

The Role of β -Glucan Particles in Driving Type I Interferon Induced Trained Immunity



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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, unless stated otherwise in the text. I confirm that the work has been completed in full compliance with relevant university policies, including the Academic Integrity policy, the Trinity Policy on Good Research Practice, and the guidance on AI and GenAI in Teaching, Learning, Assessment and Research.

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Cian Horneck Johnston B.A. (1st Class Honours)

For Mum, Dad & Sunniva

Abstract

Effective antiviral immunity requires rapid early pathogen control while maintaining a balanced inflammatory response. Trained immunity, whereby innate immune cells develop enhanced secondary responses through sustained metabolic and epigenetic reprogramming, represents a potential strategy to improve host resilience. While fungal β -glucans are well established in antibacterial and antitumour contexts, their role in antiviral defence, particularly following dietary delivery, remains unclear.

In this thesis, I investigated whether yeast-derived β -glucan can induce antiviral trained immunity and defined the underlying mechanisms. Using *Saccharomyces cerevisiae*-derived whole glucan particles (WGP), I demonstrate that WGP induced durable metabolic reprogramming alongside stable epigenetic modifications. These effects required Dectin-1 engagement and phagocytosis.

Following stimulation with viral ligands or live viral infection in vitro, trained cells mounted amplified type I interferon (IFN-I) and interferon-stimulated gene responses, resulting in improved viral control. Mechanistically, β -glucan training inhibited fumarate hydratase (FH), a tricarboxylic acid cycle enzyme that regulates antiviral immunity. FH inhibition promoted mitochondrial nucleic acid release, triggering IFN-I signalling and priming macrophages into an antiviral state.

To establish in vivo relevance, mice were orally supplemented with WGP for up to four weeks. Dietary β -glucan induced central and peripheral features of trained immunity, including expansion of bone marrow progenitors, epigenetic reprogramming of circulating monocytes, and remodelling of lung alveolar macrophages. These effects were IFN-I dependent and persisted following β -glucan withdrawal, as demonstrated by enhanced responses to intranasal poly(I:C) challenge.

In summary, this thesis identifies a previously unrecognised mechanism of β -glucan-induced antiviral trained immunity, termed **Interferon-Dependent immune Training (IDT)**, providing a framework for exploiting dietary fungal components to enhance host preparedness against emerging viral infections.

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Publications

Recognition of yeast β -glucan particles triggers immunometabolic signaling required for trained immunity.

Horneck Johnston CJH, Ledwith AE, Lundahl MLE, Charles-Messance H, Hackett EE, O'Shaughnessy SD, Clegg J, Prendeville H, McGrath JP, Walsh AM, Case S, Austen Byrne H, Gautam P, Dempsey E, Corr SC, Sheedy FJ. *iScience*, 2024

β -glucans from *Agaricus bisporus* mushroom products drive Trained Immunity.

Case S, O'Brien T, Ledwith AE, Chen S, **Horneck Johnston CJH**, Hackett EE, O'Sullivan M, Charles-Messance H, Dempsey E, Yadav S, Wilson J, Corr SC, Nagar S, Sheedy FJ. *Front Nutr*. 2024

Dietary β -glucan reprograms myeloid immunity to reverse pbesity-induced dysfunction through trained haematopoiesis.

Ledwith AE, Prendeville H, **Horneck Johnston CJH**, McGrath JP, Corkish C, Cunningham S, Sulera MV, Ní Chasaide C, Mills K.H, O'Farrelly C, Finlay DG, Lynch L, Roche HM, Sheedy FJ. 2025 [Manuscript in preparation]

Patent on file No. 18/995,990; Beta-Glucan Particles for Trained Immunity

Ledwith Anna, Charles-Messance Hugo, Mitchelson Kathleen, Hackett Emer, **Johnston Cian Horneck**, Roche Helen, Sheedy J. Frederick

Abbreviations

MAMPs	Microbial Associated Molecular Patterns
PRR	Pattern Recognition Receptors
APCs	Antigen Processing Cells
DCs	Dendritic Cells
HSCs	Haematopoietic Stem Cells
BM	Bone Marrow
LT-HSCs	Long-Term HSCs
IL	Interleukin
TNF	Tumour Necrosis Factor
IFN	Interferon
CSF	Colony Stimulating Factor
M-CSF	Monocyte-CSF
GM-CSF	Granulocyte/Monocyte-CSF
TLR	Toll-Like Receptor
LPS	Lipopolysaccharide
OxPhos	Oxidative Phosphorylation
ST-HSC	Short-Term HSC
MPP	Multi-Potent Progenitor
Sca-1	Stem Cell Antigen 1
GMPs	Granulocyte-Monocyte Progenitors
LCPCs	Lineage Committed Progenitor Cells
CMPs	Common Myeloid Progenitors
CLPs	Common Lymphoid Progenitors
PAMPs	Pathogen Associated Molecular Patterns
MEPs	Megakaryocyte-Erythrocyte Progenitors
MDP	Macrophage-Dendritic cell Precursor
cMoP	Common Monocyte Precursor
MDMs	Monocyte Derived Macrophages
CCL2	C-C motif Ligand 2
CCR2	C-C Chemokine Receptor type 2
DAMPs	Damage Associated Molecular Patterns
TCA	Tricarboxylic Acid Cycle
ATP	Adenosine Triphosphate
ROS	Reactive Oxygen Species
NOS	Nitrous Oxide Species
Arg1	Arginase 1
FAO	Fatty Acid Oxidation
AMs	Alveolar Macrophages
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NKT Cell	Natural Killer T cell
IIM	Innate Immune Memory
TI	Trained Immunity
BCG	Bacillus Calmette-Guérin
mTb	Mycobacterium Tuberculosis
Ca	Candida Albicans
SCID	Severe Combined Immunodeficiency Disease
RAG1	Recombination Activating Gene 1

HSPCs	Haematopoietic Stem and Progenitor Cells
COVID-19	Corona Virus Induced Disease 19
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
IAV	Influenza Virus A
BMoAMs	Bone Marrow Monocyte derived Alveolar macrophages
FeMoAMs	Foetal Yolk-Sac Derived Alveolar Macrophages
WGP	Whole Glucan Particle
IP	Intraperitoneal
ILC	Innate Lymphoid Cells
PDH	Pyruvate Dehydrogenase
GADPH	Glyceraldehyde 3-Phosphate Dehydrogenase
HIF-1 α	Hypoxia Inducible Factor-1 α
Pi3k	Phosphoinositide 3-Kinase
AKT	Protein kinase B
mTORC	Mammalian Target of Rapamycin Complex
cKO	Conditional Knock-Out
WT	Wild-Type
ETC	Electron Transport Chain
NADH	Nicotinamide-Adenine Dinucleotide
FADH ₂	Flavin Adenine Dinucleotide
IMS	Inter Mitochondrial Membrane Space
$\Delta\phi_m$	Mitochondrial Membrane Potential
CoQ	Ubiquinone
SDH	Succinate Dehydrogenase
Cyto C	Cytochrome C
BMDMs	Bone Marrow Derived Macrophages
CMS	Citrate-Malate Shunt
ACOD1	Aconitate Decarboxylase 1
α -KG	Alpha Ketoglutarate
α -KGDD	Alpha Ketoglutarate Dependent Dioxygenase
MMF	Mono-Methyl Fumarate
DMI	Dimethyl Itaconate
MDH2	Malate Dehydrogenase 2
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
oxLDL	Oxidised Low Density Lipoprotein
MTA	5'-Methylthioadenosine
Set7	Lysine Specific Methyltransferase 7
TADs	Topology Associated Domains
HI-C	High-throughput chromosome conformation capture
IPLs	Immune gene-priming lncRNAs
UMLILO	Upstream master lncRNA of the inflammatory chemokine locus
JAK1	Janus Kinase 1
Tyk2	Tyrosine Kinase 2
STAT	Signal Transducer & Activator of Transcription
IRF	Interferon regulatory Factor
ISG	Interferon Stimulatory Gene
IRSE	IFN-stimulated Response Element
NALT	Nasal associated lymphoid tissue
BALT	Bronchus-associated lymphoid tissue

ER	Endoplasmic Reticulum
NA	Neuraminidase
SA	Sialic Acid
RIG I	Retinoic acid-inducible gene 1
ds	Double stranded
ss	Single stranded
HSV1	Herpes simplex virus
AIM2	Absent in melanoma 2
cGAS	Cyclic GMP-AMP synthetase
RLRs	RIG-like Receptors
MDA5	Melanoma Differentiation Associated Gene 5
MAVS	Mitochondrial antiviral signaling protein
MAM	Mitochondrial associated endoplasmic reticulum membrane
STING	Stimulator of IFN genes
BAX	BCL2 associated X protein
ASC	Apoptosis-associated speck-like protein
TF	Transcription factors
ADAR1	Adenosine deaminase acting on RNA 1
OAS1	2'-5'-oligoadenylate synthetase 1
PKR	dsRNA activated protein kinase R
HIV	Human Immunodeficiency Virus
cDC	Conventional Dendritic cells
IFNAR	IFN α/β Receptor
MOI	Multiplicity of Infection
pIC	Polyinosinic:polycytidylic acid
MSC	Mesenchymal Stromal Cells
HATs	Histone Acetyltransferases
HDACs	Histone Deacetylases
H3K4me3	Histone 3 lysine 4 tri-methylation
H3K27me3	Histone 3 lysine 27 tri-methylation
miRNA	Micro RNA
siRNA	Small interfering RNA
ncRNA	Non-coding RNA
CLR	C-type Lectin Receptor
sWGP	Soluble WGP
LCM	L929 Conditioned Media
PAM	PAM ₍₃₎ CSK ₍₄₎
HEK	Human Embryonic Kidney cells
ELISA	Enzyme-Linked Immunosorbent Assay
qPCR	Quantitative Polymerase Chain Reaction
CPH	Cyproheptadine
ECAR	Extracellular Acidification Rate
OCR	Oxygen Consumption Rate
2DG	2-deoxy-D-glucose
CytoD	Cytochalasin D
dZym	Depleted Zymosan
ODN	CpG oligodeoxynucleotide
TpIC	Transfected pIC
IFN1	Type I Interferon
G3-YSD	G3-ended Y-form short DNA

DMXAA	5,6- Dimethylxanthenone-4-acetic acid
VSV	Vesicular stomatitis virus
GO	Gene ontology
DEG	Differentially expressed gene
PCA	Principal component analysis
GSEA	Gene set enrichment analysis
KO	Knock Out

Chapter 1. Introduction

1.1 The immunological tree and its' branches

1.1.1 Historical context and discovery of the immune system

The work revolutionised by the 18th century English physician, Edward Jenner, kick started a revolution in biology which we now call Immunology. Jenner developed the first modern, vaccine, called such due to its origin from a relatively mild cow disease known as vaccinia¹. He has since been credited with laying the foundations of modern immunology. However, although he may have known that vaccinia was an avirulent disease he had no idea about the exact mechanism behind his work. By the late 19th century Robert Koch had discovered that microorganisms, he called pathogens, could cause disease² and Louis Pasteur furthered on this work developing vaccines for deadly diseases such as Rabies and Cholera. The search for an immunological mechanism had begun! In the 1890s, Emil von Behring and Shibasaburo Kitasato discovered that in animals immune to diphtheria and tetanus their serum contained anti-toxic molecules, now known as antibodies. This response was specific to the pathogen these animals were immune to, and the response was shown to evolve or “adapt” over time, laying the foundations for the adaptive immune system and immune memory. Around the same time Russian microbiologist, Elie Metchnikoff discovered that certain cells could attack pathogens and engulf them through a process he termed “phagocytosis” and these cells he termed macrophages or big eaters from the Greek makros (big) and phagein (to eat). Importantly, the ability of these cells to respond to pathogens did not require prior exposure and thus the first component of the innate immune system was discovered.

1.1.2 The two arms of the immune response

The discoveries made in the 18th and 19th century laid the very foundations for the field we now call immunology. Our bodies are constantly exposed to potential pathogens and their toxins, yet it is only in rare instances that one becomes sick and even more rare dies. Even more interesting, it is well studied that our own bodies must deal with self-containing microbiomes full of bacteria, fungi and

viruses³. The ability of our immune system to fight off infection while also maintaining tolerance is a complex process. This is, in part, due to specialised cells known as leukocytes which make up the immune system. As alluded to above, mammalian immune systems are split into two arms or branches: the innate response and the adaptive response. The innate response is always ready to respond and does not require prior exposure. It is composed of immune cells such as granulocytes, polymorphonuclear cells known as neutrophils and cells, like those which Elie Metchnikoff discovered such as macrophages. These cells respond to common ligands on pathogens known as microbial associated molecular patterns (MAMPs) and are recognised by (germline encoded) receptors on these cells known as pattern recognition receptors (PRRs). In contrast, the adaptive response requires time to establish, generating immunological memory to specific patterns on pathogens known as epitopes or antigens. This response is comprised of cells known as lymphocytes that express a myriad of genetically heterogeneous antigen-specific receptors which are made up of B cells which generate antibodies (B from Bursa of Fabricius) and T cells (T from thymus) which provide specific cytotoxic responses or “help” in maturing antigen presenting cells (APCs) such as dendritic cells (DCs). Together the two arms work in tandem to provide protection from pathogens. The innate response often buys time for the adaptive response to become effective. The initial adaptive immune response requires time due to its’ ability to generate long lasting memory T and B cells which develop antibody and T cell cytotoxic responses to specific epitopes⁴. In this way the adaptive immune response provides long-lasting protection from pathogens that have been previously encountered.

1.1.3 Maintaining immunity- Haematopoiesis

In contrast to the long-lived memory cells of the adaptive immune response, innate immune cells need constant maintenance and renewal. Many of these cells are short-lived, with monocytes having an average life span of 2 days whereas neutrophils only last less than 24hrs⁵. In contrast, certain innate cells, such as DCs and tissue resident macrophages have relatively longer half-lives and may also self-renew⁵. However, these cells are often the first casualties during infection and must

be replenished⁶. The short-lived cells are kept in circulation and only enter tissues when they receive distress signals, sometimes from either sites of damage or infection⁷. These signals include cytokines induced during inflammation and act as “push” factors that attract innate immune cells to the site they are needed in⁸. However, loss of both red and white blood cells in the peripheries, coupled with the short half-lives of these cells, begs the question; how are these cells maintained? The replenishment of these cells is known as haematopoiesis and occurs within the marrow of bones. Haematopoiesis is maintained by a group of stem cells known as haematopoietic stem cells (HSCs). Initially, these cells are multipotent before being influenced by different factors and conditions to become more committed to specific lineages (oligopotent) and finally becoming committed to a single mature immune cell lineage. I shall discuss the various factors that impact this development later, but for now I shall discuss HSCs and haematopoiesis in more detail.

