA expression in SD GM-DCs, but only *Tnf* in HFD GM-DCs, following SeV stimulation Scatter plot of (A) *Tnf* mRNA expression relative to *Actb* following 6-hour SeV stimulation in the presence or absence of 2-DG (n=4). (B) *Il12b* mRNA expression relative to *Actb* following 6-hour SeV stimulation in the presence or absence of 2-DG (n=4). (C) *Il6* mRNA expression relative to *Actb* following 6-hour SeV stimulation in the presence or absence of 2-DG (n=5). (D) *Ifnb1* mRNA expression relative to *Actb* following 6-hour SeV stimulation in the presence or absence of 2-DG (n=5). Statistical analysis performed using Two-way ANOVA with Tukey's correction, ns= $p>0.05$, *$p\leq0.05$, **$p\leq0.01$.

5.3.8 2-DG reduced cytokine production in both SD and HFD GM-DCs

As glycolysis inhibition had varied effects on cytokine mRNA expression, we next determined its effect on cytokine production. We stimulated GM-DCs from both SD and HFD mice with SeV for 18 hours, in the presence or absence of 2-DG. Cytokines were measured in the aspirated supernatants. All cytokine levels, TNFα, IL-6 and IFNβ, were normalised to their respective stimulated sample. In the presence of 2-DG, TNFα concentration more than halved for both SD and HFD GM-DCs (Figure 5.8A). TNFα was the only cytokine which HFD GM-DCs produced less of, in the presence of 2-DG, compared to SD mice. IL-6 concentration approximately halved for both SD and HFD GM-DCs, in the presence of 2-DG (Figure 5.8B). IFNβ concentration was completely abrogated for both SD and HFD GM-DCs in the presence of 2-DG (Figure 5.8C). These results demonstrate the importance of glycolysis for GM-DC cytokine production.

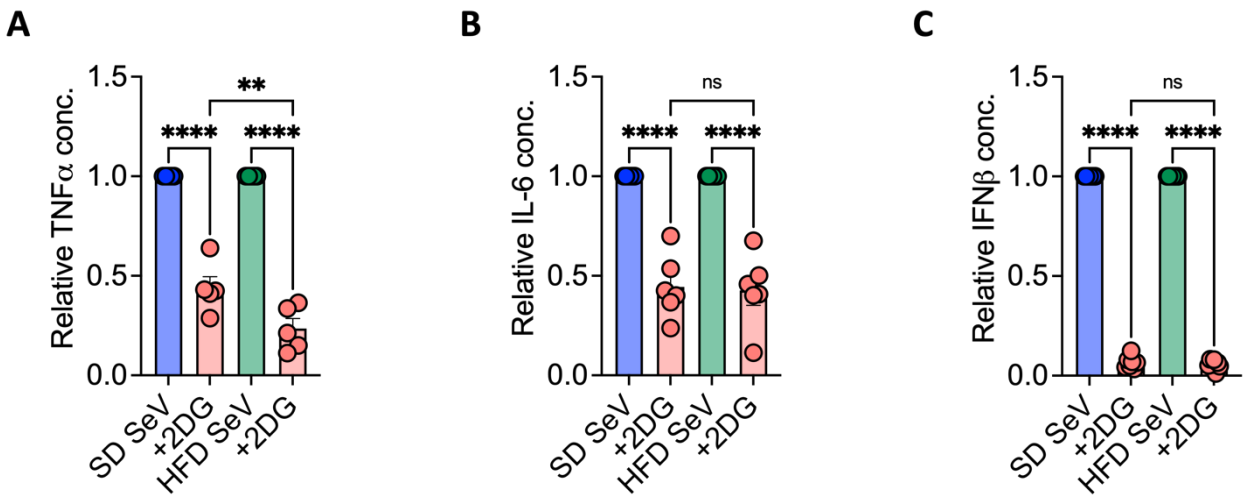


Figure 5.8 Hexokinase inhibitor 2-DG reduced SD and HFD GM-DC cytokine production to a similar extent, except for TNFα Scatter plot of relative (A) TNFα production (pg/ml) following 18-hour SeV stimulation in the presence or absence of 2-DG (n=5). (B) IL-6 production (pg/ml) following 18-hour SeV stimulation in the presence or absence of 2-DG (n=6). (C) IFNβ production (pg/ml) following 18-hour SeV stimulation in the presence or absence of 2-DG (n=7). Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction, ns= $p>0.05$, **$p\leq0.01$, ****$p<0.0001$.

5.3.9 Heptelidic acid reduced cytokine production in both SD and HFD GM-DCs

Heptelidic acid inhibits the fifth step of glycolysis, its target is depicted in Figure 4.8A. Inhibition of glycolysis with 2-DG demonstrated the importance of this metabolic pathway on cytokine production for both SD and HFD GM-DCs. Utilising other glycolytic pathway inhibitors, we wanted to confirm this finding. We stimulated GM-DCs from both SD and HFD mice with SeV for 18 hours, in the presence or absence of heptelidic acid. Cytokines were measured in the aspirated supernatants. All cytokine levels, TNFα, IL-6 and IFNβ, were normalised to their respective stimulated sample. In the presence of heptelidic acid, TNFα concentration was more than halved for both SD and HFD GM-DCs compared to SeV stimulated only (Figure 5.9A). A similar finding was observed for IL-6, where IL-6 concentration was more than halved for both SD and HFD GM-DCs, in the presence of heptelidic acid compared to SeV stimulated only (Figure 5.9B). Contrary to 2-DG (Figure 5.8C), heptelidic acid did not result in complete inhibition of IFNβ, the concentration was halved instead for both SD and HFD GM-DCs compared to SeV stimulated only (Figure 5.9C). Therefore, in the presence of heptelidic acid, cytokine production was reduced to the same extent in both SD and HFD GM-DCs following SeV stimulation.

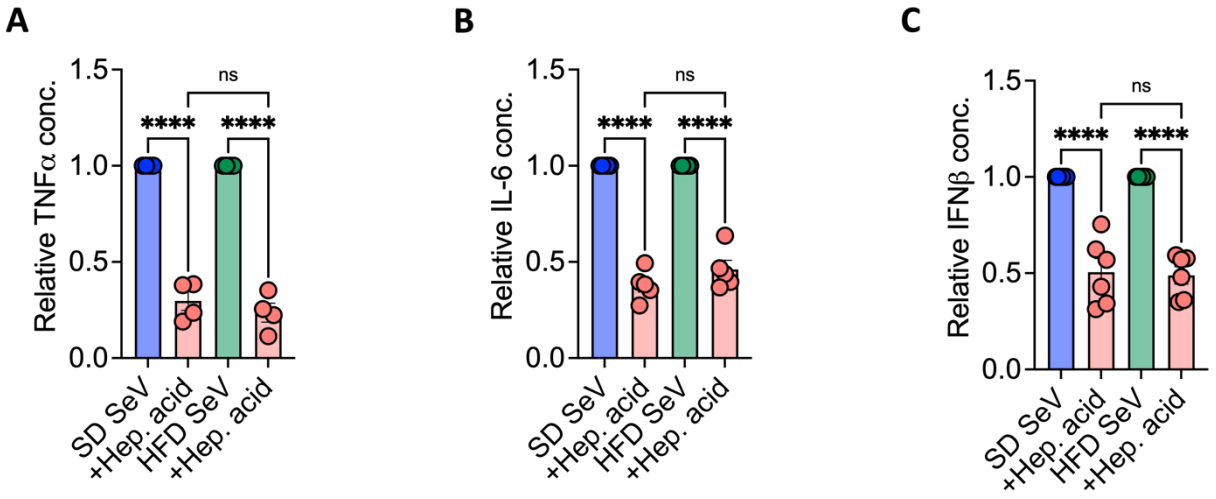


Figure 5.9 GAPDH inhibitor Heptelidic acid reduced SD and HFD GM-DC cytokine production to the same extent Scatter plot of relative (A) TNFα production (pg/ml) following 18-hour SeV stimulation in the presence or absence of heptelidic acid (n=4). (B) IL-6 production (pg/ml) following 18-hour SeV stimulation in the presence or absence of heptelidic acid (n=5). (C) IFNβ production (pg/ml) following 18-hour SeV stimulation in the presence or absence of heptelidic acid (n=6). Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction, ns= p>0.05, ****p<0.0001.

5.3.10 AZ67 had no effect on cytokine production in both SD and HFD GM-DCs

To further demonstrate the importance of glycolysis, we utilised another inhibitor, AZ67 indirectly inhibits the third step of glycolysis, its target is depicted in Figure 4.9A. We stimulated GM-DCs from both SD and HFD mice with SeV for 18 hours, in the presence or absence of AZ67. Cytokines were measured in the aspirated supernatants. All cytokine levels, TNFα, IL-6 and IFNβ, were normalised to their respective stimulated sample. For all cytokines, AZ67 had no significant impact on SD or HFD GM-DC cytokine production (Figures 5.10A – C). No differences were observed between SD and HFD GM-DC cytokine production, in the presence of AZ67.

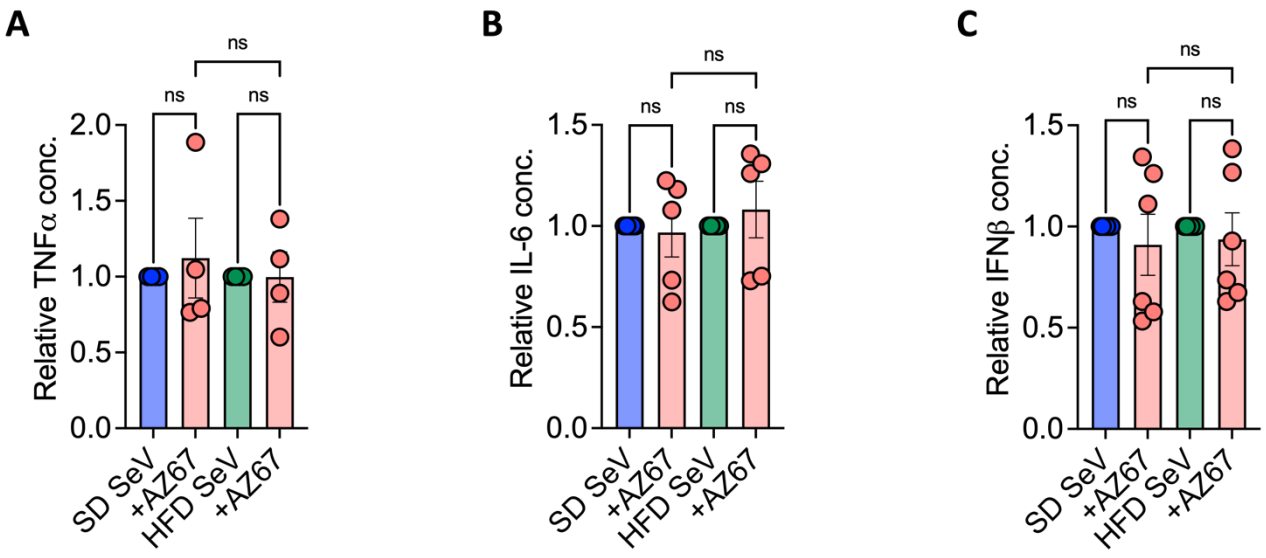


Figure 5.10 PFKFB3 inhibitor AZ67 had no effect on SD and HFD GM-DC cytokine production
Scatter plot of relative (A) TNFα production (pg/ml) following 18-hour SeV stimulation in the presence or absence of AZ67 (n=4). (B) IL-6 production (pg/ml) following 18-hour SeV stimulation in the presence or absence of AZ67 (n=5). (C) IFNβ production (pg/ml) following 18-hour SeV stimulation in the presence or absence of AZ67 (n=6). Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction, ns= $p>0.05$.

5.3.11 Oligomycin increased only *Il12b* mRNA expression in SD GM-DCs, but both *Il6* and *Il12b* in HFD GM-DCs, following SeV stimulation

Oligomycin inhibits OXPHOS, its target is depicted in Figure 4.3B. We stimulated GM-DCs from both SD and HFD mice with SeV for 18 hours, in the presence or absence of oligomycin. RNA was isolated and cytokine mRNA expression was measured, to determine the importance of OXPHOS.

For both SD and HFD GM-DCs, *Tnf* expression trended upwards, in the presence of oligomycin (Figure 5.11A). Whereas *Il12b* expression clearly demonstrated the effect of oligomycin, where its addition resulted in approximately twice as much *Il12b* expression compared to SeV stimulation only for both SD and HFD GM-DCs (Figure 5.11B). For SD GM-DCs, no clear difference in *Il6* expression was observed in the presence of oligomycin, as opposed to HFD GM-DCs where mean *Il6* expression doubled in the presence of oligomycin compared to SeV stimulation only (Figure 5.11C). *Ifnb1* expression trended upwards for SD GM-DCs in the presence of oligomycin compared to SeV only, a similar trend is possibly observed in HFD GM-DCs, however, due to the lower *Ifnb1* expression in HFD GM-DCs, the difference is not as stark as SD GM-DCs (Figure 5.11D).

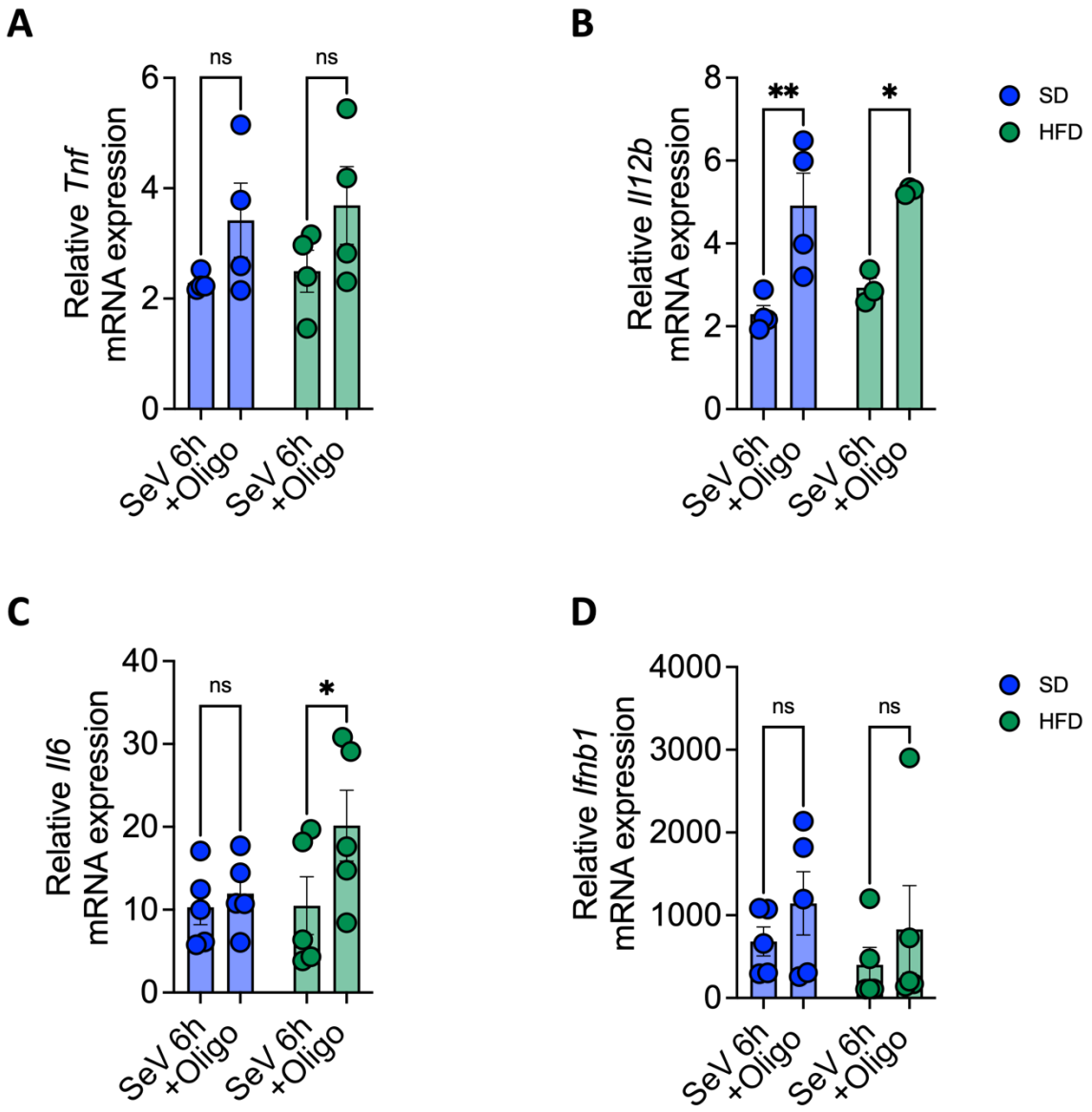


Figure 5.11 ATP synthase inhibitor Oligomycin increased only *Il12b* mRNA expression in SD GM-DCs, but both *Il6* and *Il12b* in HFD GM-DCs, following SeV stimulation Scatter plot of (A) *Tnf* mRNA expression relative to *Actb* following 6-hour SeV stimulation in the presence or absence of oligomycin (n=4). (B) *Il12b* mRNA expression relative to *Actb* following 6-hour SeV stimulation in the presence or absence of oligomycin (SD n=4, HFD n=3). (C) *Il6* mRNA expression relative to *Actb* following 6-hour SeV stimulation in the presence or absence of oligomycin (n=5). (D) *Ifnb1* mRNA expression relative to *Actb* following 6-hour SeV stimulation in the presence or absence of oligomycin (n=5). Statistical analysis performed using Two-way ANOVA with Tukey's correction, ns= p>0.05, *p≤0.05, **p≤0.01.

5.3.12 Oligomycin did not alter cytokine production in both SD and HFD GM-DCs compared to SeV stimulation only

OXPHOS inhibition during 6-hour SeV stimulation resulted in the increased expression of cytokine mRNA expression (Figure 5.11). Next, we investigated the impact of oligomycin on cytokine protein production, to determine whether the increase in certain cytokine mRNA expression correlated with the protein levels. We stimulated GM-DCs from both SD and HFD mice with SeV for 18 hours, in the presence or absence of oligomycin. Cytokines were measured in the aspirated supernatants. All cytokine levels, TNFα, IL-6 and IFNβ, were normalised to their respective stimulated sample (Figure 5.12).

No significant difference was observed in TNFα concentration for both SD and HFD GM-DCs in the presence of oligomycin, compared to SeV stimulation only (Figure 5.12A). However, HFD TNFα concentration trended upwards in the presence of oligomycin, possibly correlated with the same trend observed in HFD *Tnf* mRNA expression (Figure 5.11A). This trend in TNFα production was not observed in SD GM-DCs (Figure 5.12A). HFD GM-DC IL-6 production was higher and SD GM-DC IL-6 production was lower compared to their respective SeV stimulation only (Figure 5.12B) – no significant difference – but this demonstrated a difference between SD and HFD GM-DCs, in the presence of oligomycin. This correlated with *Il6* mRNA expression, as an increase was observed for HFD GM-DCs with the addition of oligomycin, and not for SD GM-DCs (Figure 5.11C). Relative IFNβ production was not different for both SD and HFD GM-DCs in the presence of oligomycin, and both appeared to decrease slightly compared to their respective stimulated only groups (Figure 5.12C).

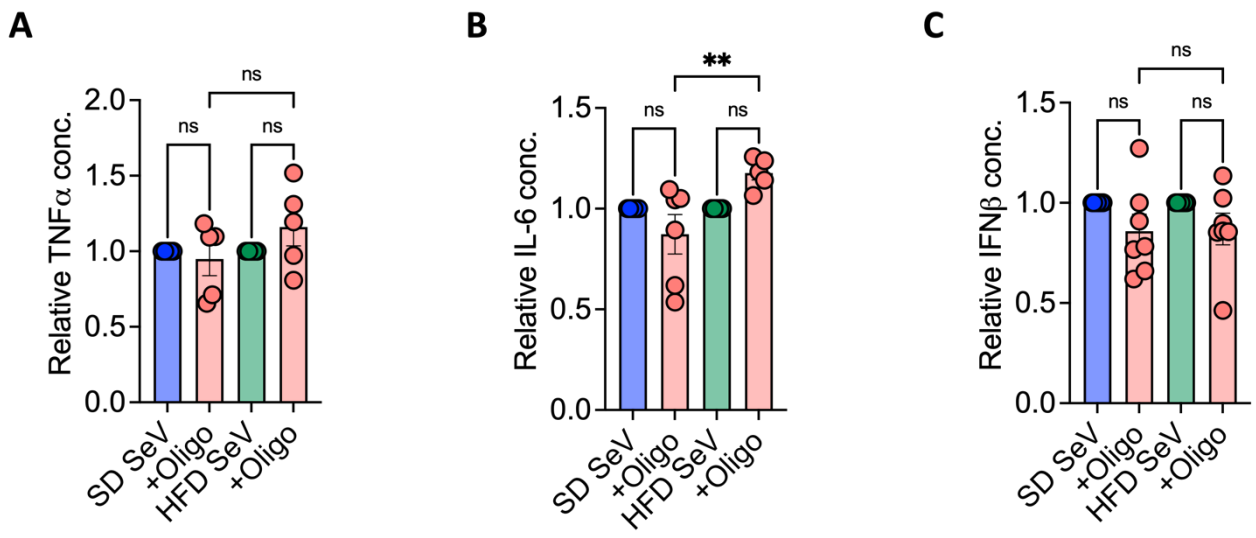


Figure 5.12 ATP synthase inhibitor Oligomycin did not alter cytokine production in both SD and HFD GM-DCs compared to SeV stimulation only Scatter plot of relative (A) TNFα production (pg/ml) following 18-hour SeV stimulation in the presence or absence of oligomycin (n=5). (B) IL-6 production (pg/ml) following 18-hour SeV stimulation in the presence of absence of oligomycin (SD n=6, HFD n=5). (C) IFNβ production (pg/ml) following 18-hour SeV stimulation in the presence or absence of oligomycin (n=7). Statistical analysis performed using Ordinary one-way ANOVA with Tukey's correction, ns= $p>0.05$, **$p\leq0.01$.