1.1.4 The haematopoietic tree- Haematopoietic stem cells

I have just introduced the idea of haematopoiesis, however, now I shall explore the cells that make up this haematopoietic tree in more detail (**Fig 1.1**). As mentioned, the HSCs are found within bone marrow (BM) in a specific microenvironment controlled by many cell types such as endothelial cells, mature immune cell populations and mesenchymal stem cells. These cell types work together to control the homeostasis of HSCs, suppressing their maturation until signs of inflammation⁹. At the top of the haematopoietic tree reside a subpopulation of HSCs known as long-term HSCs (LT-HSCs). These cells are defined based on their self-renewing ability, relative quiescence and multipotent nature^{10, 11, 12}. LT-HSCs themselves are capable of reconstituting the immune system in lethally irradiated mice, showing their multipotent potential¹³. This is due to their self-renewal capacity which is marked by their quiescent state enabling them to have relatively long life spans¹¹. The time of this has been shown to range from a few months to 20-30% of the HSC population cycling every 150-200 days^{11, 14}. Interestingly, these stem cells have also been shown to express both PRRs as well as cytokine and chemokine receptors, meaning they can respond to pathogenic infections both directly and indirectly. LT-HSCs express receptors for cytokines such as interleukin (IL)-1 β , tumour necrosis

factor (TNF), IL6 and both type I and II interferons (IFN) as well as chemokines such as colony stimulating factors (CSFs) monocyte (M)-CSF and granulocyte/monocyte (GM)-CSF^{15, 16}. Similarly, LT-HSCs can respond directly to pathogens. For example, these cells express receptors known as Toll-like receptors (TLR), TLR-2 and TLR-4, which can respond to bacterial ligands such as peptidoglycan or lipopolysaccharide (LPS) respectively¹⁷. These factors, cytokines and direct sensing of pathogens, are known as “push” factors¹⁸. Coupled with the quiescent and self-renewing nature of LT-HSCs the hypoxic environment of the BM means that these cells rely more heavily on glycolysis and less on oxidative and mitochondrial metabolism known as oxidative phosphorylation (OxPhos)¹⁹.

However, in order for these cells to repopulate the immune system they must mature and differentiate, rather than self-renew. LT-HSCs differentiate into short-term (ST) HSCs and then into multi-potent progenitor (MPP) cells. Importantly, only LT-HSCs and ST-HSCs maintain the potential to completely repopulate the haematopoietic tree after lethal irradiation²⁰. ST-HSCs differ in their shorter self-renewal potential, roughly 1 month, and cannot revert to become LT-HSCs and thus are thought to be terminally differentiated from LT-HSCs²¹. Taken together LT-HSCs, ST-HSCs and MPPs are defined as HSCs and in mice are classified by expression of the stem cell factor surface receptor CD117 more commonly called cKit, stem cell antigen-1 (Sca-1) and lack of expression of mature cell markers known we characterise as lineage negative (LIN-). Therefore, these cells are defined throughout this thesis as LIN-cKit+Sca-1+ or LKS+ cells.

1.1.5 Multipotent progenitor cells

In contrast to LT- and ST-HSCs, MPPs lack the ability of self-renewal and have thus been shown to be inadequate in replenishing the haematopoietic tree post lethal irradiation^{22, 23}. Similarly, MPPs differ in their capacity for entering cell cycle, they are less quiescent and have higher differentiation activity^{22, 23}. The dawn of single cell transcriptomics and fate mapping has further characterized cellular differences between stem cells in the bone marrow²³. For example, MPPs in general were thought to be a homogenous population of cells, however, single cell

transcriptomics have identified that within the MPP population there are a number of sub-populations which have their own relative bias for differentiating down a particular haematopoietic branch²⁴. These unique MPP populations span from MPP1 to MPP4. MPP2/3 progenitors have a higher probability of becoming cells of the myeloid lineage (myeloid bias) whereas MPP4 progenitors have a higher probability of become cells of lymphoid lineage (lymphoid bias)²⁵. This was highlighted by single-cell transcriptomic analysis which demonstrated that myeloid biased MPPs had higher expression of myeloid lineage associated genes, such as *Irf4*, *Csf2* and *Tirap*, whereas lymphoid biased MPPs expressed lymphoid genes, such as *Cd24a* or *Flt3*²⁵. However, both subsets express genes that are common for granulocyte/monocyte differentiation, *Irf8* and *Cepba*, highlighting that although they have a higher probability/bias, they still retain the potential to differentiate into a different cell type²⁵.

I have previously mentioned that HSCs express PRRs and cytokine receptors that allow them to respond to “push” factors. Similarly, MPPs also express these receptors. For example, it has been shown that these cells can respond to LPS stimulation which encourages the specific expansion of myeloid cells, termed myelopoiesis, due to activation of TLR-4¹⁷. However, these cells also express the machinery necessary for production of cytokines. The release of cytokines, such as IL6, from these cells can enhance myeloid differentiation and proliferation of HSCs in a paracrine manner²⁶. Sepsis induced ‘emergency’ granulopoiesis, induced by LPS (and other bacterial factors during infection), can cause the production of G-CSF from endothelial cells which triggers the expansion of granulocytes through differentiation of granulocyte-monocyte progenitors (GMPs)²⁷. Similarly, HSCs and MPPs may respond to changes in systemic IFN levels either through viral infection (type I) or bacterial infection (type II), triggering granulopoiesis, myelopoiesis or both^{28, 29}. At least for now this hierarchical concept of HSCs and how they differentiate is accepted, however, as our understanding of infection and homeostasis increases, as well as the technology at our disposal, this already intricate model may further change.

1.1.6 Lineage committed progenitor cells

When these factors start the terminal differentiation of HSCs the next step after MPP is the lineage committed progenitor cells (LCPCs). These consist of cells such as common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs) as well as common granulocyte/monocyte progenitors (GMPs). These are more biased towards their respective lineages and once are at this point cannot switch lineage, unlike MPP2/3 and MPP4s. CLPs give rise to cells of lymphoid lineage, T cells, B cells and natural killer (NK) cells. CMPs can give rise to myeloid cells such as monocytes, macrophages and DCs as well as erythrocytes and megakaryocytes. GMPs, can give rise to granulocytes, monocytes and DCs. There is a natural bias towards the myeloid lineage, hence the increased number of myeloid cells in the body, due to preferential differentiation of MPPs³⁰. This makes sense in the context of immunity, since innate immune cells have shorter half-lives, they need to be preferentially replenished. In contrast, lymphocytes proliferate upon activation and mature in peripheral lymphoid organs and tissues. The ability of the BM niche to respond to changes in peripheral cell numbers is referred to as “pull” factors¹⁸. Therefore, “push” and “pull” factors effect the BM niche (**Fig 1.2**).

Like HSCs and MPPs, LCPCs also express receptors. Of particular importance is the expression of receptors for cytokines such as GM-CSF and M-CSF. Both cytokines are particularly important in driving differentiation and proliferation of CMPs down/along the myeloid lineage and have also been shown to enhance survival of these cells³¹. The knowledge that these cytokines have an impact on HSPC populations has been used for different treatments and therapies. For example, prior to BM donation, donors are given recombinant G-CSF to expand their bone marrow cells and patients who suffer from leukopenia, during cancer therapy, are also given G-CSF to expand their cell numbers^{32, 33}. Similarly, the BM can positively select for lineage specific cell types due to the abundance of circulating cytokines. For example, there is an expansion of myeloid biased progenitors in response to BCG vaccination dependent on IFN γ signalling³⁴, expansion of granulocytes post β -glucan injection (intraperitoneal) is dependent on

type I IFN signalling³⁵ and progenitor cells can respond to PAMPs of helminths and IL5 to induce the expansion of eosinophils during parasitic infections³⁶.

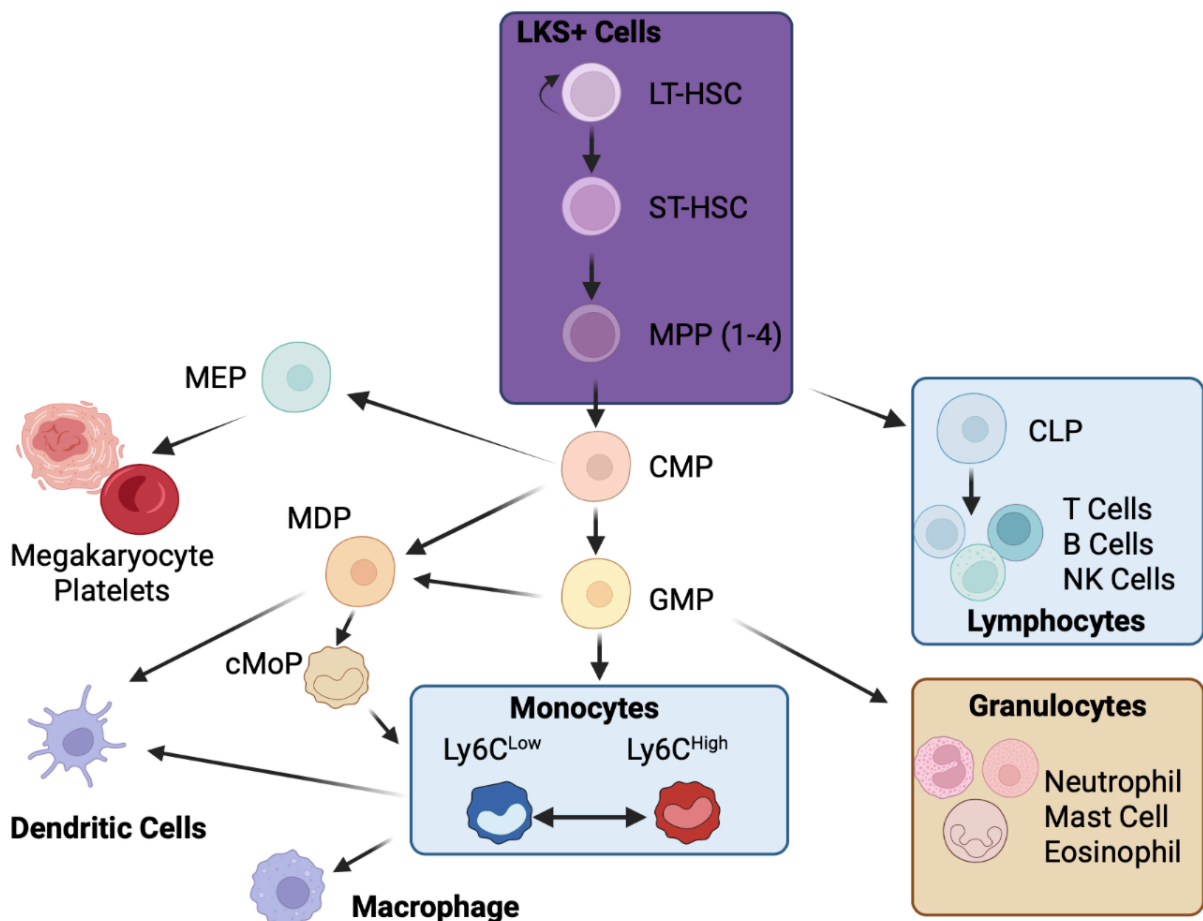


Figure 1.1 The cells of the haematopoietic tree from LT-HSCs to macrophages

Figure shows the hierarchical system which makes up the immunological tree. At the very start stem cells known as LKS cells reside in the bone marrow. This grouping is made up of LT-HSCs, which possess the ability of self-renewal, ST-HSC which lose the ability to self-renew but maintain the ability to differentiate into multiple immune cell lineages and MPPs, which are biased towards a specific immunological branch, lymphoid vs myeloid. These cells may be “pushed” or “pulled” down the various paths due to various immunological stressors, infection, cytokine etc. MPP4 stem cells can differentiate into CLPs, which can further differentiate into T, B and NK cells. MPP1 and MPP3 cells may differentiate into CMPs, GMPs or MDPs which allow for the further differentiation into monocytes, granulocytes or dendritic cell subtypes respectfully. Long-term haematopoietic stem cell (LT-HSC), short-term haematopoietic stem cell (ST-HSC), multipotent progenitors (MPPs), common myeloid progenitors (CMPs), granulocyte-monocyte progenitors (GMPs), common lymphoid progenitors (CLPs), megakaryocyte-erythrocyte progenitors (MEPs), macrophage-dendritic cell precursor (MDPs), common monocyte precursor (cMoP), patrolling monocyte (Ly6C^{Low}) and infiltrating monocyte (Ly6C^{High}).

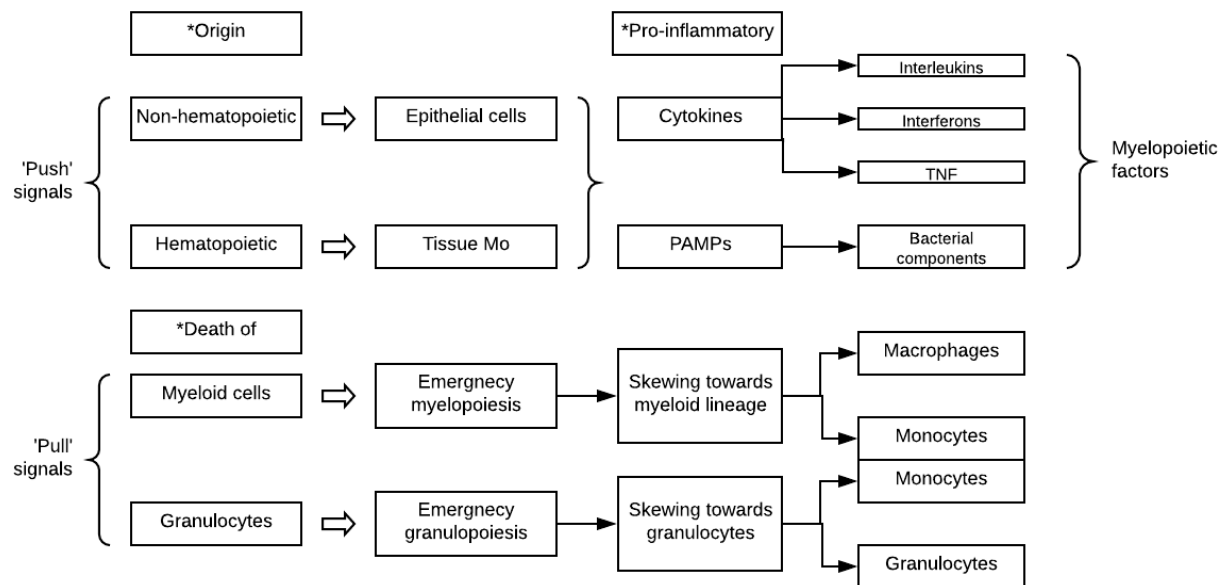


Figure 1.2 Bone marrow push and pull: Signals for haematopoiesis

Graph shows the two main sources that drive haematopoiesis: “push” and “pull” factors. Push factors stem from a cellular or pathogenic origin. Tissue cells or immune cells sense PAMPs or DAMPs and produce cytokines which feed on the bone marrow to push HSCs towards a specific fate. In contrast, pull factors occur in the peripheries due to cellular death. During infection cells die and this pulls the bone marrow towards a specific lineage in order to maintain homeostasis and fill the void in the niche.