5.3.13 NF-κB signalling was required for HFD GM-DC activation by SeV

Upon SeV stimulation, IKBα is phosphorylated (Figure 3.11), this process enables the release of NF-κB and its subsequent translocation to the nucleus for pro-inflammatory gene expression (Beg *et al.*, 1993). As we show that obesity impacts certain cytokine mRNA expression, especially *Ifnb1* expression (Figure 5.5H), and subsequent cytokine production (Figure 5.6), we next wanted to investigate the signalling pathways which result in these downstream processes. We stimulated GM-DCs from both SD and HFD with SeV for 6 hours, then prepared them for immunoblotting as per section 2.10.

We blotted the samples for phospho-IKBα. SeV-stimulated GM-DCs from SD and HFD mice were compared. β-Actin was used as the loading control, ensuring the bands were consistent between all samples (Figure 5.13A). All samples, SD and HFD, expressed p-IKBα upon 6-hour SeV stimulation (Figure 5.13A), to varying degrees. Densitometry analysis was performed on the generated blots, p-IKBα bands were normalised to β-Actin. Mean relative levels of p-IKBα appeared to be higher in SD GM-DCs, however with the numbers investigated, this was not statistically significant (Figure 5.13B). Obesity could possibly impact signalling pathways required to activate the antiviral response, except further investigation is required, this could be a potential explanation of downstream differences observed.

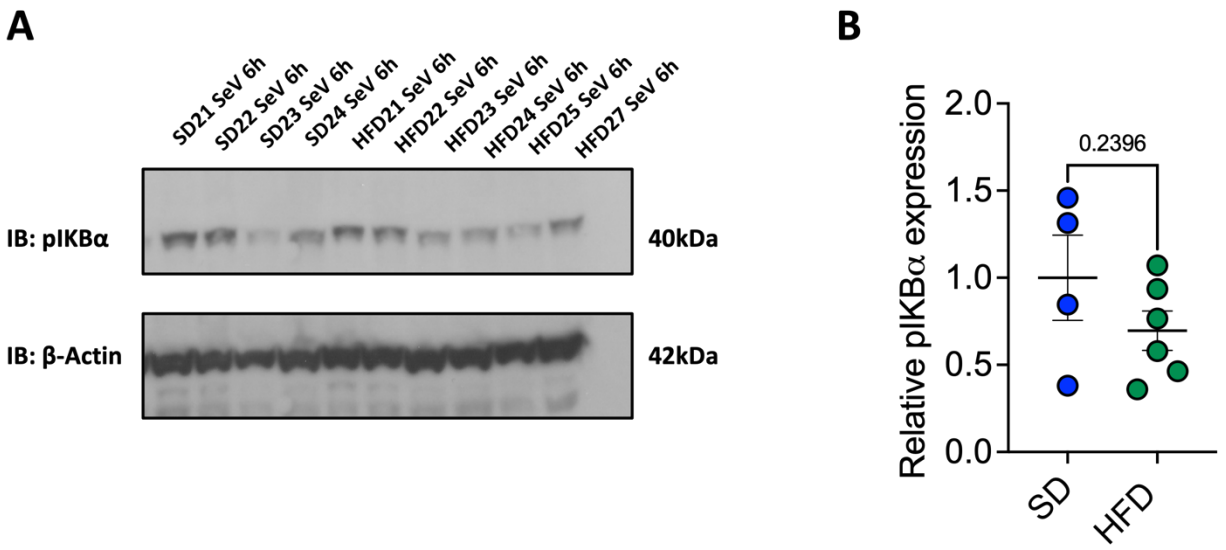


Figure 5.13 The NF-κB pathway was activated following SeV stimulation of SD and HFD GM-DCs (A) Immunoblot of phospho-IKBα (40kDa) and β-Actin (42kDa) expression basally and following 6-hour SeV stimulation (SD n=4, HFD n=6). (B) Densitometry displaying phospho-IKBα expression relative to β-Actin (SD n=4, HFD n=6). Statistical analysis performed using unpaired student's t-test, ns=p>0.05.

5.3.14 Obesity resulted in reduced protein synthesis

We demonstrated that obesity reduced cytokine production (Figure 5.6), therefore, next we wanted to determine the levels of protein synthesis occurring within GM-DCs, via the SCENITH assay (see section 2.11.4). Puromycin incorporation is equivalent to protein translation levels (Argüello *et al.*, 2020). Cellular energy, generated by the cells, fuels protein synthesis, therefore, protein synthesis can be used as a surrogate for metabolic activity. We stimulated GM-DCs from both SD and HFD mice with either SeV or LPS stimulation for 18 hours. Both stimulants were used as previously translation levels were shown to be different for both (Figure 4.2C &D).

We demonstrated that HFD GM-DC puromycin incorporation (geometric mean fluorescence intensity) was lower in all conditions: untreated (Figure 5.14A), SeV (Figure 5.14B) and LPS (Figure 5.14C) stimulation, compared to SD GM-DCs. Similar to SD GM-DCs (Figure 4.2C), HFD GM-DCs following SeV stimulation, increased protein translation levels (Figure 5.14D). Whereas no significant increase was observed following LPS stimulation for HFD GM-DCs (Figure 5.14E), similar to SD GM-DCs (Figure 4.2D). Next, protein synthesis levels were compared between SD and HFD GM-DCs and we observed significantly less protein translation in HFD GM-DCs when compared to SD GM-DCs (Figures 5.14F & G).

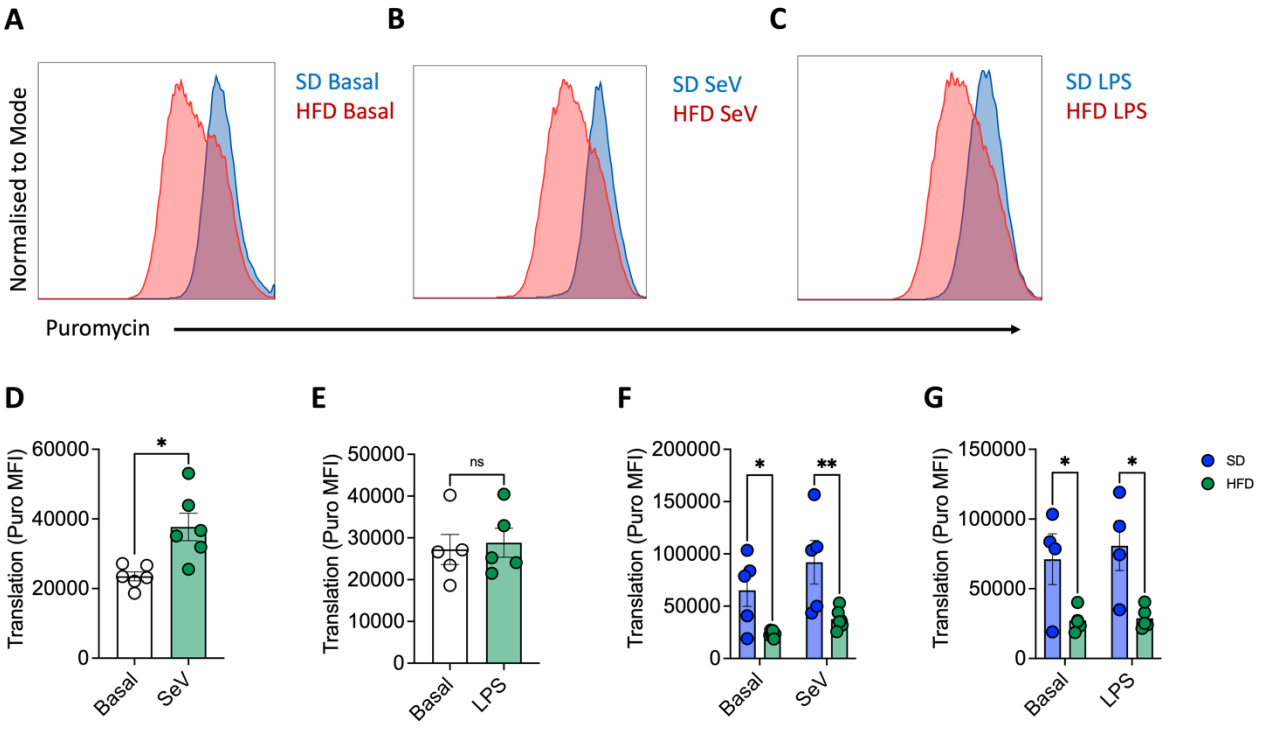


Figure 5.14 Protein translation was reduced in HFD GM-DCs compared to SD GM-DCs (A-C) Representative histograms of puromycin mean fluorescence intensity (MFI) in SD vs HFD GM-DCs basally (A), following 18-hour SeV stimulation (B) or following 18-hour LPS stimulation (C). Scatter plot of puromycin incorporation (MFI) in (D) HFD GM-DCs following 18-hour SeV stimulation (n=6), (E) HFD GM-DCs following 18-hour LPS stimulation (n=5). Puromycin incorporation (MFI) in (F) SD and HFD GM-DCs following 18-hour SeV stimulation (SD n=5, HFD n=6), (G) SD and HFD GM-DCs following LPS stimulation (SD n=4, HFD n=5). Statistical analysis performed using paired student's t-test or Two-way ANOVA with Bonferroni's correction, ns=p>0.05, *p≤0.05, **p≤0.01.

5.3.15 SD and HFD GM-DCs favoured the same metabolic pathways following SeV or LPS stimulation

We calculated the metabolic dependencies and capacities for each group, following stimulation with SeV or LPS, as per Table 2.9. The metabolic pathway favoured was shown to be stimulant-dependent (Figure 4.4). Glucose dependence refers to the proportion of protein synthesis (and energy production) reliant on oxidation of glucose. Mitochondrial dependence refers to the proportion of protein synthesis (and energy production) reliant on OXPHOS. Glycolytic capacity is when OXPHOS is inhibited, the maximum capacity of the cell to support protein synthesis. Fatty acid and amino acid oxidation (FAO & AAO) capacity is when glucose oxidation is inhibited, the capacity of the cell to support protein synthesis/ATP production using fatty acids and amino acids (Argüello *et al.*, 2020).

We showed that HFD GM-DC glucose dependence was similar to SD GM-DCs (Figure 4.4A & B) for untreated, SeV stimulation and LPS stimulation (Figure 5.15A & B respectively). Depicting a 70-90% reliance on glucose oxidation for GM-DC maintenance and function. Next, we showed that in SeV-stimulated conditions, there were no differences between SD and HFD mitochondrial dependence compared to their respective untreated samples (Figure 5.15C). Depicting a 70-80% reliance on OXPHOS for GM-DC maintenance and function, for basal and SeV-stimulated GM-DCs. Similar to SD GM-DCs (Figure 4.4D), mitochondrial dependence decreased following LPS stimulation (Figure 5.15D). Correlating with the observed increase in glycolytic capacity following LPS stimulation for both SD GM-DCs and HFD GM-DCs (Figure 5.15F). Demonstrating less of a reliance on OXPHOS for LPS stimulated GM-DCs. Following SeV stimulation, there was no change in glycolytic capacity for SD and HFD GM-DCs (Figure 5.15E). Therefore, SeV stimulation requires both glucose and mitochondrial oxidation for GM-DC function.

As the calculation for FAO & AAO capacity is the inverse of glucose dependence, we observed a slight decrease following SeV stimulation compared to untreated, for both SD and HFD GM-DCs (Figure 5.15G). The decrease is more noticeable for SD GM-DC LPS stimulation, however, for HFD GM-DCs, there seemed to be a slight increase for LPS stimulated compared to untreated (Figure 5.15H), but we did not observe a significant difference between SD and HFD.

The percentages calculated are all relative to their respective sample, therefore, these results demonstrate that obesity does not alter which metabolic pathways GM-DCs utilise, despite overall reduction in protein synthesis in HFD GM-DCs (Figure 5.14).

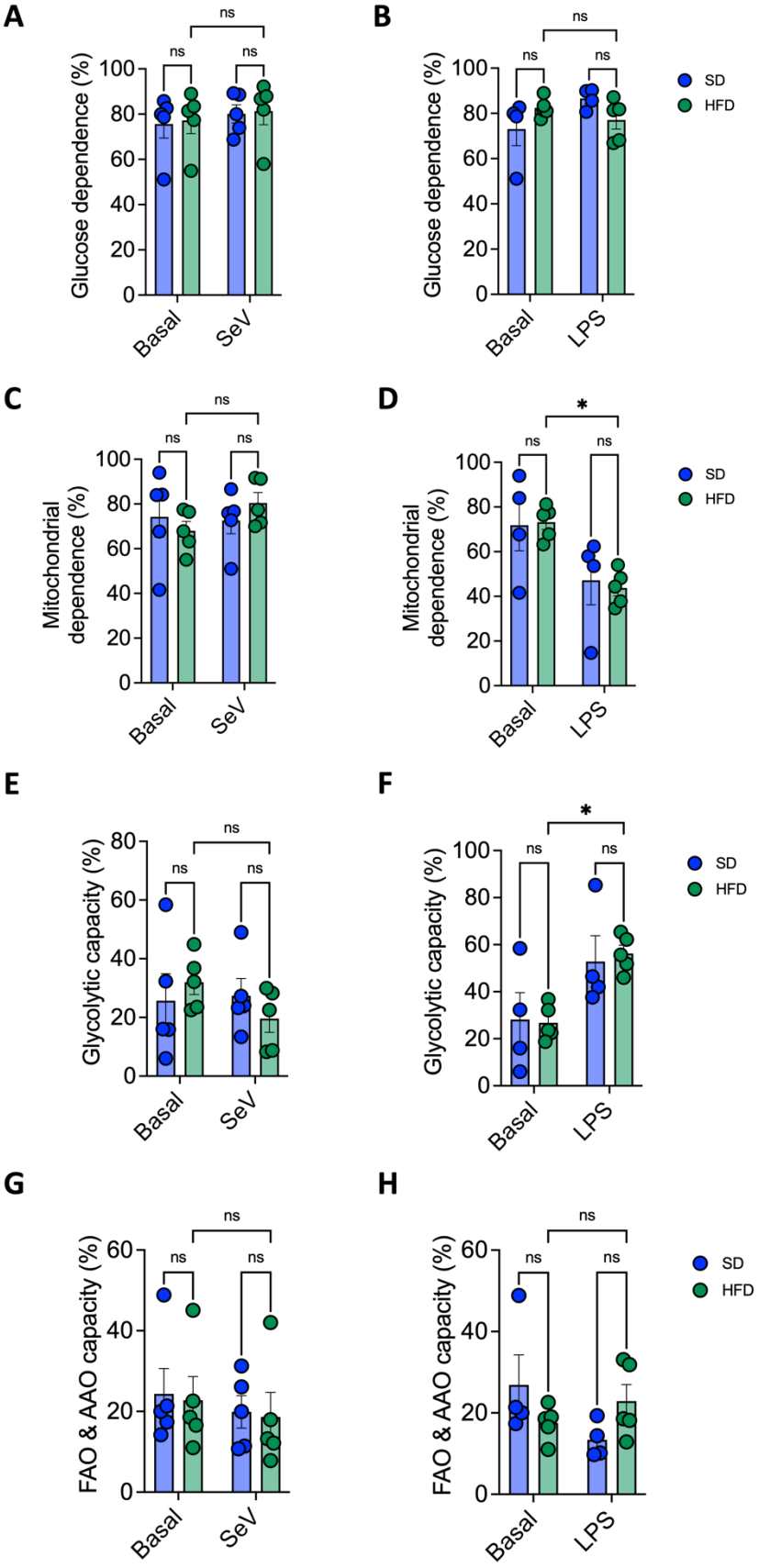


Figure 5.15 (Figure legend on the next page)

Figure 5.15 SD and HFD GM-DCs favoured similar metabolic pathways following SeV or LPS stimulation Scatter plot of (A-B) Glucose dependence of SD and HFD GM-DCs following 18-hour SeV stimulation (n=5) (A) or LPS stimulation (SD n=4, HFD n=5) (B). (C-D) Mitochondrial dependence of SD and HFD GM-DCs following 18-hour SeV stimulation (n=5) (C) or LPS stimulation (SD n=4, HFD n=5) (D). (E-F) Glycolytic capacity of SD and HFD GM-DCs following 18-hour SeV stimulation (n=5) (E) or LPS stimulation (SD n=4, HFD n=5) (F). (G-H) Fatty acid oxidation (FAO) & amino acid oxidation (AAO) capacity of SD and HFD GM-DCs following 18-hour SeV stimulation (n=5) (G) or LPS stimulation (SD n=4, HFD n=5) (H). Statistical analysis performed using paired student's t-test or Two-way ANOVA with Tukey's correction, ns=$p>0.05$, *$p\leq0.05$.

5.4 Discussion

Obesity is a multifactorial disease which is associated with over 200 chronic comorbidities (WHO, 2021; Horn, Almandoz and Look, 2022), including an increased susceptibility, morbidity, and mortality to infection (van Kerkhove *et al.*, 2011; Popkin *et al.*, 2020). Previous work from our lab found decreased circulating levels of dendritic cells in PWO, along with reduced expression of the maturation marker, CD83, on untreated and LPS- or Poly(I:C)-stimulated DCs from PWO compared to controls (O'Shea *et al.*, 2013). This observation depicted obesity-induced defects in DCs which could contribute to infection susceptibility in PWO. DCs are important cells in bridging the innate and adaptive immune response and are key APCs for naïve T cell activation and differentiation into effector and memory T cells (Sallusto and Lanzavecchia, 2002). In this chapter, we aimed to generate a murine model of diet-induced obesity to investigate the impact of obesity on GM-DC function and metabolism. As metabolism is intrinsically linked to cellular function (O'Neill, Kishton and Rathmell, 2016), we wanted to determine whether any changes in DC function was a consequence of altered metabolism.

Adipose tissue composition is regarded as a better indicator of obesity (Oliveros *et al.*, 2014; Franco, Morais and Cominetti, 2016). In our murine diet-induced model of obesity, we observed a very clear and distinct increase in epididymal adipose tissue, a 100% increase, in mice on the HFD compared to mice on the SD measured at cessation of the 16-week diet. This also coincided with an increase in body weight of HFD mice compared to SD mice. To gain a global view of the impact of obesity on GM-DC biology, we performed RNA sequencing on cells from SD and HFD mice. We observed that obesity had clear impacts on GM-DCs, prior to any stimulation, as untreated GM-DCs had 227 differentially expressed genes (DEGs) between the two groups. 77% of these genes were decreased in expression in HFD mice. Building on this approach, we also examined the effect of SeV on global RNA expression of GM-DCs in both SD and HFD groups, this had not previously been investigated. In SeV-stimulated GM-DCs, there were 316 differentially expressed genes between the two groups. 88% of these genes were decreased in expression in HFD mice. Differential gene expression already hints at the possible dysfunction in HFD GM-DCs. Chen *et al*. (2022) performed RNA sequencing on DCs from the spleen of mice on a HFD, compared to SD. This study demonstrated

transcriptional alterations between SD and HFD splenic DCs. Genes involved in activation, differentiation and migration of leukocytes were upregulated in SD splenic DCs, compared to HFD splenic DCs where genes involved in cell respiration, lipid and amino acid metabolism, and mitochondrial function were upregulated (Chen *et al.*, 2022). Gene alterations, in this case, correlated with metabolic dysfunction in HFD splenic DCs which impeded their T cell stimulatory capacity. Jaiswal *et al.* (2020) also demonstrated transcriptomic alterations in DC progenitors isolated from the bone marrow of HFD mice compared to SD. Transcriptomic analysis displayed alterations in signalling pathways which correlated with increased oxidative stress in the HFD mice that led to increased DC expansion and accumulation in mice lungs and exacerbated inflammatory responses upon HDM exposure, compared to SD mice (Jaiswal *et al.*, 2020). Therefore, alterations in bone marrow-derived DC progenitors can downstream result in the dysfunction we observe. Here we demonstrate that there are also alterations in gene expression between GM-DCs generated from SD and HFD mice. Analysis of the DEGs, several genes involved in diverse pathways, from anti-viral responses to signalling, emerged. We focused on certain genes involved in metabolism and anti-viral responses to determine how DC function could be affected.