1.1.7 Monocyte and macrophage precursors

Following on from committed progenitor cells are more dedicated precursors. Down the myeloid lineage, CMPs have a few options for further differentiation and development. Firstly, these cells can differentiate into cells known as megakaryocyte-erythrocyte progenitors (MEPs) which give rise to red blood cells and platelets. These have been shown to be distinguishable from other maturing CMP subsets due to lacking expression of IL-3Ra and CD45Ra³⁷. However, more relevant for this thesis CMPs can differentiate into granulocyte/monocyte progenitors (GMPs) or macrophage-dendritic cell precursors (MDPs). MDPs give rise to dendritic cells and macrophages. The transcription factor *Irf8* is vital for MDP differentiation into macrophages since mouse models lacking this factor fail to produce monocyte intermediates for macrophage development³⁸. GMPs commonly differentiate into granulocytes, such as neutrophils. However, it has also been shown that GMPs can directly differentiate into monocytes that are neutrophil like³⁹. Both pathways can yield monocytes which are Ly6C^{High} CCR2^{High} yet, yield a different transcriptional profile depending on their origin. Interestingly, the authors showed that monocytes derived from GMPs arose after LPS in-vivo administration, whereas monocytes derived from MDPs were induced after CpG DNA in-vivo administration³⁹. This again gives weight to the Push/Pull hypothesis.

However, the most commonly accepted model now for monocyte differentiation is an intermediate step between MDPs and monocytes, the common monocyte precursor (CMoP). Unlike mature monocytes these cells lack the ability to induce phagocytosis and have a transcriptional profile focused on cell cycle and proliferation^{40, 41}. Both GMPs and MDP/CMoPs are also induced differently depending on the stimulation. GMPs have been shown to be induced after TLR4 antagonism, whereas MDP/CMoPs were induced after TLR9 antagonism³⁹. As previously mentioned, monocytes that were derived from GMPs behave functionally like neutrophils, whereas MDP/CMoPs monocytes were shown to be more patrolling in nature and differentiate into DCs.

1.1.8 Monocytes: Expression to recruitment

Monocytes make up between 1 to 10% of the leukocytes in the blood of healthy adult humans and are vitally important in fighting infections. These cells can be split into subpopulations based on their expression of CD14 and CD16. In mice this is correlated with the expression of Ly6C. Inflammatory “classical” monocytes are classified as being Ly6C^{High}, corresponding to CD14^{High}CD16^{Low} in humans, whereas patrolling monocytes are classified as being Ly6C^{Low}, corresponding to CD14^{Low}CD16^{High} in humans. Monocytes are vital in infection due to their ability to differentiate into monocyte derived macrophages (MDMs). In *Mycobacterium tuberculosis* infection this differentiation is vital in controlling bacterial growth with CD4⁺ T cells recruiting monocytes to the lung via IFN γ where monocytes can control bacterial replication due to metabolic and inflammatory gene changes⁴².

The ability of monocytes to leave the BM, enter the blood and traverse into tissues is dependent on the chemokine, chemokine (C-C motif) ligand 2, CCL2. It has been shown that during *Listeria monocytogenes* infection, the ability of monocytes to control bacterial growth was dependent on CCL2 signalling. CCL2 signals through its' receptor C-C chemokine receptor type 2 (CCR2). Models of CCR2 deletion showed that monocytes accumulated in the BM and were lower in the blood circulation⁴³. They also showed that monocytes from CCR2 knock out mice had no issue infiltrating tissue if already in the circulation. This showed that CCL2 signalling plays a role in monocytes emigrating from the BM. It was also shown that mice lacking CCR2 do significantly worse to bacterial challenges⁴⁴, but did significantly better and were protected from atherosclerosis and experimental autoimmune encephalitis^{45, 46}.

1.1.9 Macrophages: Resident vs recruited

After leaving the vasculature in response to inflammatory signals, monocytes enter tissues and once in tissues, monocytes can differentiate and mature into macrophages. Macrophages themselves are found in all tissues of the body and come in two main forms: BM-derived or yolk-sac derived. The ability to distinguish

differences in BM derived and yolk-sac derived was greatly enhanced by antibodies used in flow cytometry and fate mapping. It was shown that tissue-resident macrophages had two major subgroups of cells. Yolk-sac derived macrophages were shown to be F4/80^{High} CD11b^{Low} whereas BM derived recruited macrophages were F4/80^{int} CD11b^{Low}. These cells were further split based on the expression of *C-myb* which was required for BM-derived macrophage development but not for yolk-sac derived development⁴⁷. However, it is well accepted that tissue resident macrophages are often a heterogeneous group with both yolk-sac derived and BM-derived cells occupying this space. This arose from studies examining embryonic development which identified two distinct waves of haematopoiesis. In mice the first wave is known as the primitive wave, which occurs in the blood islands of the yolk-sac and is restricted to the production of erythrocytes and macrophages⁴⁸. After day 8 these early yolk-sac derived progenitors then exit via the circulatory system and seed the foetal liver, which now became the main source of haematopoiesis⁴⁹. By day 10, a second wave of haematopoiesis occurs, known as the definitive wave, in the major atrial vessels. This is where the first HSCs with multi-lineage potential develop⁵⁰. Finally, by day 16 these early HSCs peak in the foetal liver and then transition to the BM where haematopoiesis occurs for the rest of the adult life⁵¹.

Macrophages are vital in the immune response due to their phagocytic ability as well as their unique activation status. These cells are uniquely shaped by their environment where cytokine levels and various PAMPs and damage associated molecular patterns (DAMPs) effect their immune phenotype. They have unique plasticity, although often oversimplifying their responses, being classified into M1/classically activated or M2/alternatively activated macrophages (**Fig 1.3**). The M1/M2 classification namely distinguishes macrophages based on in-vitro polarisation, M1 being LPS/IFN γ activated and M2 being IL4/IL13 activated, their ability to produce certain cytokines, their metabolic phenotype as well as changes in gene expression. This simplified profile of macrophages allow us to classify macrophages in-vivo based on their responses to infection, classifying responses into M1-like or M2-like.

M1 macrophages are classified as producing proinflammatory cytokines such as TNF, IL1 β , IL6 and IL12⁵². They are often associated with heightened inflammatory potential, with breaks in Tricarboxylic Acid (TCA) cycle and glycolytic reprogramming that enables the mitochondria to focus on anabolic metabolism generating adenosine triphosphate (ATP) from glycolysis and not OxPhos. These cells also utilise arginine metabolism to generate reactive oxygen species (ROS) through the upregulation of nitrous oxide synthase (NOS) genes such as *Nos2*. M1 macrophages also upregulate expression of antigen processing genes and, although are not classically associated as the main professional APCs type, can present antigen to CD4+ T cells through major histocompatibility complex (MHC) II. M1 macrophages are activated and primed to this inflammatory state through recognition of PAMPs such as LPS or through direct stimulation with cytokines such as IFN γ ⁵³.

In contrast M2 macrophages have been traditionally viewed as inducing tissue remodelling and promote resolution of inflammatory responses. These cells are typically associated with producing anti-inflammatory or type II cytokines, such as IL-1Ra, IL10 and TGF β ⁵⁴. Due to their anti-inflammatory nature, they have a different metabolic phenotype, relying on arginine metabolism to generate substrates such as ornithine through upregulation of arginase (*Arg1*) which enables tissue repair and remodelling. These cells also rely heavily on fatty acid oxidation (FAO) and TCA cycle for metabolic responses and generate ATP through OxPhos⁵⁵. Instead of antigen presentation, M2 macrophages focus on upregulating genes involved in efferocytosis, the process of clearing cellular debris to resolve inflammation⁵⁶. These genes involve scavenger receptors such as CD36, CD163 and CD206. M2 macrophages are often induced due to the abundance of anti-inflammatory cytokines such as IL4, IL10 or IL13⁵⁷.

However, this M1/M2 classification often is too simplistic. For example, tissue resident macrophages derived from the foetal yolk-sac are often viewed as anti-inflammatory M2 like cells. In the intestine for example macrophages play a key role in maintaining immunological tolerance by their production of IL10 therefore maintaining the integrity of the gut⁵⁸. Similarly in the lung the tissue resident alveolar

macrophage (AM) maintains a M2-like state. Here, AMs release growth factors like TGF β to induce the proliferation of epithelial cells, while at the same time regulating fluid transport and clearing pulmonary surfactant and cellular debris^{59, 60}. However, importantly during infection these cells can adopt a very different phenotype. During bacterial infections these cells can upregulate the production of IL1 β , switch their cellular metabolism to upregulate nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase and ROS production while also having the capacity to induce pro-inflammatory type I IFN responses during viral infections^{61, 62, 63}. However, as with our rudimentary understanding of the haematopoietic tree, the M1/M2 characterisation of macrophages is still a useful tool in understanding the role macrophages play in homeostasis and disease.

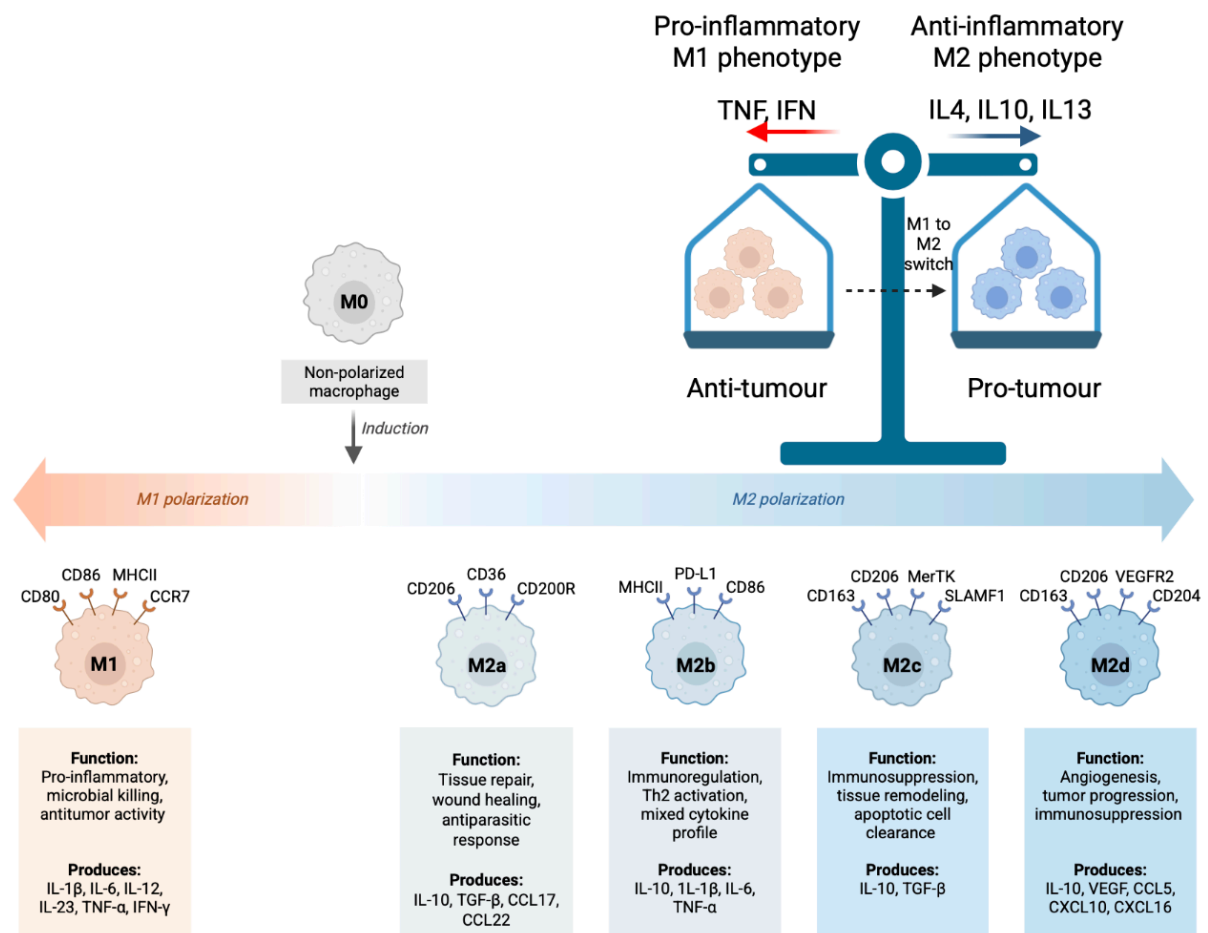


Figure 1.3 Macrophage plasticity: Polarisation from M1 to M2

Graph shows the process of macrophage polarisation. Macrophages can exist on a spectrum ranging from activated inflammatory macrophages (M1) to alternatively activated macrophages (M2). Within this spectrum exists macrophages that are very anti-inflammatory (M2d) that can drive tumour progression and immune suppression to anti-inflammatory macrophages that can still produce inflammatory cytokines (M2b) and that can fight helminth infection (M2a). Importantly, these cells are kept at a balance to maintain homeostasis, with M1 macrophages often being replaced by M2 macrophages during the course of infection.

1.2 Immunological memory & Innate Immune memory

1.2.1 Immunological memory: Changes to the dogma

The dogmatic view of the immune system I have previously described has recently been challenged with the discovery that certain cell types break immunological norms and display an overlap of both innate and adaptive features, such as natural killer T cells (NKT cells) and $\gamma\delta$ T cells⁶⁴. Recently, a similar phenomenon has been identified in myeloid cells. This is a form of innate immune memory (IIM) termed 'trained immunity' and was first described by Netea, M.G. *et al.*,⁶⁵. Trained Immunity (termed TI from herein) is defined as "*the long-term functional reprogramming of innate immune cells, which is evoked by exogenous or endogenous insults and which leads to an altered response towards a second challenge after the return to a non-activated state*"⁶⁶.

IIM similarly ranges in a spectrum based on the length between the primary and secondary immunological responses, as well as between the direction of the secondary response (**Fig 1.4**). For example, it has been shown that TLR2 agonist stimulation in the murine lungs leads to enhanced anti-viral responses, due to priming of the epithelial cells⁶⁷. This example of priming does not allow the cells to return to a resting state, after TLR2 stimulation, since transcriptionally they are still active and thus the priming stimulus acts in synergy with the secondary viral response to cause enhanced cytokine responses⁶⁸. In contrast, it has long been known that patients suffering from late-stage sepsis have weakened immune responses known as innate immune tolerance⁶⁹. Unlike priming, here the response is impaired compared to patients that have recovered or do not have sepsis. Finally, the most recently recognised example of IIM is TI which I have previously described.

Unlike classical adaptive memory, TI is non-specific and can be induced by a wide range of stimuli such as low dose LPS, the *Bacillus Calmette-Guérin* (BCG) vaccine, β -glucan, and more recently various cytokines such as IFN β . Importantly, unlike the genetic changes that occur for V(D)J recombination in adaptive immune cells, the functional reprogramming of TI is induced via immunometabolic and epigenetic

changes. Importantly, TI involves the metabolic reprogramming of innate immune cells which provides metabolic intermediates enabling chromatin modifications at the loci of inflammatory/innate immune genes, such that upon a secondary insult they can respond more rapidly and with heightened kinetics. Similarly, it has also been shown that TI is relatively reversible, and its phenotype is generally short lived, lasting on average 3 months to up to a year^{70, 71}.

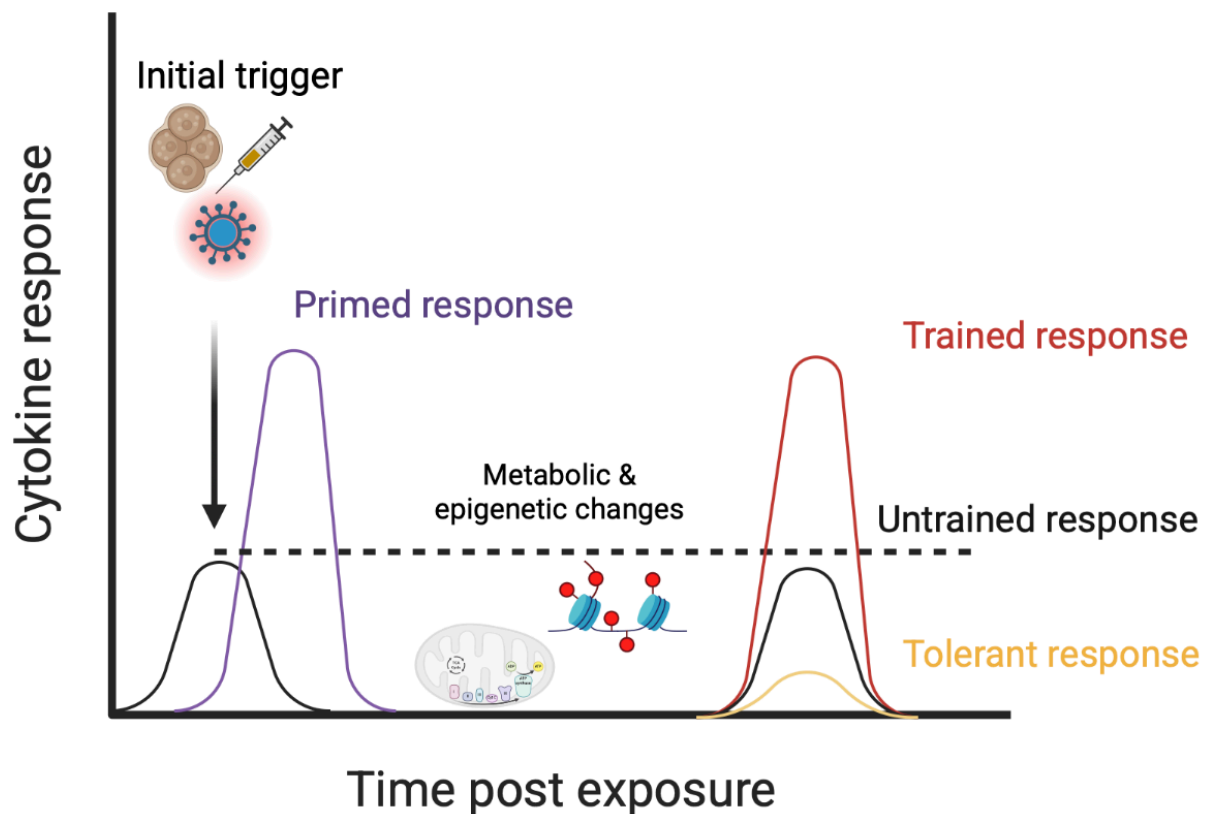


Figure 1.4 Innate immune memory spectra

Graph represents the three main forms of innate immune memory. Immune cells, such as macrophages, can respond to an initial challenge be it vaccine, infection or β -glucan. During a primed response (purple) the cytokine response is heightened relative to a normal response. However, there is no time for the primed cell to return to a resting state (black). However, during a training or tolerising response the cell returns to a relatively inert state but undergoes metabolic and epigenetic changes. However, when challenged a second time trained cells (red) respond with greater cytokine responses versus untrained (black right). In contrast, tolerised cells (yellow) respond with lower cytokine response relative to the normal response (black right).

1.2.2 Evolution of innate immune memory

Although the concept of TI is relatively new we now know that memory effects of the innate immune response are relatively conserved across the tree of life. Only about 3% of organisms on Earth have evolved an adaptive immune response, meaning that 97% of life on Earth only have the innate response to deal with pathological threats⁷². In fact, the original concept of TI traces back to the idea that plants and invertebrate species possess a form of innate memory whereby upon an initial infection these species are 'primed' to deal with a secondary infection^{73, 74}. Due to the success of these species it was theorised that innate memory was evolutionary conserved. The original concept for trained immunity in vertebrates was developed in the late 1920s by Swedish scientist Carl Naeslund. This ground breaking research was observed in a study involving BCG immunisation which showed a reduction in early childhood deaths⁷⁵. Since these deaths could not be due to reduction in the *Mycobacterium tuberculosis* (mTb) infection rate alone he postulated that "*One could evidently be tempted to find an explanation for this much lower mortality among vaccinated children in the idea that BCG provokes a non-specific immunity...*"

It wasn't for almost another 100 years that a mechanism was proposed to explain this non-specific or heterologous immunity. The fungal cell wall component β -glucan and other immunomodulatory agents such as CpG oligonucleotides were shown to confer non-specific protection against both *Staphylococcus aureus*, *Streptococcus pneumoniae* and *rotavirus* respectively^{76, 77, 78}. Similarly, BCG vaccination in mice were shown to not only protect against mTb infection but also non-specific infections by pathogens such as *Candida albicans* (Ca) and *Schistosoma mansoni*^{79, 80, 81}. This large body of evidence highlighted the ability of immunostimulatory agents to confer both specific and non-specific protection. Further, studies were performed in both *severe combined immunodeficiency disease* (SCID) mice and in recombination-activating gene 1 (RAG1) deficient mice which lack functional adaptive immune cells. These mice were first primed with either BCG or Ca leading to protection from lethal candidiasis^{82, 83}. Importantly, this protection was later shown to be dependent on both pro-inflammatory cytokine production as well as macrophage recruitment, major hallmarks of TI. As well as animal models there is

also a growing body of evidence that supports the conservation of innate immune memory and TI in humans. Many live vaccinations, such as BCG and the oral polio vaccine, have been shown to have non-specific and beneficial protection against a broad range of diseases^{84, 85}. Further, controlled human in-vivo infection studies have shown that BCG can confer protection against live yellow fever vaccination and malaria vaccination through induction of monocyte recruitment and pro-inflammatory cytokine production^{86, 87}. All this information points towards the conserved nature of TI and the importance it serves in the immune response to pathogens. The cellular basis in how TI is established and maintained is through a complex interactions between epigenetic changes and metabolic intermediates, which I shall describe later.

1.2.3 Stimuli involved in trained immunity

A plethora of ligands, including both PAMPs and DAMPs, have now been shown to be capable of driving TI. After the initial description of trained immunity a lot of focus was on the ability of vaccines, particularly BCG, to drive a systemic training response. BCG was capable of driving TI through the expansion of HSPCs in an IFN γ dependent manner leading to enhanced immunity against bacterium, fungal and viral infections^{34, 88, 89}. Mechanistically this was found to be due to the changes in HSPCs, particularly epigenetic changes in innate immune genes that were maintained in the progeny of these stem cells⁹⁰. However, it is not just BCG vaccines that can confer this protection. Recently, it has been shown that the adenoviral vector vaccine against corona virus induced disease (COVID)-19 enhances the responses of peripheral monocytes to mTb infection as well as altering monocyte cell number in the circulation and increasing monocyte specific production of cytokines such as IL1 β and IFN γ basally⁹¹.

However, it is not just vaccines that can induce TI. Great emphasis has been placed on investigating the role of infections on the immune system, particularly on the development of TI. Studies have shown that non-lethal Ca infection can enhance the immune response to lethal Ca infection occurring through the epigenetic alteration of monocytes downstream of the Dectin-1 receptor, which specifically

detects fungal wall components known as β -glucan⁸³. Similarly viral infections, such as *Influenza A virus* (IAV) or *Severe acute respiratory syndrome Corona virus* (SARS-CoV)-2, have both been shown to lead to an altered immune response. Infection with IAV leads to enhanced anti-tumour function through altered AM function dependent on IFN γ and NK cells⁹². Similarly, IAV infection leads to altered AM responses to bacterial infection. Here however, the authors argue that the altered responses are due to functionally different AM subpopulations. They argue that IAV infection leads to the cellular death of resident AMs, encouraging the expansion of myeloid cells in the BM (“Pull”) to repopulate the lungs with bone marrow monocyte derived AMs (BMoAMs) which have a better response to bacterial infections^{93, 94}. Similarly, SARS-CoV2 infection has been shown to ameliorate responses to secondary viral infection. Here the authors show that SARS-CoV2 infection leads to epigenetic changes in AMs, independent of expansion of BMoAMs and leading to enhanced type I IFN responses to IAV infection⁹⁵. However, it was also shown that SARS-CoV2 may impact human health through increasing inflammatory haematopoiesis which triggers epigenetic changes in circulating monocytes driving inflammation in the brain in an IL6 dependent manner, potentially exacerbating the phenomenon of Long-COVID⁹⁶. This highlights the double-edged sword represented by TI. This has further been highlighted by the role DAMPs play in driving abhorrent TI linked to the development of inflammatory diseases. For example, it has recently been shown that oxidised low-density lipoproteins (ox-LDLs) play a role in the development of atherosclerosis⁹⁷.

Studies of Ca induced TI demonstrated a dependency on the C-type lectin Dectin-1, which responds to β -glucan in fungal cell walls and this is now one of the most widely studied ligands used to study TI. These products belong to a family of glucose/glyco polysaccharides found in the cell walls of yeast, fungi, bacteria and algae. Fungal β -glucan is linked by β -1,3 and β -1,6 glycosidic bonds which are specifically recognised by Dectin-1 to initiate an innate immune response, whereas inert β -glucans like those derived from grains and oats are linked by β -1,3 and β -1,4 bonds rendering them less immunogenic⁹⁸. The ability of fungal β -glucans to drive TI has been shown to be influenced by variations in branching which occur during preparation, as well as in introduction of contaminating TLR4 and TLR2

ligands that can impact TI through over activation of NF κ B signalling pathway⁹⁹. More recently, attention has turned to whether β -glucan derived from common yeasts like *Saccharomyces cerevisiae*, including the commonly available β -glucan known as whole glucan particle (WGP) and zymosan preparation. WGP has been shown to induce trained immunity protecting mice from tumours in the pancreas as well as metastasis of tumours into the lung^{100, 101}. This compound is also sold as a commercial dietary supplement and we have shown that dietary feeding induces myelopoiesis⁹⁹. Similar products like Zymosan can drive TI in monocytes and macrophages, however we have demonstrated that purity of β -glucan and lack of contaminant TLR2/4 ligands is crucial for this⁹⁹, which shall be further explored in this thesis.

1.2.4 Cell types involved in trained immunity

TI was originally shown to occur in both monocytes and macrophages¹⁰² as already discussed. This process is associated with opening of chromatin and enhancing the transcriptional potential of pro-inflammatory genes¹⁰³. This original observation suggested that monocytes and macrophages were the main carriers of the TI phenotype. However, this alone could not explain the long-term memory effects of TI since monocytes and recruited macrophages are relatively short lived versus the memory effects of TI (in-vivo and in humans) which can last for a few months^{101, 103}. Therefore, another cell type must be involved that enables longer lasting innate memory. It was then discovered that both BCG and β -glucans were capable of inducing changes at the bone marrow level^{79, 104}. Specifically, these agents are capable of modulating HSCs as well as their progenitors. It was shown by Kaufman et al., (2018) that transplantation of these BCG-trained HSCs alone could infer training in the recipient mice. Therefore, researchers have now classified TI into two parts: central training and peripheral training (**Fig 1.5**). It is not well known whether there is cross communication between peripheral and centrally trained cells, but it has been shown that intraperitoneal injection (IP) of β -glucan leads to trafficking to the pancreas¹⁰¹. This potentially shows that peripheral macrophages or monocytes may be trained and/or might induce cytokines that feedback and signal to bone marrow cells to initiate central training. As well as these cell types, other immune

cells such as DCs, NK cells and innate lymphoid cells (ILCs) have also been shown to demonstrate a form of immunological memory^{105, 106, 107, 108}.

It is not just the cells of the innate immune system that can undergo TI, but also cells that have been classically viewed as non-immune, such as epidermal and stromal cells. These cell types have been shown to harness a memory phenotype through epigenetic changes upon tissue damages¹⁰⁹. The importance of this inflammatory memory in a clinical setting is that these cells are the major drivers of healing and regeneration during most pathologies. This inflammatory memory has also been shown to be important in the context of anti-viral immunity, whereby it has been shown that fibroblasts can be trained through IFN β . This training was due to increased RNA polymerase II recruitment at the gene loci of interferon stimulated genes¹¹⁰. This importantly highlights the link between peripheral trained immunity of immune cells with the potential to train non-immune stem cells.

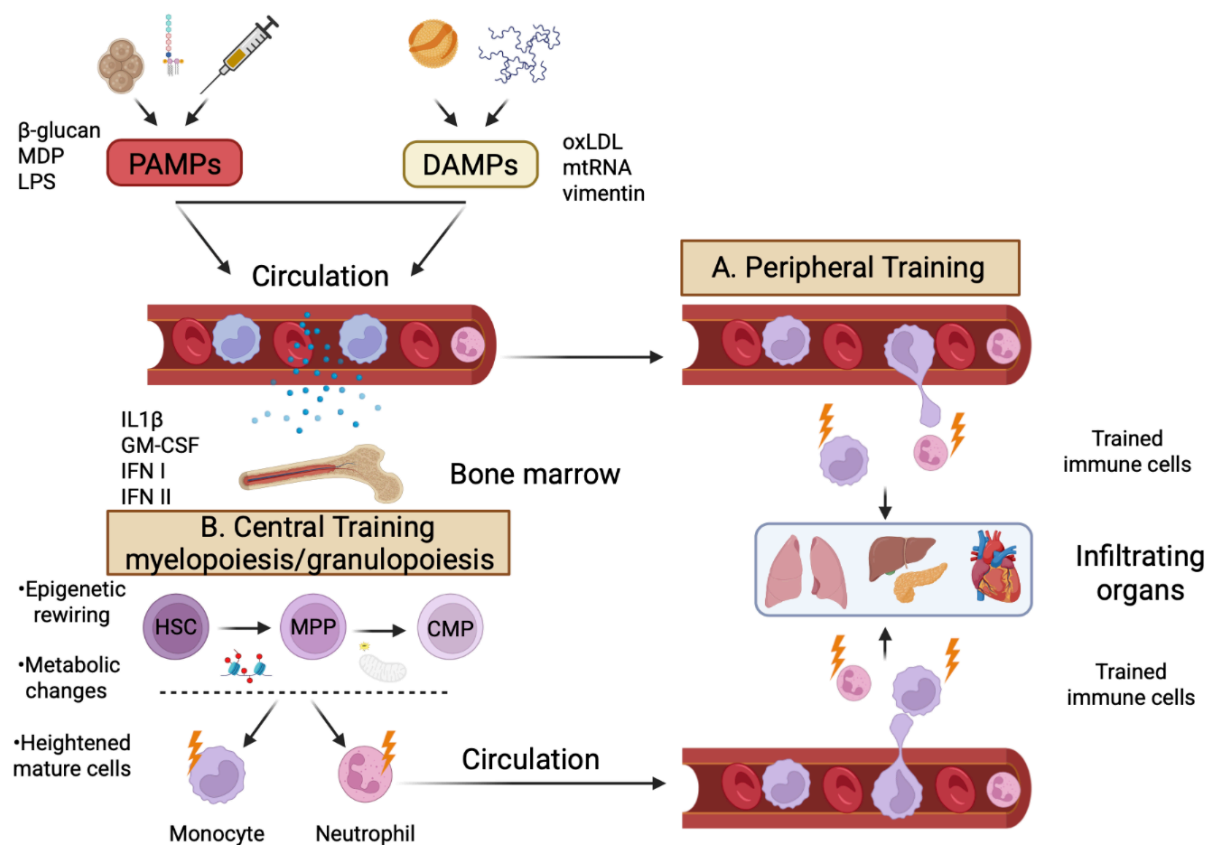


Figure 1.5 The cells of trained immunity: Central to peripheral trained immunity

Graph represents the systemic process of trained immunity. Ligands that can induce trained immunity PAMPs or DAMPs can enter the circulation. (A) The cells that come into contact with the training ligands can undergo trained immunity, either in the peripheral organs or in the circulation, known as peripheral trained immunity. (B) In other cases, cytokines produced by cells interacting with training ligands feed back onto bone marrow cells, this is known as central trained immunity. These cytokines cause the expansion of HSC populations and bias these cells to differentiate into myeloid cells, known as myeloopoiesis. These are epigenetic and metabolically altered and mature into monocyte or neutrophils which re-enter the circulation. These trained cells, as well as peripherally trained cells, can seed various organs around the body.