One gene, *Slfn4*, encodes a member of a family of proteins, named Schlafens, and most of their functions have not been elucidated. These genes are evolutionarily conserved between different vertebrates and are ISGs – activated by type I interferons (Katsoulidis *et al.*, 2010). These genes encode proteins with diverse roles within cells, ranging from differentiation, proliferation, inhibition of viral replication, and protein translation (Schwarz, Katayama and Hedrick, 1998; Geserick *et al.*, 2004; Brady *et al.*, 2005; Ding *et al.*, 2022; Jitobaom *et al.*, 2023). One of the members, SLFN11, has roles in inhibiting mTOR (Zhou *et al.*, 2020) and has been shown to selectively inhibit viral protein synthesis of another single-stranded RNA virus, human immunodeficiency virus (HIV) (Li *et al.*, 2012). *SLFN11*-KO cells displayed increased ER stress and unfolded protein accumulation (Murai *et al.*, 2021). These are just some roles of these proteins. *Slfn4* has been shown to be upregulated upon TLR ligand activation of bone marrow-derived macrophages and their upregulation is induced by IFNβ (van Zuylen *et al.*, 2011), with roles in myelopoiesis (van Zuylen *et al.*, 2011). A key observation in this chapter, is that SeV stimulation of HFD GM-DCs resulted in decreased cytokine production, including IFNβ, when compared to SD controls. Therefore, decreased IFNβ may play a role in decreased

Slfn4 expression in SeV-stimulated GM-DCs. Several SLFN proteins have been found to inhibit viral protein synthesis (Li *et al.*, 2012; Kobayashi-Ishihara *et al.*, 2023) or have roles in anti-tumour immunity (Gu *et al.*, 2021). As the roles of SLFN proteins continue to be elucidated, the importance of *Slfn4* can be investigated in anti-viral immunity and obesity.

IL-27 can induce T cell polarisation, it is involved in early Th1 differentiation (Owaki *et al.*, 2005) and can act in concert with IL-12 to enhance IFNγ production by $CD4^+$ and $CD8^+$ T cells (Takeda *et al.*, 2003; Morishima *et al.*, 2005). The addition of IL-27 can also augment NK cell cytotoxicity (Choi *et al.*, 2019). IL-27 is produced by activated APCs, including DCs (Siegemund *et al.*, 2008; de Groot *et al.*, 2012), and evidently has effects on numerous immune cells. Importantly, production of IL-27 by poly(I:C) activated $CD1c^+$ DCs enhanced the cytotoxic function of $CD8^+$ T cells in co-culture (de Groot *et al.*, 2012). Here, we show *Il27* mRNA expression was decreased at 6-hour SeV stimulation in HFD GM-DCs compared to SD controls. This could affect IL-27 production downstream, in turn, hindering the activation of effector cells, including the anti-viral functions of $CD8^+$ T cells. Another polarising cytokine, IL-6, is required for Th17 differentiation and maintenance (Harbour *et al.*, 2020). We observed reduced IL-6 production in HFD GM-DCs compared to SD. TNFα production trended downwards in HFD GM-DCs compared to SD, but levels were not significantly different. Previous work from Cha *et al.* (2020) demonstrated reduced *Il12b* mRNA expression in HFD GM-DCs compared to SD, following LPS activation. IL-12 is vital for Th1 cell differentiation (Heufler *et al.*, 1996). *Cd40* and *Cd83* mRNA expression was also reduced on LPS-activated HFD GM-DCs, compared to SD (Cha *et al.*, 2020). Altogether, these show alterations in GM-DCs due to obesity, resulting in reduced expression or production of co-stimulatory molecules or cytokines, which can impair downstream T cell stimulation by DCs. As co-stimulation and cytokines are important second and third signals for T cell activation, proliferation, and differentiation (Medzhitov and Janeway, 1997; Curtsinger and Mescher, 2010). Obesity has been shown to result in dysfunctional DCs with impaired T cell stimulatory capacity (Macia *et al.*, 2006; James *et al.*, 2012; Boi *et al.*, 2016). Collectively, these data highlight potential mechanisms through which obesity could impact host responses to infection.

We demonstrate that obesity impacts anti-viral function of DCs, impacting their ability to produce the anti-viral cytokine IFNβ. Upon SeV stimulation, there is both reduced IFNβ

cytokine mRNA expression and protein production. As mentioned above, there is a decrease in other cytokines, IL-6, involved in T cell polarisation. The decrease in type I interferon, impacted expression of one type of ISG, there could be others affected. HFD mice following influenza infection had a greater mortality rate accompanied by dramatically reduced mRNA expression of the genes encoding IFNα and IFNβ in the lung, compared to LFD mice (Smith *et al.*, 2007). Reduced expression of the genes encoding the pro-inflammatory cytokines, IL-6, TNFα and IL-1β, was observed 3 days after influenza infection in HFD mice compared to LFD controls. Along with a reduction of the anti-inflammatory cytokine, IL-10, also 3 days post-infection in HFD mice, compared to controls (Smith *et al.*, 2007). A follow-up study by the same group observed delayed infiltration of immune cells into the lungs of HFD mice compared to LFD, with decreased DCs throughout the period in the HFD mice (Smith *et al.*, 2009). Splenic DCs from the HFD mice had a reduced ability to elicit IFNγ by $CD8^+$ T cells, compared to LFD (Smith *et al.*, 2009). Chen *et al.* (2022) also demonstrated reduced antigen stimulatory capacity by HFD splenic DCs compared to LFD. Type I IFNs, along with other cytokines, are required for mediating innate and adaptive immune cell activities and impeding viral replication (Braun, Caramalho and Demengeot, 2002; Swann *et al.*, 2007; Cheney and Mcknight, 2010). Therefore, a reduction in type I IFNs by GM-DCs can hinder the activation of innate and adaptive immune cells, including NK cells which have important anti-viral functions. Reduced NK cell cytotoxicity was observed in the lung and spleen of influenza-infected HFD mice compared to LFD (Smith *et al.*, 2007). These are all potential mechanisms through which obesity can impact anti-viral responses and contribute to increased infection susceptibility and morbidity.

One caveat is that the decrease in some of the cytokines is not observed at the mRNA level. Lawless *et al.* (2017) demonstrated the upregulation of glycolytic enzymes or glucose transporter, *Slc2a1,* at 24 hours. We performed RNAseq following 18-hour SeV stimulation, no major changes in the glycolytic enzymes were observed between SD and HFD. It was a good timepoint to observe changes in genes differentiated later during stimulation, such as the ISG *Slfn4*. However, based on mRNA expression of the different cytokines, peak cytokine mRNA expression occurred earlier, as the 6-hour stimulation had greatest cytokine mRNA expression. *Ifnb1* mRNA expression was reduced in HFD GM-DCs compared to SD at 6-hour SeV stimulation, but not for any of the other cytokines (*Il12b* and *Il6*), *Tnf* expression did trend

downwards at this timepoint (and the same was observed in TNFα production). The reduction in *Ifnb1* expression, correlated with decreased protein production following overnight SeV stimulation. mRNA expression may not have been altered for all cytokines, such as *Il6*, but the differences in protein production could be due to another major finding we observed – protein translation was significantly decreased in GM-DCs from HFD mice compared to SD mice, in untreated, SeV- and LPS-stimulated conditions. Approximately half of cellular energy is utilised for protein synthesis (Buttgereit and Brand, 1995; Argüello *et al.*, 2020). Therefore, whether this finding is due to an energy deficit or protein synthesis/translation, still needs to be determined. Based on the metabolic dependencies and capacities of GM-DCs from both groups, the cells utilise the same pathways for SeV and LPS responses. We show that obesity did not alter which pathway is utilised following viral infection or ligand stimulation, as obesity has been shown to affect lipid metabolism (Michelet *et al.*, 2018; Chen *et al.*, 2022). Rates of metabolism have been shown to be decreased in other cell types due to obesity, such as NK cells (Michelet *et al.*, 2018) or MAIT cells (O'Brien *et al.*, 2019). Splenic DCs were observed to increase fatty acid oxidation (Chen *et al.*, 2022). Along with our SCENITH data, to study the metabolism differences, mRNA and cytokine levels were normalised to their respective sample to determine whether there was a greater effect of an inhibitor between the groups. The effects of different metabolic inhibitors on SD GM-DC effector cytokine production in Chapter 4 was largely replicated in HFD GM-DCs, demonstrating the reliance on the same metabolic pathways for both SD and HFD GM-DCs. Therefore, despite reduced cytokine production and protein translation, these HFD GM-DCs were able to utilise the same metabolic pathways. The only exception was *Il6* expression which was greater in oligomycin treated HFD GM-DCs compared to SD GM-DCs and this translated to the protein level, where IL-6 production was higher in oligomycin & SeV-stimulated HFD GM-DCs compared to SD. Production of the other cytokines (TNFα and IFNβ) were unaffected in the presence of oligomycin for both groups. This difference could be due to sample variation, and that OXPHOS inhibition potentially provides a glycolytic boost, which increased expression of mRNA during 6-hour stimulation but at a later stage, glycolysis would have been active and maintains the same levels of cytokine as uninhibited samples. Altogether, these data demonstrate that HFD and SD GM-DCs utilise the same metabolic pathways upon SeV stimulation for cytokine production and their inhibition by metabolic inhibitors, affects cytokine production by the same proportions. However, cytokine production in HFD mice is lower than SD mice,

therefore, the rates at which these processes are occurring could be lower in HFD mice than SD mice, the Seahorse assay could be used to determine the rates of glycolysis and OXPHOS to determine differences in SD and HFD GM-DCs. Another potential cause of decreased protein synthesis could be due to the fact that obesity has been shown to induce ER stress (Kawasaki *et al.*, 2012) and the unfolded protein response (Chen *et al.*, 2016) which could contribute to the dysfunctional protein synthesis. The exact mechanism resulting in reduced protein synthesis within HFD GM-DCs requires further investigation.

From the RNAseq data, another gene which was altered was *Oas2* (2'-5'-Oligoadenylate Synthetase 2). *Oas2* encodes an interferon-induced protein, important for anti-viral responses (Sadler and Williams, 2008). It is involved in the activation of RNase L, an enzyme which degrades RNA within the cell, thereby, preventing viral replication (Sadler and Williams, 2008). RNA degradation by RNase L can activate RIG-I or MDA-5, resulting in IFNβ production (Malathi *et al.*, 2007). We demonstrate a reduction in *Oas2* mRNA expression following 18-hour SeV stimulation of HFD GM-DCs, compared to SD. Reduction in *Oas2* could impact the production of its protein, hence, also affecting the production of IFNβ. The decrease in expression of *Oas2*, involved in activation of the cell and signalling, and the decreased mRNA expression and protein production of type I IFN measured made us question whether there were any issues upstream, during SeV activation of the cells. Therefore, we examined the signalling pathway involved in SeV activation of GM-DCs. As demonstrated in Chapter 3, the transcription factor NF-κB is activated during SeV signalling. The phosphorylation of its inhibitor, IκBα, was measured and a trending decrease in phospho-IκBα in HFD GM-DCs was observed compared to SD GM-DCs, but not a significant difference, therefore it remains unclear if the differences in cytokine production are as a result of altered signalling (transcription factor activation). Teran-Cabanillas *et al.* (2013) demonstrated a reduction in NF-κB transcriptional levels from poly(I:C)-activated PBMCs of PWO, compared to controls, which also correlated with decreased IL-6, IFNα and IFNβ production from PBMCs of PWO. In our study, an increase in sample numbers could highlight whether the trend is significant or not.

Another gene, *Ptgs2*, also known as *COX2*, encodes the inducible COX enzyme involved in prostaglandin production which serves to control inflammation (Hla and Neilson, 1992). COX-

2 has been found to have dual or opposing functions in the context of obesity. *COX-2* was highly expressed in adipose tissue from PWO and corresponded with increased prostaglandin E_2 (PGE_2) in the AT from PWO (García-Alonso *et al.*, 2016). But exposure of adipose tissue from PWO to PGE_2 displayed anti-inflammatory effects, with a reduction in pro-inflammatory cytokine mRNA expression (García-Alonso *et al.*, 2016). COX-2 expressed by adipose tissue macrophages was also shown to decrease adipose tissue dysfunction in HFD mice (Pan *et al.*, 2022). Therefore, the decrease in *Ptgs2* we observe may not have beneficial effects for HFD GM-DCs. PGE_2 was also shown to be important for DC migration, PGE_2 augmented CCR7 expression on Mo-DCs stimulated with CD40 ligand and enhanced their migration to the respective CCR7 ligands, CCL19 and CCL21 (Scandella *et al.*, 2002). Migration of DCs to lymph nodes is regulated via these chemokines and receptor axis (Alt, Laschinger and Engelhardt, 2002; Scandella *et al.*, 2004). We demonstrate reduced *Ptgs2* expression in HFD GM-DCs, following 6-hour and 18-hour SeV stimulation, compared to SD, therefore, if the corresponding protein is downregulated, prostaglandin synthesis could be impaired, hence affecting the effector function of these molecules, such as affecting DC migration.

Certain DC subsets, such as cDC1s, have important roles in protecting against obesity (Hernández-García *et al.*, 2022). Obesity can result in DC dysfunction which can result in inappropriate immune responses (O'Shea *et al.*, 2013; Macdougall *et al.*, 2018). In summary, we observed a decrease in the production of pro-inflammatory and anti-viral cytokines from GM-DCs generated from HFD animals. This was accompanied by a decrease in protein synthesis within HFD GM-DCs basally and upon stimulation (illustrated in Figure 5.16), perhaps due to reduced rates of metabolism. Therefore, we can conclude obesity is causing a defect in bone marrow precursors which are extracted from mice fed a high fat diet, and the defective phenotype is maintained within the differentiated GM-DCs and consequently affect their function. Cytokine production is a key effector function of DCs, it allows them to activate immune cells of the adaptive immune response (Sallusto and Lanzavecchia, 2002). A decrease in cytokine production would presumably impact the subsequent activation of interacting immune cells, a potential mechanism of how obesity could impact immune responses in the host. Dysfunctional immune responses would increase host susceptibility to infection and impair vaccine efficacy.

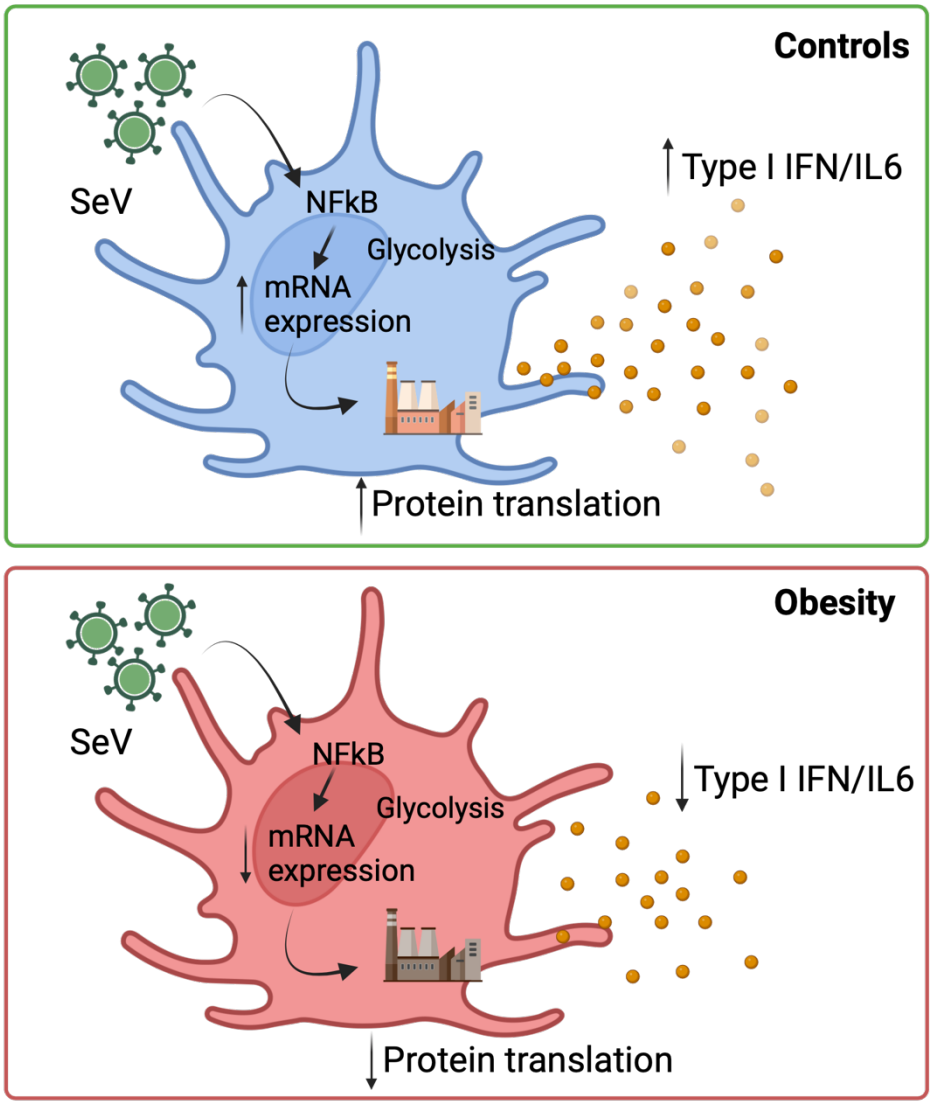


Figure 5.16 – Summary of the impact of obesity on GM-DCs compared to controls. In control SD mice, Sendai virus (SeV) infection of GM-DCs activated the transcription factor, NF-κB, which translocated to the nucleus and activated expression of various genes involved in the inflammatory or anti-viral response. Transcribed mRNA is translated in the cytosol and pro-inflammatory and anti-viral cytokines are secreted from the cells. Obesity resulted in decreased mRNA expression of various genes in the inflammatory and anti-viral response, with impaired protein synthesis and subsequent cytokine production.

Chapter Six

General Discussion

6. Discussion

Dendritic cells are innate immune sentinels which patrol the body to detect any intruders or signs of danger (Banchereau *et al.*, 2003; Steinman, 2003). DCs express a wide range of receptors which enable recognition of pathogens or infected/damaged cells (Lundberg *et al.*, 2014). During steady state, DCs display an immature phagocytic phenotype, then following pathogen recognition, DCs undergo maturation where they downregulate their phagocytic machinery and upregulate the components required for antigen presentation, and T cell instruction (Nagorsen, Marincola and Panelli, 2004; Kim and Kim, 2019). DCs are professional APCs ideally located at sites of pathogen entry (Banchereau *et al.*, 2003; Steinman, 2003), with efficient migration capacities to areas rich in T cells (Scandella *et al.*, 2004) and exceptional ability to subsequently activate naïve T cells into effector and memory T cells (Sallusto and Lanzavecchia, 2002). DCs are described as a bridge between the innate and adaptive immune responses. Upon activation, DCs present antigen on MHCs (Banchereau and Steinman, 1998) and upregulate the expression of costimulatory markers on their cell surface and secrete a myriad of cytokines (Akira, Uematsu and Takeuchi, 2006). DCs interpret signals from pathogens, other cells, or danger signals to ensure the appropriate immune responses are generated (Gallo and Gallucci, 2013). DCs express a range of PRRs which allow the detection of these signals.

SeV is a single-stranded RNA virus (Brabb *et al.*, 2012), and many disease-causing viruses also contain RNA as their genomic material, e.g., influenza virus or HIV (Poltronieri, Sun and Mallardo, 2015). SeV is non-pathogenic to humans (Matveeva *et al.*, 2015), therefore, it has been used to deduce the effects of RNA viruses on cells/tissues where pathogen entry is likely and on immune cells. Additionally, SeV has been studied as a potential vaccine adjuvant due to their immunostimulatory properties (Martínez-Gil *et al.*, 2013). SeV can activate the cytosolic PRR, RIG-I (Baum, Sachidanandam and García-Sastre, 2010). The resulting signalling cascade induces antiviral responses, such as the production of type I IFNs (Seth *et al.*, 2005). The Cantell Strain of SeV in a potent inducer of IFN-β (López *et al.*, 2006). LPS is a component of Gram-negative bacteria outer cell membrane (Rietschel *et al.*, 1994), and this exogenous ligand activates TLR4 on the cell surface (Ryu *et al.*, 2017). The resulting signalling cascade induces pro-inflammatory responses (Wan *et al.*, 2016). LPS has been widely used due to its

strong ability to promote inflammatory responses. Both SeV and LPS were used in this thesis to investigate dendritic cell antiviral and inflammatory responses, respectively.

DCs are a rare population of cells, which present technical difficulties in their investigation due to their scarcity in the host (Orsini *et al.*, 2012), therefore, differentiation of murine DCs from bone marrow precursors have been extensively used to study DC function and metabolism (Gilliet *et al.*, 2002; Everts *et al.*, 2012, 2014). Murine GM-DCs resemble inflammatory Mo-DCs from humans (Naik *et al.*, 2006; Zhang *et al.*, 2018). Mo-DCs are a subset of DCs which differentiate from monocytes during a state of inflammation (Naik *et al.*, 2006). FL-DCs give rise to the conventional subsets of DCs, cDC1s and cDC2s, and plasmacytoid DCs (Gilliet *et al.*, 2002; Onai *et al.*, 2007; Zhang *et al.*, 2018). These are the cells which circulate the body in the steady state (Orsini *et al.*, 2012). Hence this study has its limitations, as the impact of SeV or obesity on these cell subsets may not necessarily represent the other DC subsets. However, it provides a foundation with GM-DCs, onto which experiments can be translated into other DC subsets to determine whether similar differences are observed.