1.3 Initiation and maintenance of innate immune memory

1.3.1 General introduction to immunometabolism

The ability for innate cells to maintain TI is in part thanks to shifts in the cells metabolic state. The interaction between immune responses and metabolism has given birth to the field of immunometabolism. Cells adapt to their environment which can contain differing levels of nutrients and metabolites leading to changes in immune effector function. During infection, quiescent cells must upregulate their machinery necessary for proliferation, migration and production of effector molecules leading to activated cells. The activation of immune cells and the cellular process needed to generate effector molecules all require energy. This need leads to a switch in immune cells from catabolic metabolism (breakdown of complex metabolites to simpler metabolites) to anabolic metabolism (simple metabolites are used to build more complex molecules)¹¹¹. Each immune cell population has a unique metabolic phenotype which is influenced by tissue location as well as activation status, all of which enables for different proliferative and effector function effects¹¹². It is also important to note that individual metabolites can act as immunomodulatory signals akin to cytokines, directing cells to a specific activity status or driving signalling cascades¹¹³. Here, I will discuss the key metabolic changes that occur during initiation and maintenance of TI (**Fig 1.6**).

1.3.2 Glycolysis overview

The ability to breakdown glucose molecules into simpler metabolites, culminating in the formation of pyruvate is known as glycolysis. Under standard homeostatic conditions, glucose is converted to pyruvate and in the presence of O₂ this is further metabolised to acetyl-CoA via the enzyme pyruvate dehydrogenase (PDH). Acetyl-CoA can then enter the mitochondrial TCA cycle¹¹⁴. However, when O₂ is limited or not present cells can convert pyruvate into lactic acid via the enzyme lactate dehydrogenase and export it out of the cell. Interestingly, in some immune cells and in cancer cells this process can occur even in the presence of O₂^{115, 116}. This was

given the title of Warburg effect after its' discovery by Otto Warburg in 1924. This process is not as efficient as oxidative metabolism only producing 2 molecules of ATP for every molecule of glucose whereas OxPhos cycling can generate up to 32 molecules of ATP per glucose. However, rapid upregulation of glycolysis allows for the fast and sufficient supply of energy as well as metabolites involved in biogenesis of several macromolecules for the cell. Not only does glycolysis provide energy for the cell through ATP generation, but through increased availability of various intermediates it can also supply the cells with a source of carbon allowing for the generation of fatty acids, nucleotides, and amino acids. An example of the anabolic benefits of Warburg metabolism is the generation of ribose-5-phosphate and NADPH (via glycolysis linked PPP) which are used for nucleotide synthesis and a reducing agent for lipid synthesis respectively. Glycolysis is also maintained by reducing pyruvate to lactate allowing for the replenishment of NAD⁺ levels, which is a required co-factor for glyceraldehyde 3-phosphate dehydrogenase (GADPH) a key glycolytic enzyme. In this way glycolysis is essential for immune cell activation.

1.3.3 Glycolysis in trained immunity

The first major metabolic pathway identified to play a role in TI was glycolysis. As noted above, the upregulation of glycolysis allows for rapid changes in metabolite abundance, as well as a more rapid, albeit, less efficient production of ATP. The increased glycolytic pathway has been shown to be present in many different forms of monocyte TI, such as BCG¹¹⁷ and β -glucan¹¹⁸. It has also been shown that glycolytic reprogramming of HSCs is vital for configuring the protective response of β -glucan TI at the bone marrow level¹⁰⁴. The authors identified the upregulation of genes associated with glycolysis (*Hk3*, *Pfkfb*, and *Ldha*) as well as cholesterol biosynthesis pathways (*Pmvk*, *Mvk* and *Mvd*) in LT-HSC that had been sorted for RNA-sequencing. Similarly, they observed increases in glycolytic flux after sorting cKIT⁺ cells (total LKS⁺ BM progenitors) using extracellular flux analysis (Seahorse). These results highlighted a bias for β -glucan treated HSCs to undergo glycolytic reprogramming. The role of glycolysis in β -glucan haematopoiesis was further shown at earlier timepoints. Mice that had been IP injected with β -glucan for 24hrs showed heightened glucose uptake as well as increased lactate production by LKS⁺

cells¹¹⁹. This shift in glycolysis was dependent on type I IFN signalling and blockade of IFN lead to a reduction in the glycolytic phenotype as well as a loss of LKS+ entering cell cycling. Taken together this data is consistent with glycolysis being a key pathway that allows the proliferation of HSC which is associated with commitment to lineages and differentiation and thus enhanced haematopoiesis.

In monocytes, TI induces glycolytic reprogramming through a process that is dependent on the stabilisation of hypoxia inducible factor (HIF)-1 α . This occurs through activation of the Phosphoinositide 3-Kinase (Pi3k) and protein kinase B (AKT) which leads to the activation of the mechanistic target of rapamycin complex (mTORC). It was shown in-vitro that monocytes pre-treated with rapamycin, prior to β -glucan stimulation, lost their ability to drive the functional responses seen in TI¹¹⁸. Similarly, in monocytes that lacked the Dectin-1 receptor, β -glucan was not able to induce mTOR activation. The authors also showed that inhibition of the Pi3k-AKT pathway led to reduced activation of mTOR and the blocking of the enhanced responses due to training. This highlighted the important relationship between the activation of the Pi3k-AKT pathway downstream of dectin-1 and mTORC activation for β -glucan induced TI. Similarly, they also showed that in myeloid cell specific HIF-1 α conditional knockouts (cKOs) β -glucan trained mice lost the ability to mount the heightened functional responses seen in wild type (WT) β -glucan trained mice. This highlighted the importance of glycolytic reprogramming in the protective effects of TI.

However, importantly these experiments were carried out in β -glucans derived from CA. More recently, other sources of β -glucan have been explored for their potential in driving TI. As noted above, yeast-derived WGP, has recently been gaining attention in the literature^{100, 101}. Interestingly, this β -glucan does not seem to signal through the canonical AKT-mTOR-HIF-1 α pathway described above. It was shown that in raptor cKOs (functional KOs for studying mTOR) β -glucan still induced TI. Similarly, they also showed that cKOs of HIF-1 α had a significant decrease in the training effect relative to the WT, but still had heightened responses relative to the untrained control in the cKOs¹⁰⁰. Therefore, they showed that in WGP TI HIF-1 α , but not mTOR, may play some sort of role in inducing TI. Our lab have also shown

that in murine macrophages β -glucan trained immunity is independent of mTORC1 signalling⁹⁹ Taken together this information highlights the functional differences in glycolytic reprogramming of different β -glucan induced TI.

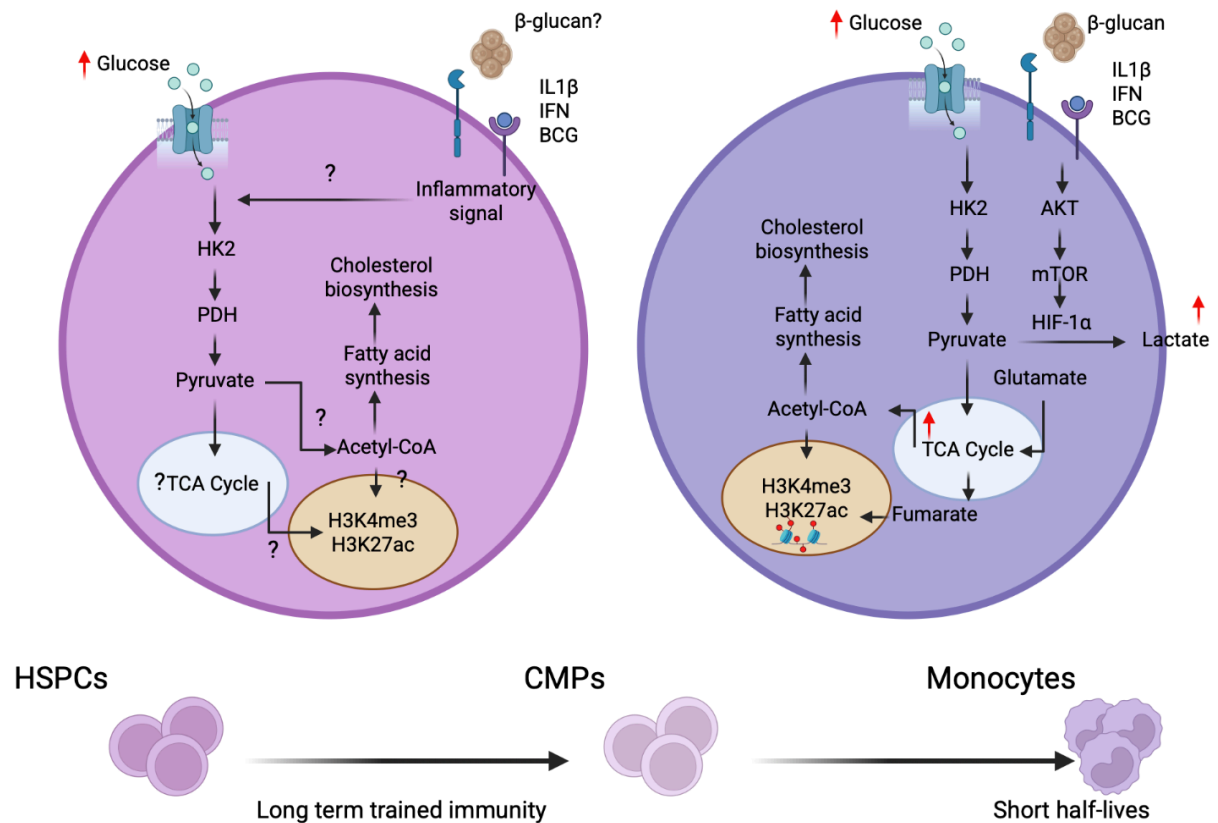


Figure 1.6 The mechanism to maintain innate immune memory: Central & peripheral alterations

Graph represents the cellular process whereby innate immune memory is maintained by HSPCs and mature innate cells, such as monocytes. Consistent in both cells there is an increase in glycolytic metabolism which allows increased glucose uptake and shuttling to TCA cycle. The mechanism by which this occurred is poorly defined in HSPCs, but in cells such as monocytes occurs through HIF-1 α stabilisation downstream of AKT-mTOR signalling. In monocytes TCA derivatives such as fumarate and acetyl-CoA induce epigenetic changes and acetyl-CoA can be utilised by the cell to directly impact epigenetic changes or can be converted to mevalonate during cholesterol biosynthesis to amplify glycolytic metabolism or act on epigenetic changes. This pathway seems to also occur in HSPCs, although the exact mechanism has not been fully worked out yet.

1.3.4 Oxidative phosphorylation and the TCA cycle

As previously mentioned, the most efficient way to generate the energy necessary for cells is through the mitochondria. Mitochondrial metabolism combines the TCA cycle with the electron transport chain (ETC) to generate ATP in a process known as OxPhos. I have mentioned that the mitochondria can generate 32 ATP molecules from one glucose molecule. However, the completion of the TCA cycle generates other intermediates that can be used by the cells including nicotinamide-adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide (FADH₂). These molecules are energy rich and are utilised by the mitochondria through the ETC. The ETC contains 5 major complexes located in the inner mitochondrial membrane, each of which are important in the transport of electrons to generate a proton gradient. The first complex links the TCA to the ETC. This complex is known as NADH dehydrogenase or complex I. NADH is oxidised to NAD⁺ by this complex allowing for the effective pumping of 4 H⁺ ions into the inter mitochondrial membrane space (IMS). The differences between the H⁺ ions in the inter and outer mitochondrial membrane generates a H⁺ membrane potential known as mitochondrial membrane potential ($\Delta\phi_m$). This occurs alongside the transfer of electrons from NADH to ubiquinone (CoQ) in the IMS. This leads to the second major complex (Complex II) which is also linked to the TCA since it also acts to convert succinate into fumarate via the enzyme succinate dehydrogenase (SDH). Complex II converts succinate, FADH₂ and CoQ into fumarate, FAD⁺ and CoQH₂ via the transfer of electrons. In the phospholipid bilayer electrons from complexes I and II are transferred to complex III by CoQ. Complex III is also called cytochrome C reductase as electrons are transferred from CoQ to cytochrome C (Cyto C) leading to another 4 H⁺ ions entering the IMS. Electrons are then finally transferred from Cyto C to oxygen via complex IV also known as cytochrome C oxidase. This leads to the reduction of oxygen in the presence of 2 H⁺ ions to generate water on the side of the mitochondrial matrix. This also allows for the further pumping of 2 H⁺ ions to the IMS. The $\Delta\phi_m$ generated then allows for the final complex, ATP synthase or complex V, to push H⁺ ions back across the membrane to generate ATP. In order to feed the electron hungry ETC the reducing equivalents, NADH and FADH₂, must be generated. These are generated by the TCA cycle. The TCA cycle and ETC through OxPhos are the primary metabolic pathways that are utilised by cell types

that must maintain longevity and quiescence, such as memory T cells. It is now appreciated that The TCA cycle also generates a number of metabolites that can be utilised by immune cells for effector functions.