We found that SeV infection induced major transcriptional changes in GM-DCs, ranging from signalling to metabolism. Many components of the TNFA signalling pathway via NFKB were increased in expression following SeV stimulation which coincided with the activation of the NF-κB transcription factor by SeV. NF-κB activation results in cytokine expression (López *et al.*, 2006) and we observed increased expression of cytokine mRNA and protein by SeV. These cytokines are involved in antiviral or inflammatory responses. As expected, LPS stimulation also resulted in an increase in inflammatory cytokine mRNA expression and protein production. A key consideration arising from this thesis was the importance of choosing the appropriate housekeeping gene. Housekeeping gene levels are meant to be consistent despite the condition, however, we demonstrated alterations in the levels of *Hprt* in GM-DCs at different SeV stimulation timepoints which necessitated changing our housekeeping gene for all SeV RT-PCR assays. *Hprt* is an enzyme required to salvage purine nucleotides released during DNA breakdown (Stout and Caskey, 2003), especially guanine to produce guanosine monophosphate (GMP) (Carter-Edwards, Fung and Snyder, 1989). GMP is involved in a multitude of processes, as it can be converted to guanosine triphosphate (GTP) and cyclic GMP (cGMP) (Dasgupta *et al.*, 2021; Pasmanter, Iheanacho and Hashmi, 2022), these are all vital

cell molecules. These molecules can serve: as energy sources; as building blocks of RNA and DNA; for protein synthesis; as signalling molecules and second messengers (Bhagavan and Ha, 2015; Pasmanter, Iheanacho and Hashmi, 2022). *HPRT* has been found to be altered in tumour cells and normal host cells (Townsend *et al.*, 2017, 2019). Cancer cells and viral cells alter cellular metabolism and function similarly to sustain their proliferation. Viruses have been shown to hijack the metabolism of cells to enable their propagation (Aragón *et al.*, 2000; Connor and Lyles, 2002; Thaker, Ch'ng and Christofk, 2019). Therefore, the reduction of *HPRT* could serve as a mechanism through which cells dampen viral replication and propagation. Host cells have other mechanisms through which they counteract viral hijacking of protein synthesis (Stern-Ginossar *et al.*, 2019), but whether *Hprt* is targeted, directly or indirectly, would require further investigation. To summarise, in Chapter 3 we show that SeV and LPS both activate the NF-κB signalling pathway and produce certain cytokines in common, albeit to different extents.

Immunometabolism is the interplay between metabolism and immunology (O'Neill, Kishton and Rathmell, 2016). Reprogramming of immune cell metabolism has been shown to be vital for cell differentiation, proliferation, fate, and function. Immune cells utilise ATP and different metabolites for these processes (O'Neill, Kishton and Rathmell, 2016). As metabolism has been shown to link to cellular function, we next wanted to determine whether the two activations induced similar metabolic pathways within GM-DCs. Resting DCs have a distinct metabolic phenotype compared to activated DCs (Krawczyk *et al.*, 2010). GM-DCs have been extensively used to investigate DC metabolism. GM-DCs utilise fatty acid oxidation (breakdown of fatty acids) and glutaminolysis (breakdown of glutamine) to supply OXPHOS in the resting state of GM-DCs (Krawczyk *et al.*, 2010; Adamik *et al.*, 2022). Upon TLR stimulation, DCs upregulate glycolysis and lactate production (termed aerobic glycolysis) (Krawczyk *et al.*, 2010). Glycolysis is upregulated at a faster rate than OXPHOS (Pfeiffer, Schuster and Bonhoeffer, 2001) and produces an array of biosynthetic precursors required by the cell (O'Neill, Kishton and Rathmell, 2016). Early LPS stimulation was observed to increase FAS to provide the building blocks for the expansion of cellular organelles, i.e., Golgi apparatus and ER (Everts *et al.*, 2014). These organelles are required for protein production, folding and transport; their expansion is required to accommodate the heightened protein production induced by DC activation (Everts *et al.*, 2014). As the field of immunometabolism has

progressed, so have the techniques which can be used to study immune cell metabolism (Voss *et al.*, 2021). Alterations in cellular metabolism during different states provides potential targets for therapy, as the metabolic state of DCs is important during their differentiation from precursors, during their resting and activated state and during their polarisation of T cells (Krawczyk *et al.*, 2010; Everts *et al.*, 2014; Thwe *et al.*, 2017; Patente, Pelgrom and Everts, 2019).

We demonstrated by puromycin incorporation, an increase in protein synthesis in GM-DCs following SeV activation, which coincided with increased mRNA expression and production of various cytokines. A similar observation was found in LPS-stimulated GM-DCs, although the increase in translation levels following LPS stimulation was not significant. Viruses employ immune evasion strategies to prevent their detection or destruction (Alcami and Koszinowski, 2000). As mentioned previously, viruses can hijack different processes within the cell, such as protein production machinery, to allow their propagation (Aragón *et al.*, 2000; Connor and Lyles, 2002; Thaker, Ch'ng and Christofk, 2019). This may contribute to the increase in protein synthesis in SeV-stimulated GM-DCs. A novel finding of this thesis was that these two PRR activators may favour different metabolic pathways. After 18 hours of stimulation, LPS-stimulated GM-DCs had decreased mitochondrial dependency and increased glycolytic capacity, whereas these same dependency and capacity in SeV-stimulated GM-DCs were not altered. LPS stimulation results in long-term favouring of glycolysis (Everts *et al.*, 2012) but here we demonstrate that SeV stimulation was observed to still require the mitochondria. To study this further, the seahorse assay could be performed to determine the OCR and extracellular acidification rate (ECAR) following stimulation. Glycolysis however is still observed to be important for both stimulations, as the addition of the glycolytic inhibitor, 2-DG, decreased protein synthesis, observed by both SCENITH and ELISAs. However, the inhibition of glycolysis had a more profound effect on SeV-stimulated GM-DCs, particularly the production of IFNβ. Using various metabolic inhibitors, we demonstrated the impact of glycolysis inhibition on GM-DC cytokine production following SeV stimulation. Glycolysis was required and the effect of OXPHOS inhibition long-term did not alter cytokine production for both LPS and SeV activation of GM-DCs, signifying that the cells can utilize other pathways in the presence of oligomycin (OXPHOS inhibitor) to support cytokine production. The difference in activation of metabolic pathways by either the LPS ligand or with a viral infection, further

shows that *in vitro* stimulation may not correlate with what occurs *in vivo* and that ligands may act differently to the pathogen itself. Future work should investigate the impact of these ligands and SeV particularly, on GM-DC ability to phagocytose antigen, to express co-stimulatory molecules and to subsequently prime T cells. Understanding the metabolism during infection could allow the targeting of specific pathways to mitigate viral replication. However, the global effect of the metabolic inhibitors must be considered, as other cells could be impacted.

A key characteristic of DCs is their ability to migrate to different tissues around the body (Austyn *et al.*, 1988), where they are exposed to a multitude of microenvironments with oscillating levels of nutrients or oxygen (Gottfried *et al.*, 2006; Yang *et al.*, 2009). We sought to investigate the effects of different carbon sources on DC function. We observed no differences in cytokine production in GM-DCs stimulated in the presence of glucose, galactose, fructose or in glucose-deprivation. As mentioned previously, LPS-stimulated GM-DCs in normal media, containing glucose, and in the presence of oligomycin had no differences in cytokine production. However, inhibition of OXPHOS with oligomycin in GM-DCs in fructose or no glucose conditions resulted in cell death, which depicted a large dependency on OXPHOS to fuel GM-DCs when glucose is lacking. This effect of the carbon source contrasted other studies which replaced the media with galactose (Everts *et al.*, 2012). However, Lawless *et al.* (2017) found that switching the media to galactose-containing media after 8 hours of LPS stimulation, resulted in no effect on cell viability and increased ability to prime T cells. We cultured the GM-DCs in the media with the alternative carbon source for the duration of the stimulation (18 hours) and mostly found no difference in cytokine production compared to normal glucose-containing media. Jaiswal *et al.* (2019) cultured Mo-DCs in high concentrations of glucose or fructose (15mM) for varying timepoints (24 to 72 hours) and demonstrated a more pro-inflammatory phenotype from the fructose-cultured Mo-DCs. Therefore, these different studies and our own depict the importance of the timing of when the cells are exposed to the different carbon sources. This study depicts that GM-DCs are metabolically flexible and can utilise alternative carbon sources when glucose is not present by favouring other metabolic pathways to support the cell. Future work should investigate the effect of GM-DCs stimulated in these different conditions on T cell priming, whether similar T cell responses are generated. Cellular metabolism is a complex web where different pathways are

interlinked and can affect each other intrinsically but there is also the added component of extrinsic signals from the surroundings. DCs need to be able to sense these changes in the environment and the nutrient levels within the cells and act accordingly. In conditions, such as obesity, where different microenvironments can be altered (Sam and Mazzone, 2014), the impact on immune cells need to be further unravelled.

Obesity is a multifactorial, chronic and progressive disease, with various genetics and environmental factors playing their roles in the development and progression of the disease (Bray, Kim and Wilding, 2017; Sbraccia and Dicker, 2023). Determining the cause and impacts of obesity are of great importance to human health. Providing preventative measures is always the best means of tackling disease. However, due to the exponential increase in the cases of obesity worldwide over the past 40 years (WHO, 2021), determining how best to treat the illness is also essential. Obesity alters different immune cells, resulting in their dysfunction. PWO were shown to have reduced numbers of DCs in circulation along with reduced expression of the maturation marker, CD83, on untreated and LPS- or Poly(I:C)-stimulated DCs compared to controls (O'Shea *et al.*, 2013). A key DC effector function is their ability to produce cytokines (Nagorsen, Marincola and Panelli, 2004) and stimulate cells in their surroundings and the cells which they interact with (i.e., T cells) (Charles A Janeway *et al.*, 2001; Mattei *et al.*, 2001; Hervas-Stubbs *et al.*, 2014; Huntington, 2014). Cytokine release can induce naïve T cell activation, proliferation, and differentiation (Geginat, Sallusto and Lanzavecchia, 2001). O'Shea *et al.* (2013) also showed decreased IL-12 production in untreated and Poly(I:C) stimulated DCs from PWO compared to controls. IL-12 is important in inducing Th1 differentiation (Heufler *et al.*, 1996), therefore, a reduction in this cytokine would impact their subsequent differentiation. An increase in the immunosuppressive cytokine, IL-10 (Haase, Jørgensen and Michelsen, 2002; Mittal *et al.*, 2015), was observed in LPS- and Poly(I:C)-stimulated DCs from PWO compared to controls (O'Shea *et al.*, 2013). The Th2 cytokine, IL-4, was increased in T cell co-cultures with DCs from PWO compared to control DCs (O'Shea *et al.*, 2013). Favouring a more immunosuppressive phenotype would impair immune responses to pathogens, where clearance of pathogens and infected cells is required. Splenic DCs have previously been demonstrated to be higher in mice on a HFD compared to controls and these HFD DCs displayed reduced T cell stimulatory capacity (James *et al.*, 2012; Boi *et al.*, 2016). Reduced DC function would affect downstream T cell activation, and lower splenic $CD4^+$

T cells were observed in these HFD mice (Boi *et al.*, 2016). The impact of obesity on DCs requires further elucidation.

The reduction of cytokine production from SeV-stimulated HFD GM-DCs, which we demonstrated, has several implications. Firstly, this could be a potential contributing factor as to why obesity is associated with an increased susceptibility to infection (van Kerkhove *et al.*, 2011; Popkin *et al.*, 2020), as our murine DCs were not able to produce the same levels of cytokines as controls. Therefore, during viral infection, these murine DCs would not elicit the appropriate responses, potentially affecting the subsequent activation of the adaptive immune response. Interferon production is essential for host anti-viral responses. During the COVID-19 pandemic, type I interferon production was associated with disease severity, particularly, the timing and location of its production. Late type I IFN production was associated with greater immunopathology and early production ameliorated severe acute respiratory syndrome-coronavirus (SARS-CoV) pathology (Channappanavar *et al.*, 2016). Blunted type I IFN was associated with increased viral load in the blood of patients with increased disease severity and greater inflammatory markers (Hadjadj *et al.*, 2020). Therefore, reduced type I interferons from DCs would impair viral disease resolution.

Secondly, the diminished DC response could correlate with the reduced vaccination efficacy observed in obesity (Sheridan *et al.*, 2011), where there were reduced frequencies and function of memory $CD8^+$ T cells from HFD mice following a secondary influenza infection (Karlsson, Sheridan and Beck, 2010). Reduced DC cytokine could impact T cell priming and T cell memory generation, hence, affecting vaccine efficacy in PWO. Decreased cytokine production following SeV infection could also indicate diminished responses to vaccine adjuvants, which also target PRRs, possibly signifying a reduction in immunostimulatory properties of SeV as an adjuvant in PWO. This would affect vaccine development, in turn, efficacy once again. It has already been demonstrated that even following influenza vaccination, PWO have almost double the risk of influenza infection or influenza-like illness compared to vaccinated controls (Neidich *et al.*, 2017).

Thirdly, bone marrow precursors from both SD and HFD mice were extracted from the mice and cultured *in vitro* in the same media, therefore, the HFD GM-DCs were not exposed to the

obesogenic environment during the 10 days of culture. The dysfunction we witnessed in the GM-DCs may be epigenetically imprinted. Obesity has been previously demonstrated to cause chromatin remodelling in murine liver tissue (Leung *et al.*, 2014) and a switch in diet (from HFD to control diet) did not reverse the changes, as the epigenetic modifications were maintained (Leung *et al.*, 2016). Additionally, obesity has been shown to affect haematopoiesis. Obesity is being recognised as a state of accelerated aging. Similar to aging (Pang *et al.*, 2011), obesity causes skewing of haematopoiesis towards a myeloid lineage (Nagareddy *et al.*, 2014) and can result in a reduction in lymphoid subsets (Adler *et al.*, 2014), which would impact the adaptive arm of the immune response. Obesity has ranging effects on the bone marrow compartment and dysfunction in bone marrow subsets were found to be due to oxidative stress (Lee *et al.*, 2018). Lee *et al.* (2018) demonstrated that oxidative stress caused by obesity altered the compartment of HSCs, including the expression of a transcription factor, *Gfi1*. *Gfi1* is involved in transcriptional repression of HSCs, inhibiting their proliferation, and promoting quiescence (Hock *et al.*, 2004). Switching the HFD mice, with high *Gfi1* expressing HSCs, back to the chow diet did not reduce the expression of this gene, depicting sustained cell alterations due to obesity (Lee *et al.*, 2018). Alterations in HSCs, the bone marrow precursors, can potentially result in the dysfunction downstream in the generated dendritic cells. Haematopoiesis gives rise to the diverse immune cell subsets (Parekh and Crooks, 2013) and DCs and their precursors are replenished by bone marrow progenitors (Naik *et al.*, 2007). Therefore, if the bone marrow environment is altered by obesity, the subsequent cells and their functions possibly will be too.

Another novel finding of this thesis is that the decrease in cytokine production caused by obesity coincided with a decrease in protein synthesis in HFD GM-DCs basally, in SeV- or LPS-treated GM-DCs. A limitation of SCENITH is that it is a surrogate marker of metabolism but does not deduce how or why protein translation is altered. How protein translation is being affected requires further elucidation, whether it is due to dysfunction in organelles associated with protein production or due to inadequate levels of metabolic activity. The reduction in protein synthesis would infer a reduction in energy usage, which in turn depicts a lower rate of overall metabolism. This is supported by studies in other cell types, for example, natural killer cells from people with obesity displayed significantly reduced rates of both glycolytic and oxidative metabolism (Michelet *et al.*, 2018) and the unconventional T cell, MAIT cell, from

PWO also displayed reduced rates of glycolysis (O'Brien *et al.*, 2019). We demonstrated that the metabolic pathways stimulated by SeV are the same in SD and HFD GM-DCs, but these were data normalised to the respective sample. HFD GM-DC cytokine production was lower than that of SD. Future work should determine whether other proteins required by GM-DCs to function (such as co-stimulatory molecules) are also reduced. Transcriptomic analysis of 18-hour SeV-stimulated GM-DCs provided novel insights into gene alterations in HFD GM-DCs compared to SD. Delving into this data and investigating whether the subsequent protein synthesis is also altered is another avenue to pursue to determine the impact of obesity. As *Ptgs2* is one of the enzymes involved in prostaglandin synthesis (Hla and Neilson, 1992), PGE_2 has been demonstrated to have several impacts on DCs, including the ability to induce CCR7 expression on Mo-DCs (Scandella *et al.*, 2004), hence influencing their migratory capacity, or PGE_2 can influence DC cytokine production (Harizi *et al.*, 2002), in turn, affecting subsequent T cell activation (Kabashima *et al.*, 2003) or polarisation (Hilkens *et al.*, 1995). Therefore, whether the reduced *Ptgs2* mRNA expression observed in HFD GM-DCs translates to the protein level, would have implications on prostaglandin synthesis, in turn, affecting DC functions. Despite elevated prostaglandin levels being observed in obesity (García-Alonso *et al.*, 2016). However, similar to above, the dysfunction we observe in our GM-DCs may be epigenetically imprinted. This would require further investigation.

Future work should investigate metabolism (including carbon flexibility) and the impact of obesity on conventional DC subtypes, FL-DCs could be used. The original goal of this thesis was to translate the presented studies into humans, to determine whether obesity impacts human DCs in the same manner. However, after starting this PhD in February 2020, the world changed dramatically with the COVID-19 pandemic. This resulted in the closure of our research laboratory, and more importantly, closure of our obesity clinic for nearly 12 months. It highlighted however, in the most awful manner, the vulnerability of people living with obesity, and the absolute need for investigations like the one undertaken here. We had also aimed to investigate pDCs, which are the largest producers of type I IFNs and are important during viral infection (Siegal *et al.*, 1999; Hervas-Stubbs *et al.*, 2014), therefore, the potential of obesity impacting interferon production or protein synthesis could explain the increased susceptibility of PWO to infection (especially viral infection) (van Kerkhove *et al.*, 2011).

This research was performed using murine samples *in vitro* only, in media, which does not entirely replicate physiological conditions. The complete RPMI media which was utilised in the *in vitro* experiments contained approximately 11mM glucose levels, which is much higher than physiological glucose concentrations of 5mM (Wojtaszewski and Richter, 1998). Additionally, the cells are cultured on their own but in the body, DCs would be exposed to many different cells and factors, where cell-cell interactions and environmental factors could influence DC function. Therefore, mimicking physiological conditions is of great importance, possibly through the usage of different media such as human plasma-like media for any human translational work. In this research, the various metabolic inhibitors used were dissolved in certain solvents (molecular grade water or DMSO). More vehicle controls need to be incorporated in additional experiments, to ensure the effects observed are due to the inhibitor itself and not the solvent or vehicle. Additionally, in certain cases where there was either an increasing or decreasing trend in results, these trends could be ameliorated with an increase in sample numbers to determine whether the effect of the stimulation, inhibitor, or diet was significant.

People with obesity experience a decreased quality of life and reduced life expectancy (Peeters *et al.*, 2003; Kolotkin and Andersen, 2017). The physical, mental, and emotional tolls of the disease on the individual are profound and further research is necessary to determine how to treat the vulnerabilities caused by obesity. As weight loss may not always reverse the dysfunction observed (De Barra *et al.*, 2023; Frasca *et al.*, 2023). In addition to the chronic illnesses associated with obesity, a subpar immune system can result in more frequent infection or the inability to clear infection in PWO (Maier *et al.*, 2018). Pharmaceutical treatments are showing natural killer cell improvements (De Barra *et al.*, 2023), further elucidation of these treatments on other immune cells is required, for example, investigating DC function pre- and post-treatment. Additionally, the effects of obesity on immune cell compartments are clear (Lynch *et al.*, 2009; Carolan *et al.*, 2015) and these data need to be heeded by governments, and strategies must be implemented worldwide to curb the growing figures of obesity. As the disease has major economic and social impacts within countries (Anekwe *et al.*, 2020).

This study has provided novel insights of a potential mechanism as to how obesity induces susceptibility to infection in their host. We demonstrated DCs from mice on a HFD had impaired global protein synthesis, along with reduced cytokine production. We propose that a defect in their ability to perform one of their key effector functions impacts their subsequent activation of T cells, which would render the host more susceptible to infection and render vaccines more ineffective. This study has advanced the knowledge of SeV infection on DCs, its impact on DC metabolism and function. We also demonstrate the ability of DCs to favour different metabolic pathways to allow flexibility in fuel usage. The work performed in these GM-DCs can provide a foundation onto which additional experiments can be performed to determine the cause of reduced protein synthesis or to translate into humans. Determining these alterations broadens the understanding of how obesity impacts DC function and how it could be targeted to help people with obesity.

Chapter Seven

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