1.3.5 Oxidative phosphorylation and the TCA cycle in trained immunity

A more recently appreciated fundamental alteration in β -glucan TI is the reprogramming of mitochondria and its linked metabolic processes. It was originally observed that in monocytes treated with β -glucan, TI enhanced aerobic glycolysis which was coupled with a decrease in oxygen consumption and thus decreased oxidative metabolism¹¹⁸. However, more recently dose dependant stimulation of the *Candida albicans* β -glucan as well as WGP stimulation has shown that both glycolysis and OxPhos are highly upregulated in trained macrophages and monocytes^{100, 120}. It has also been shown that single nucleotide polymorphisms in genes related to OxPhos were associated with degrees of responsiveness to β -glucan training highlighting its importance in inducing TI in human cells¹²⁰. It has also been shown that the ability to drive enhanced OxPhos in bone marrow derived macrophages (BMDMs) treated with WGP was dependant on mitochondrial fission¹⁰⁰. This fission of the mitochondria was vital for inducing the protective effects of WGP induced trained immunity. Collectively, this data highlights that overall enhanced metabolism, as opposed to Warburg aerobic glycolysis, is vital for the induction of TI.

TCA cycle metabolites are not only used to fuel the energetic needs of the cell but can also be used to generate signalling macromolecules and substrates for immune cell reprogramming. Similarly, trained cells may also utilise the TCA cycle to provide the necessary macromolecules for their functional reprogramming. Three major metabolites have been shown to be accumulated in TI: Citrate, succinate and fumarate^{120, 121}. The accumulation of citrate is very important in the metabolic reprogramming of immune cells such as macrophages. This is because citrate can be exported from the mitochondria via the citrate-malate shunt (CMS). Citrate can then be converted into acetyl-CoA in the cytosol via the enzyme ACLY. The

accumulation of cytosolic acetyl-CoA may be important for use of trained cells in acetyl-transferases, providing the machinery necessary for epigenetic modifications in TI. Citrate can also be metabolised in the mitochondria into itaconate via the enzyme aconitate decarboxylase 1 (ACOD1), which converts cis-aconitate (downstream of citrate in the TCA) into itaconate. It has been shown that in human monocytes itaconate and its derivatives can induce TI¹²². Here the authors showed that extracellular itaconate can drive epigenetic changes as well as metabolic changes that are dependent on TCA cycle and glutathione metabolism.

Secondly, succinate and fumarate possess the ability to drive HIF-1 α stabilisation^{123, 124}. This could potentially be another process by which trained cells induce HIF-1 α stabilisation independently of the AKT-mTOR axis. HIF-1 α stabilisation leads to the increase in histone demethylation expression which plays a fundamental role in epigenetic reprogramming, pointing towards another fundamental dynamic for TCA cycle metabolites and HIF-1 α in TI¹²⁵. However, both fumarate and succinate themselves can both act as negative regulators of a group of enzymes known as α -ketoglutarate (α -KG) dependant dioxygenases ((α -KGDD)¹²⁶. This group of enzymes include Jumonji (Jmj)-KDM5 family enzymes which drive the demethylation of histones and DNA. Importantly, Jmj-KDM5 acts on both H3K4me3 and H3K27m3, two major epigenetic markers for trained immunity^{103, 127}. Fumarate alone has been shown to increase H3K4me3 marks in human monocytes via the application of monomethyl fumarate (MMF) a cell permeable form of fumarate. These marks were found on the promoters of IL6 and TNF genes and MMF was also shown to inhibit KDM5 activity¹²¹. This highlights the importance of mitochondrial metabolite accumulation in cells for the epigenetic modifications associated with TI, as mentioned earlier.

Two fundamental pieces of evidence point towards the importance of linking TCA cycle and OxPhos to trained immunity. These both revolve around SDH. Firstly, it has been shown that stimulation with the cell membrane permeable dimethyl itaconate (DMI) was capable of inhibiting the trained response of β -glucans¹²⁸. Similar to its' derivative, DMI, itaconate is a competitive inhibitor of SDH since it can occupy the succinate binding pocket. Secondly, it has also been shown that single

nucleotide polymorphisms in the β -subunit of SDH (SDHB) can affect the responsiveness of human monocytes to TI. Coupled with this, it has been shown that in human monocytes various subunits of SDH were also upregulated 6 days post β -glucan trained immunity¹²⁰. This upregulation was linked with the lysine methyltransferase Set7. It was shown that Set7 regulated SDHB and malate dehydrogenase 2 (MDH2) transcriptional expression and functional activity via epigenetic modification at distal enhancers sites for the transcription of these genes.

1.3.6 Introduction to epigenetics

Trained immunity deals with a more rapid, strong and sometimes altered transcriptional response to non-specific stimuli. This altered response is in turn due to changes at the chromatin level known as epigenetic changes. The Greek prefix 'epi' means above and thus epigenetics deals with the functional changes that occurs above the basic level of the nucleic acid sequence, i.e. the DNA and RNA level¹²⁹. There are three functional mechanisms by which epigenetics effects cellular responses, including DNA methylation, histone modifications and non-coding RNAs (ncRNA).

Each of these epigenetic mechanisms impact the structure of DNA or chromatin making it easier or more difficult for transcriptional machinery to access the coding sequences. For example, DNA methylation involves the transfer of methyl groups onto the DNA backbone, usually at the C5 region of cytosine. This makes it more difficult for transcription factors (TFs) to bind to the DNA and thus has a silencing effect on transcription¹³⁰.

In contrast, these changes can also occur on the histones surrounding the DNA and thus do not impact the DNA sequence directly. Histone modifications in TI occur mainly via methylation or acetylation and often lead to phenotype changes in the cell. For example, histone acetylation is regulated by histone acetyltransferases (HATs) or histone deacetylases (HDACs)¹³¹. As mentioned previously, the ability to shuttle acetyl-CoA into the cytosol is vitally important for providing the substrate necessary for HATs to operate. HATs transfer the acetyl group from acetyl-CoA to

lysine (K) side-chains, more specifically the ϵ -amino group. The transfer of the acetyl group causes a change in the electrical charge of the histone, neutralising the positive lysine charge, and thus causes weakening of the histone-DNA interaction¹³¹. Therefore, when acetylation occurs on histone of promoters it causes increased gene transcription potential. HDACs in contrast oppose this mechanism. Here the positive charge is restored by removing the acetyl group. Thus, deacetylation is often associated with tighter histone-DNA interactions and thus shut down of transcription.

Histones may also be modified via methylation modifications. Similar to histone acetylation, this also normally occurs on lysine side chains in TI. In contrast to acetylation, methylation does not impact the charge of the histone and can also occur with different number of methylation marks; mono, di or tri¹³¹. These modifications normally occur within the N-terminal tails and the area of the enzymatic activity of the methylation enzyme is often contained within a region known as the SET domain. Unlike histone acetylation, the degree and location of histone methylation impacts gene transcription. For example, histone 3 lysine 4 tri-methylation (H3K4me3) leads to more active transcription whereas histone 3 lysine 27 tri-methylation (H3K27me3) leads to more repressed chromatin¹³².

Finally, non-coding (nc)RNAs can also impact epigenetic changes either through silent state epigenetic modifications at the promoter regions of gene transcription or through silencing via targeted micro (mi)RNA at the promoter¹³³. For example, small interfering (si)RNA can target promoter regions and recruit epigenetic markers, such as histone or DNA methylation, in order to silence transcription¹³⁴. In contrast, miRNAs can also target anti-sense ncRNAs themselves, when the anti-sense ncRNA is in abundance, which can release the mRNA promoter from the suppressive ncRNA and enable active transcription¹³³. This ncRNAs can act as both repressors and promoters of gene transcription via blocking or attracting epigenetic machinery.

These epigenetic signatures can occur in mature cell populations, however, interestingly for the maintenance of a trained phenotype it has also been shown that

they may occur in the HSC niche¹³⁵. This coupled with changes in metabolism may be the cellular mechanism via which TI maintains long-term immunological memory in innate immune cells which will be expanded upon below.

1.3.7 Epigenetic modifications involved in trained immunity

The ability of chromatin to act as a functional compartment of DNA is vital since it can lay in a structurally open configuration, easily accessible for gene transcription, or in a condensed form, which represses gene transcription¹³⁶. Since, these alterations, via epigenetics, alter gene responses leading to a more or less robust response they are an attractive target for explaining how memory is conserved in innate cells. In fact it has been shown that TI can occur through histone modifications and lncRNA, with emerging data on methylation of DNA in tolerance at least.

The best studied epigenetic changes in TI are histone modifications. These modifications consist of histone methylation and acetylation. In TI the two most commonly enhanced chromatin markers are H3K27ac accession of acetyl groups at the distal enhancers of genes and H3K4me3 at the promoters of genes⁶⁶. In TI H3K3me3 has been shown to be associated with BCG, β -glucan and oxLDL induced trained immunity^{82, 83, 137}. Similarly, H3K4me3 is also associated with tolerance whereby tolerized cells have enhanced H3K4me3 on enhancers of specific genes versus control cells¹³⁸. H3K4me3 is a very important distinguisher of TI since this mark is positively associated with active transcription and active enhancers in multiple models and cell types^{139, 140}. TI also relies on this tri-methylation of enhancer regions since co-culture with the methyl transferase inhibitor 5'-methylthioadenosine (MTA) inhibits the effects of trained immunity in multiple TI models^{82, 141}. More recently, a link between methyltransferases and cellular metabolism in β -glucan trained cells has been established¹²⁰. There, the SET-domain containing lysine methyltransferase SET7 was shown to be highly upregulated in β -glucan trained cells. SET7 was shown to methylate the enhancer regions of two key metabolic enzymes SHD, which converts succinate into

fumarate, and MDH2, which converts malate into oxaloacetate. This further amplified the epigenetic signature of β -glucan trained cells since fumarate can inhibit α -KG dependant histone and DNA demethylation. On top of histone methylation, TI also induces *de novo* H3K27ac. Seed et al., (2014) used an epigenome wide map of various markers in β -glucan trained cells and showed that around 500 promoters gained H3K27ac post training¹⁰³. Importantly, they were able to demonstrate that over 40% of all dynamic promoters had this *de novo* H3K27ac in cells that were treated with β -glucan. In contrast LPS tolerised cells had less subtle H3K27ac marks demonstrating the spectrum of immune memory in myeloid cells. H3K27ac has also been shown to be an important hallmark of TI, since these marks were restored in LPS tolerised cells treated with β -glucan⁷⁰. This shows how β -glucan treatment can rewrite the epigenetic landscape of myeloid cells and that H3K27ac is one of these key markers that allows for rapid transcription of inflammatory genes like *Tnf* or *Il6*.

LncRNA has also been shown to play a major role in writing the epigenetic landscape of trained cells. In order to provide a rapid transcriptional response the highly stochastic genetic environment must be reduced. Therefore, it has been suggested that in trained cells, the contact between chromatin and rapidly responding genes is in a preformed and thus a more stable shape⁶⁶. In order for this to occur both chromosomal looping and topology associated domains (TADs) have been suggested. This has been revealed in high-throughput chromosome conformation capture (Hi-C) studies showing innate immune genes are separated into these TADs¹⁴². Here, a class of lncRNA called immune gene-priming lncRNAs (IPLs) have been shown to be brought into close proximity with 'transcriptionally poised' immune genes via TADs. This close proximity enables for this rapid response seen in TI. Importantly, IPLs are capable of adding the H3K4me3 histone modifying complexes we have previously discussed to the promoters of various highly responsive genes in order to initiate TI via epigenetic reprogramming. This is due to the ability of IPLs to exploit the preformed 3D looping nature of TADs^{66, 142}. In human monocytes trained with β -glucan IPLs were shown to be important in ELR+ CXCL TADs, also known as upstream master lncRNA of the inflammatory chemokine locus (UMLILO), since ablation of this transcript disrupted training¹⁴².

Taken together the interplay between metabolite accumulation and epigenetic modifications enable innate immune cells to maintain the trained signature and thus acts like a 'book-mark' in dealing with inflammatory stimuli.

1.4 Overlap of fungal and viral immunity: Understanding the non-specific nature of trained immunity

1.4.1 The type I interferon response

Type I IFNs play a fundamental role in anti-viral immune responses. Firstly, these proteins are secreted to induce cell intrinsic states in both neighbouring and infected cells that limit viral spread. Secondly, they are vital for innate immune cell function since they promote antigen presentation and NK cell function as well as controlling pro-inflammatory cytokine production and pathways. Thirdly, the adaptive immune response is stimulated to induce protective memory via T and B cells¹⁴³. Type I IFNs are made up of IFN- α , IFN- β , IFN- ϵ , IFN- κ and IFN- ω . In this thesis, I shall focus on the most studied group: type I IFNs, IFN- α and IFN- β . Type I IFN signal through the IFN α receptor (IFNAR), which is composed of two subunits IFNAR1 and IFNAR2. Engagement of the IFNAR receptor leads to receptor associated protein tyrosine kinase Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) activation. This activation induces phosphorylation of the downstream signalling transcription factors signal transducer and activator of transcription 1 (STAT1) and STAT2¹⁴⁴. This phosphorylated STAT1 and STAT2 can then dimerise, translocate to the nucleus and bind with IFN-regulatory factor 9 (IRF9) to form a complex known as IFN-stimulated gene factor 3 (ISGF3)¹⁴³. The formation of the tricomplex ISGF3 enables its effective binding with a gene sequence known as IFN-stimulated response elements (IRSE) which directs the activation of IFN stimulated genes (ISGs)¹⁴³ (**Fig 1.7**).

Type I IFNs are tightly regulated throughout homeostasis and infection. This occurs through an autocrine loop whereby low levels of 'tonic' IFN- β can induce and

maintain high expression of STAT1 and IRF9-dependent ISG's. During homeostasis commensal flora may play a role regulating the basal expression of IFN- β , which can help regulate type I IFNs and ISGs^{143, 145}. Importantly, STAT1 can be phosphorylated by low concentrations of IFN- β , and thus can prime cells for enhanced type I IFN response¹⁴⁶. Similarly, in TNF activated macrophages low concentrations of IFN- β can lead to highly expressed ISGs¹⁴⁷. Type I IFN signalling can also be negatively regulated via the downregulation of IFNAR. This can occur through pro-inflammatory signalling or through oxidative stresses¹⁴⁸. Another important mechanism for type I IFN regulation is through chromatin remodelling and epigenetic changes. During a type I IFN response chromatin around ISGs are remodelled in order to make them more accessible to transcription machinery. This occurs through the recruitment of the ATP-dependant nucleosome remodelling complex known as BAF¹⁴⁹. BAF has been shown to be capable of driving a subset of ISG responses post TLR activation as well as being correlated with increased transcription of ISGs^{150, 151}. In this way type I IFNs are tightly regulated during an anti-viral immune response and are linked to differentiation of multiple innate immune subsets.

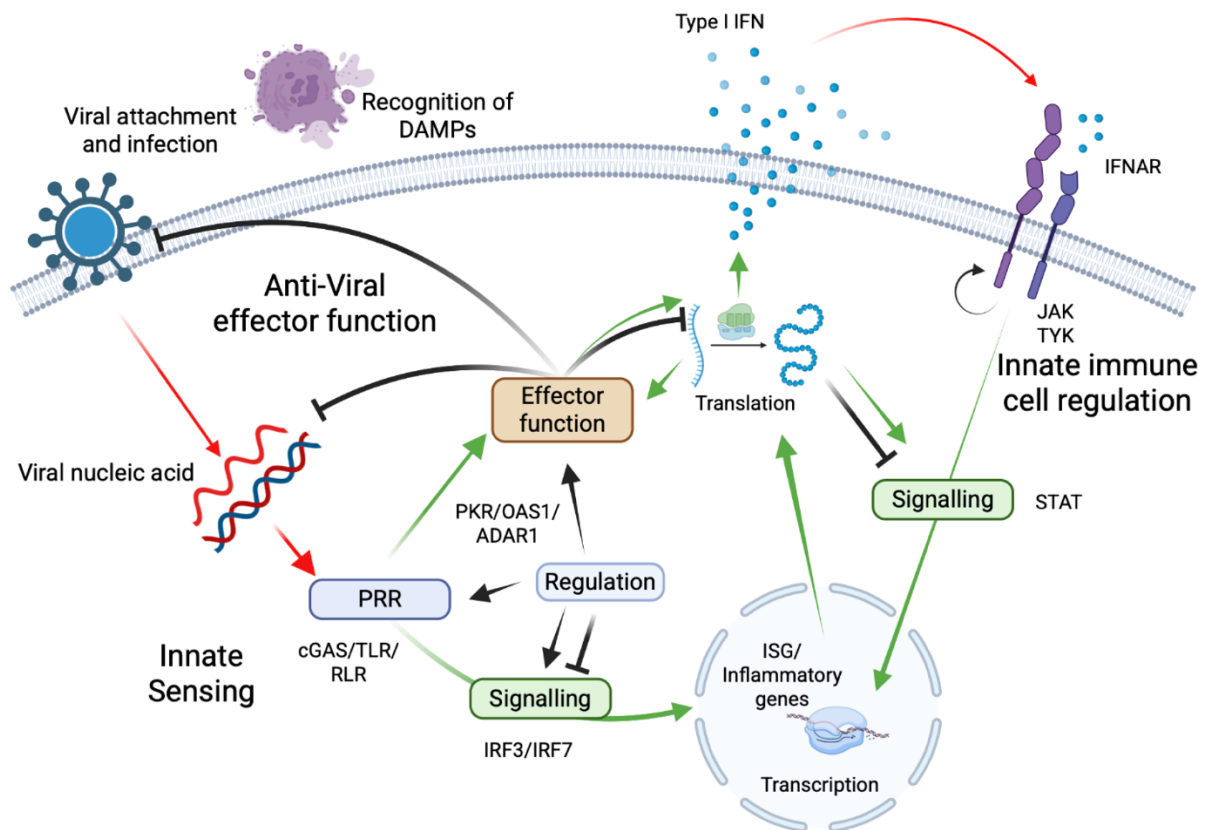


Figure 1.7 Innate anti-viral immune response and type I IFN signalling

Graph represents the cellular process whereby innate immune cells recognise viral PAMPs/DAMPs triggering anti-viral responses and IFN signalling. Viruses are recognised by direct PAMPs, such as nucleic acid, or indirectly due to cellular damage, DAMPs. This triggers activation of PRRs, such as TLRs, RLRs, or cGAS/STING pathway. PRRs signal through IRFs which trigger the activation of inflammatory genes and IFN genes. Production of IFN leads to autocrine or paracrine signalling which activates the JAK/STAT pathway. IFN signalling leads to ISG transcription and translation to prime cells for anti-viral activity.

1.4.2 Process of viral infection

Viruses are complex intracellular parasites that can induce a broad range of diseases and disorders due to their infection of host cells. Therefore, understanding of how viruses invade cells and take over host cellular machinery is vital for developing treatments and understanding host immune responses. In order for a virus to infect and spread in the body there are four major steps: 1. attachment, 2. Penetration, 3. Uncoating and replication and 4. assembly. Beforehand, however, the virus must invade the host and initiate the first round of viral replication. There are many different routes of viral entry. These include skin, respiratory tract, gastrointestinal, and bodily fluids. For simplicity we shall define viral entry via the respiratory route here. The respiratory tract is protected by a 'mucocilliary blanket', which is made up of both cilia on the surface of epithelial cells as well as mucus that is produced by goblet cells¹⁵². This mucosal layer protects alveolar cells from respiratory infections since pathogens that penetrate into the lungs are trapped and exported via the beating of the cilia to be ingested or exhaled. The respiratory tract is also protected by specialised lymphoid tissues known as nasal associated lymphoid tissue (NALT) and the tonsils and bronchus-associated lymphoid tissue (BALT)¹⁵².

However, despite the protective barriers in the respiratory tract viruses have evolved specialised mechanisms to infect lung cells. This specialised ability to infect specific regions of tissue is known as virus tropism. Critically viruses require specific receptors on host cells in order to bind and infect cells. Once inside the cell viruses utilise host machinery in order to replicate their genome and protein coats. For example, Influenza virus replication takes place within the nucleus and must first transcribe complimentary RNA (cRNA) and then new viral RNA (vRNA)¹⁵³. IAV viral translation is fully dependant on host machinery and occurs within both the cytoplasm and the endoplasmic-reticulum (ER). The new viral particle is then fully assembled and is trafficked to the cell surface where the viral protein neuraminidase (NA) facilitates viral release. It does this through the hydrolysis of sialic acid (SA) glycosidic linkages which bind SA to sugar molecules. This prevents HA binding to

the cell surface and thus viral release¹⁵³. This is one major example of how viruses can induce infections in other organisms.

1.4.3 Immune response to viral infection

We have already seen that anatomical barriers function as a first wave of defence against viral infection. However, it is important to note that if these barriers are breached proper containment of infection through the innate and adaptive immune responses are vital to prevent pathogenesis. The innate immune response can distinguish host versus non-host in three main ways. Firstly, host cells can recognise or distinguish abnormal genomic information in the form of viral DNA or RNA. For example, viral RNA normally does not contain a 5' cap and thus can be recognised by the host cytosolic receptor retinoic acid-inducible gene I (RIG-I). Similarly, dsDNA or ssDNA accumulation outside of the nucleus due to viral replication is also used to distinguish and recognising self-versus non-self. Secondly, host cells can recognise DAMPs or PAMPs which accumulate due to infection of cells, cell death and resulting tissue damage. This is referred to as the stranger/danger response. For example during viral infection with herpes simplex virus 1 (HSV1), leakage of host 5S ribosomal RNA pseudogene can occur which can be recognised as a DAMP also known as viral unmasking¹⁵⁴. Also, various proteins on the cell viral envelope can be recognised as PAMPs by germline encoded innate immune receptors such as the SARS-CoV-2 spike protein (by TLR2). Finally, viruses may alter the state of infected cells to avoid detection and thus escape immune responses. For example, viruses can induce loss of major histocompatibility complexes (MHC) class I from cell surfaces to avoid detection from CD8+ T cells. This in itself can alert the host immune response to infection and innate lymphocytes including NK cells recognise this as missing self' and induce killing of these cells.

As previously mentioned PRRs are the primary receptors used to broadly recognise foreign invaders. In the anti-viral immune response there are two main categories of "sensors" which provide innate protection¹⁵⁵. The first major group include the membrane expressed and endosomal toll-like receptors (TLRs), the cytosolic RNA sensors RIG-I like receptors (RLRs) and the cytosolic DNA sensors absent in

melanoma 2 (AIM2) and cyclic GMP-AMP synthetase (cGAS). TLRs consist of endosomal receptors TLR3, TLR7, TLR8 and TLR9, although it has been shown that in some cell types TLR3 may also be expressed on the cell surface¹⁵⁶. TLR3 recognises dsRNA whereas TLR8 recognises ssRNA. TLR7 can dual recognise both dsRNA as well as ssRNA. In contrast TLR9 recognises DNA, more specifically unmethylated DNA found within CpG-motifs¹⁵⁷. More recently, it has been shown that other TLRs such as TLR2 and TLR4 play an important role in recognising molecules on the viral envelope^{158, 159}. RLRs consist of RIG-I and melanoma differentiation associated gene 5 (MDA5). Both these receptors recognise RNA and signal via the mitochondrial antiviral signalling protein (MAVs), which is located in the outer membrane of the mitochondria as well as the mitochondrial-associated endoplasmic reticulum membrane (MAM) and peroxisomes. The final group in the first category of PRRs are the DNA sensors AIM2 and cGAS. cGAS signals through stimulator of interferon genes (STING) to activate interferon response factor 3 (IRF3) which can activate BCL-2 associated X protein (BAX) to trigger apoptosis. In contrast, AIM2 signals through the AIM2 inflammasome to induce pyroptosis of cells via apoptosis associated speck-like protein (ASC) and caspase 1¹⁵⁵. Overall, the PRRs described above can either directly or indirectly activate TFs, such as NF- κ B or IRF3. The activation of NF- κ B leads to pro-inflammatory cytokine and chemokine responses important for anti-viral effector functions, such as tumour necrosis factor (TNF), interleukin 6 (IL6) and IL8. In contrast signalling through IRF3 leads to type 1 interferon (IFN) responses. Type 1 IFNs play a dominant role in antiviral responses¹⁵⁵.

The second class of sensors revolve around nucleic acid sensors that have direct anti-viral activity. These enzymes mechanistically work via enzymatic reactions but function as sensors of viral infection and are often enhanced by type I IFNs and PRR activation¹⁵⁵. These sensors include adenosine deaminase acting on RNA 1 (ADAR1), 2'-5'-oligoadenylate synthetase 1 (OAS1) and dsRNA activated protein kinase R (PKR). These sensors don't drive TF activity but function as direct drivers of anti-viral activity. PKR, for example, is activated by dsRNA. Once activated PKR induces the phosphorylation of eIF2 which inhibits protein synthesis and thus blocks viral replication in cells¹⁶⁰. OAS proteins are another family of these type 2 class of

anti-viral sensors. These also bind to dsRNA. Once bound to dsRNA targets OAS undergo conformational changes enabling the catalytic pocket to be revealed. This can convert the targeted ATP molecule to 2'-5'A which acts as the secondary messenger¹⁶¹. This secondary messenger binds to and activates RNase-L which blocks viral replication by cleaving viral and cellular RNA¹⁶². In this way the host has many different ways of recognising and preventing the spread of viral infections.

Finally, metabolism also plays a pivotal role in the anti-viral immune response. Different viruses can induce changes in host cell metabolism in order to hijack machinery necessary for replication or to evade the host immune response. For example, infection of CD4+ T cells with Human Immunodeficiency Virus (HIV) is correlated with increased OxPhos and glycolysis in CD4+ T cells and disease spread is positively correlated with OxPhos responses¹⁶³. The authors show that blocking OxPhos using metformin reduces viral load and that the virus causes an increase in the mitochondrial innate immune receptor NLRX1, which regulates OxPhos and glycolysis in T cells. In terms of infection they showed that HIV causes an increase in this gene to promote viral replication via hijacking of nutrients provided by the enhanced OxPhos and glycolysis. Similarly, the type I IFN response to viral infection also plays a role in modifying metabolic responses. In DCs, type I IFN induces changes in oxidative metabolism leading to increased reliance on FAO to fuel TCA cycle which enhances the ability of DCs to produce type I IFN¹⁶⁴. However, they may also lead to a reduction in the cells metabolic capacity. It has been shown that type I IFNs can negatively regulate glycolysis and OxPhos making cells less responsive¹⁶⁵. This may be one way in which cells prevent increased viral replication by shutting down bystander cells via type I IFN.

1.4.4 The role of type I IFN in anti-fungal immunity

However, viral infections are not the only class of pathogen that can induce a type I IFN response. It is well been known that the cell wall component of gram negative bacteria, LPS, can induce type I IFN responses in macrophages¹⁶⁶. However, more importantly for the work in this thesis type I IFNs are now being studied in the context of antifungal immunity. In some early studies it was shown that conventional

dendritic cells (cDCs) were capable of producing both IFN α and IFN β in response to fungal infections in-vitro¹⁶⁷. These IFN responses were shown to be dependent on nucleic acid sensing, activation of TLR4 by fungal PAMPs such as O-linked mannans or through LPS contamination since treatment with alkali as well as the use of TLR9 and TLR4 knock-out cells abrogated these responses. In this early study it was suggested therefore that the fungal wall component β -glucan alone was not sufficient in driving type I IFN responses by cDCs. Importantly, this group also showed that type I IFN signalling was required for effective survival from *Candida albicans* (Ca) infection in-vivo. This occurred due to paracrine IFN signalling which primed cDCs for enhanced ROS production for effective fungal killing.

The results from this study were further followed up to explore the cellular mechanism through which cells induce type I IFN in response to fungal infection¹⁶⁸. Unlike in the previous study it was shown that BMDMs as well as cDCs could induce IFN in response to fungal infections, albeit to a lower extent in macrophages than seen in DC. Importantly, the IFN responses were shown to be induced directly downstream of dectin-1 signalling and did not require IFN autocrine signalling to enhance responses. IFN signalling was induced via a Dectin-1-CARD9-IRF5 mediated mechanism. Again, it was shown that IFN signalling was vital for in-vivo containment of fungal infections orchestrated by DCs. IFNAR knock out mice had increased susceptibility to fungal infections and failed to recruit leukocytes to the site of infection.

These results have been substantiated by work in human cells through both ex-vivo experiments and transcriptomic analysis. Firstly, IFN induction by human DCs were vital in driving non-pathogenic helper T cell 17 subsets (Th17). It was shown that fungal ligands trigger transient IFN signalling by DCs which led to the expression of the integrin $\alpha\text{v}\beta\text{8}$ leading to the release of active TGF β and polarisation of Th17 cells¹⁶⁹. Importantly, this mechanism was mediated by opposing changes in IRF1 and IRF5 transcriptional levels. IRF1 deficiency was shown to lead to uncontrolled IFN β responses and thus restrained TGF β activity via the ISG BST2 in DCs. Transcriptional analysis further added to the evidence that in humans type I IFN responses were vital for anti-fungal immunity¹⁷⁰. Ca induced a type I IFN profile in

human blood mononuclear cells. Interestingly, polymorphisms in the type I IFN pathway led to changes in inflammatory cytokine responses which also correlated with increased susceptibility to fungal infection. As well as these polymorphisms further evidence was shown that in patients with chronic mucocutaneous candidiasis there were defective type I IFN gene expression. Overall, these results highlighted the importance of type I IFN in both animal and human responses to fungal infection.

However, contrasting these results it has also been shown that IFN may play a role in driving tissue damage and inflammation during fungal infections¹⁷¹. Interestingly, these experiments showed that at high multiplicity of infection (MOI) IFNAR deficient animals had worse responses to fungal infection (as shown by other groups), however, at lower MOI IFNAR deficient animals had a higher survival rate. This was shown to be due to type 1 IFN driving granulopoiesis which recruited inflammatory neutrophils and monocytes via CCL2. These monocytes also matured into inflammatory DCs and together with neutrophils lead to lethal kidney pathology. It has also been shown that IFN signalling can lead to increased susceptibility to other fungal infections, such as *Candida parapsilosis*, due to alterations in IL27. IFN signalling leads to the induction of IL27 and animals deficient in the IL27 receptor (IL27R) had enhanced fungal clearance and inflammatory cytokine responses, showing that IL27 acts as a negative regulatory of inflammatory cytokines, downstream of IFN signalling, which leads to negative regulation of fungal clearance¹⁷². Therefore, the literature suggests that IFN signalling may play either a protective or deleterious role in anti-fungal immunity.

1.4.5 The role of type I IFN in trained immunity

Since type I IFN play such a major role in both anti-viral and anti-fungal immunity and have impacts on systemic immune responses it is likely that this cytokine can also drive trained responses. It has well been established that type I IFNs play a major role in driving a core feature of trained immunity, haematopoiesis. Firstly, Li et al, established that both type I and type II IFN play a major role in developmental haematopoiesis with mice lacking IFN α and IFN γ having less HSPCs during the

aorta-gonado-mesonephros stage of development¹⁷³. Similarly, IFN knock out embryos had less lymphoid progenitors although erythroid-myeloid progenitors were not affected. These mice also had significantly enhanced innate immune signatures showing that lack of type I IFN skewed the development of immune cells through developmental haematopoiesis¹⁷⁴. In adult haematopoiesis it was originally shown that in-vitro treatment of murine pluripotent stem cells with IFN caused a significant reduction in cells entering the S phase of the cell cycle. Similarly, in-vivo administration of pIC into mice also had a similar effect¹⁷⁵. However, more recently a dual role for IFN in adult haematopoiesis has been explored. It has been shown that at steady state HSCs retain a state of self-renewal with the occasional maturation into immune cell populations. However, during stressors such as infection, ageing or diet, shifts in IFN levels may trigger HSC differentiation impacting circulating immune cell populations (**Fig 1.8**). It was shown that type I IFN directly induced the proliferation of HSCs and chronic IFN signalling reduced the number of quiescent HSCs in-vivo. The quiescent potential of the HSC compartment was tightly regulated by the transcription factor IRF2¹⁷⁶.

Both in-vitro and in-vivo studies have shown the direct or indirect effects type I IFN have on the bone marrow. Early in-vitro studies demonstrated that direct type I IFN stimulation of HSCs led to changes in the expression of Sca-1 in different progenitor cells leading to a rapid remodelling of the BM phenotype, whereas in-vivo work with pIC stimulation showed that chronic IFN levels increase HSC proliferation and BM aplasia but does not affect the size of the HSC compartment¹⁷⁷. However interestingly, the changes observed in-vivo have never been replicated in-vitro even upon addition of stromal bone marrow cells¹⁷⁸. This is thought to be due to altering the BM niche in-vitro and thus inducing stresses on HSCs which impacts their ability to respond to IFNs¹⁷⁹.

Other cell types in the BM have been implicated in playing a role in inducing changes in haematopoiesis in an IFN dependent manner. For example, mesenchymal stromal cells (MSCs) have been shown to secrete IL6 which can indirectly impact IFN γ mediated myelopoiesis¹⁸⁰. IFN γ itself can also impact MSC phenotype inducing a more immunosuppressive response and limiting proliferation

and differentiation of these cell types¹⁸¹. It would be interesting to understand whether type I IFNs may play a similar role in MSC biology or if they induce a different response to type II IFNs. As well as changes to MSCs, endothelial cells can also be altered via changes in IFN. In-vivo work has shown that type I IFN can induce BM vessel permeability via stimulated HSCs thus showing a cross talk between stem cells and the ability of infiltrating mature cell populations via changes in blood vessel permeability¹⁸². Type I IFN can also impact extracellular matrix proteins in the BM further highlighting the role of these cytokines in BM remodelling¹⁸³.

Type I interferon stimulation has also been shown to induce epigenetic changes in different cell types, another hallmark of trained immunity¹⁸⁴. Kamada R et al, showed that upon type I IFN stimulation fibroblasts undergo epigenetic changes in certain ISGs which enhances their responses to secondary IFN stimulation but also to viral infection. These “memory” ISGs facilitate enhanced protection from viral infection. They also showed that IFN γ can induce similar epigenetic changes in mature macrophages, however, the functional implications of these changes were not examined. The epigenetic marks identified were different to those explored in trained immunity which classically investigate H3K27ac and H3K4me3 markers whereas in this fibroblast context histone H3.3 and H3K36me3 were investigated.

Finally, functional responses have been shown to be altered by type I IFN in mature cell populations. For example, Zahalka S et al, showed that inhalation of LPS was able to enhance the response mice to *Streptococcal pneumonia* infection. They showed that LPS inhalation induced changes in both metabolic phenotype of AMs, a switch away from glycolysis and TCA cycling and towards glutaminolysis and FAO, as well as in the transcriptional profile of these cells. The overall functional responses did not occur in IFNAR1-deficient mice and inhalation of recombinant IFN replicate the functional phenotype implicating the role of type I IFN in alveolar macrophage trained immunity¹⁸⁵. Similar to these results there have been further studies implicating viral infections in driving trained phenotypes. One such studied showed that inactivated SARS-CoV-2 could induce trained immunity in human monocytes in-vitro, with type I IFN being one of the highest differentially expressed

pathways¹⁸⁶. These monocytes showed heightened inflammatory cytokine responses upon bacterial ligand stimulation as well as showing heightened changes in key metabolic and epigenetic profiles. Like other training models the functional responses were abolished after pretreatment with histone methyl and acetyltransferase inhibitors during the training phase. These results have been further highlighted in-vivo with past SARS-CoV-2 infection leaving epigenetic imprints in murine AMs as well as in circulating human monocytes⁹⁵ allowing for enhanced response to secondary viral infections. Taken together this data highlights the role type I IFN has on systemic changes that can cumulate in type I IFN mediate trained immunity.

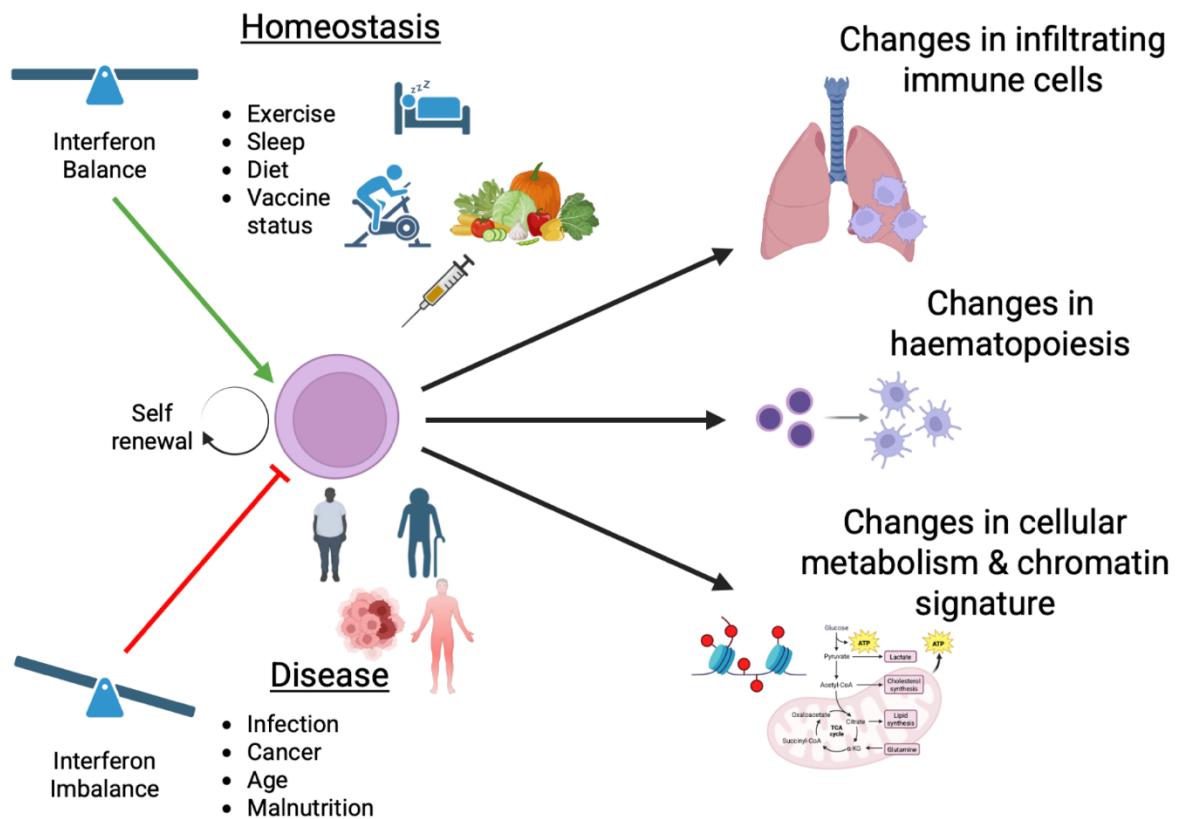


Figure 1.8 Environmental stresses can impact HSC populations

Graph represents the impact environmental stress has on the bone marrow compartment. Health and vaccine status, exercise and a proper balanced diet and sleep schedule can have a positive impact on HSCs^{90, 187, 188, 189}. In contrast, infection, cancer, aging and malnutrition can all have a long lasting 'negative' impact on HSCs^{187, 190, 191, 192}. All these environmental changes can lead to changes in haematopoiesis and bias towards lymphoid or myeloid cells. They can also lead to changes in peripheral immune cell populations in distal organs which can change the immune landscape of those organs. Finally, these changes can be long lasting due to changes in cellular metabolism which drives epigenetic modifications.

1.5 Thesis Aims

It is now established that intraperitoneal (IP) delivery of β -glucan is sufficient in inducing trained immunity against various forms of disease, such as bacterial and fungal infection, as well as cancer^{100, 102, 193}. However, little is known about the mechanism via which β -glucan induces these changes in-vivo and whether anti-viral signalling is altered. In this project, I tested the **hypothesis that β -glucan training would alter type I IFN signalling to enhance responses to viral challenges**. As well as this main hypothesis I also postulated that feeding β -glucan would drive systemic changes dependent on type I IFN signalling. Using a commercially available food-grade yeast β -glucan (Whole Glucan Particle, WGP), I aimed to provide a novel protective mechanism to bridge the gap left between an emerging viral pathogen and more specific therapeutics such as vaccines. Similarly, I also appreciated that the null hypothesis may be true: β -glucan training has no effect on IFN responses, and thus I sought to also investigate if the metabolic changes in β -glucan trained cells could be a novel source of anti-viral protection.

1.5.1 Specific Objectives

1. To investigate the cellular mechanism by which WGP induces trained immunity
2. To identify if WGP stimulation alters anti-viral signalling pathways
3. To examine whether metabolic alterations impact type I IFN responses
4. To investigate the impact of type I IFN has on WGP dietary supplementation in-vivo

Chapter 2. Material & Methods

2.1 Materials

Reagents and equipment used in this investigation and their supplier are listed in the following Tables

2.1.1 Cell Culture Reagents & Chemicals

Reagent	Supplier
Dulbecco modified eagle medium + glutamax (DMEM) 1X	Gibco
Foetal bovine serum (FBS)	Sigma-Aldrich
Penicillin-Streptomycin (P/S)	Sigma-Aldrich
Normocin	Thermofisher scientific
Zeocin	Thermofisher scientific
HEK-Blue CLR Selection	Thermofisher scientific
HEK-Blue Selection	Thermofisher scientific
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma-Aldrich
Trypan Blue	VWR Life Science
L929 conditioned medium	Generated on site (L929 murine fibroblast cell line)
Dimethyl Sulfoxide (DMSO)	Thermofisher Scientific
Red Blood Cell (RBC) Lysis Buffer Hybri-Max™	Sigma-Aldrich
Biochemical grade ethanol	Fisher Bioreagents
Seahorse XF DMEM assay media (without phenol red)	Agilent
α-D-glucose	Agilent
L-Glutamine	Agilent
Oligomycin	Sigma-Aldrich
Rotenone	Sigma-Aldrich
Antimycin A	Sigma-Aldrich
2-Deoxy-D-Glucose (2DG)	FlouroChem

Fluoro-carbonyl cyanide phenylhydrazine (FCCP)	Sigma-Aldrich
Quanti-Blue Solution	Thermofisher scientific
Seahorse XF calibrant solution	Agilent
Heat inactivated FBS (HiFBS)	Sigma-Aldrich
EDTA	Sigma-Aldrich
DMEM W/O Phenol red, glucose and glutamine (DMEM5030 powder)	Sigma-Aldrich
NaHCO₃	Sigma-Aldrich
Mili-Q water	Sigma-Aldrich
[U-13C₆]glucose	Sigma-Aldrich
Dialyzed (d) FBS	Sigma-Aldrich
Hanks' Balanced Salt Solution (HBSS)	Sigma-Aldrich
O-propargyl-puromycin (OPP)	Jena Bioscience

Table 2.1 List of cell culture reagents and chemicals

2.1.2 Cell Stimulation/Treatment Reagents

Reagent	Supplier
Dispersible whole glucan particle (dWGP)	Kerry Group
Soluble WGP (sWGP)	Kerry Group
LPS Ultrapure	Thermofisher scientific
Zymosan	Thermofisher scientific
Zymosan depleted	Thermofisher scientific
Pam3CSK4	Thermofisher scientific
Poly I:C (HMW)	Thermofisher scientific
Cytochalasin D	Sigma-Aldrich
Rapamycin	Sigma-Aldrich
Methylthioadenosine (MTA)	Sigma-Aldrich
Dimethyl malonate (DMM)	Sigma-Aldrich

Glycogen phosphorylase inhibitor (GPI)	Sigma-Aldrich
G3-YSD (Y-form DNA)	Thermofisher scientific
R848 (Resiquimod)	Thermofisher scientific
ODN 2395 (Class C CpG oligonucleotide)	Thermofisher scientific
Fumarate hydratase inhibitor 1 (Fhin1)	Medchem Express
DMXAA	Thermofisher scientific
Cyproheptadine (CPH)	Sigma-Aldrich
Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES)	Sigma-Aldrich
Apenin A5 (A5)	Sigma-Aldrich
Itaconate	Thermofisher
Lipofectimen	Thermofisher
Succinate	Sigma-Aldrich
Dimethyl Fumarate (DMF)	Sigma-Aldrich

Table 2.2 List of Cell Stimulation/Treatment Reagents

2.1.3 Non-Tissue Culture (TC) reagents and chemicals

Reagent	Supplier
Quanti-Blue Solution	Thermofisher scientific
Bovine serum albumin (BSA)	LifeSciences
Sulfuric acid (H₂SO₄)	Sigma-Aldrich
Hydrochloric acid (HCl)	Sigma-Aldrich
Immobilon western chemiluminescent horse radish peroxidase (HRP) substrate	Thermofisher scientific
Sodium chloride (NaCl)	Thermofisher scientific
Sodium hydroxide (NaOH)	Thermofisher scientific
Tween® 20	Thermofisher scientific

eBioscience™ intracellular (IC) fixation buffer	Thermofisher scientific
OneComp eBeads™ compensation beads	Thermofisher scientific
PureLink Mini RNA kit	Thermofisher scientific
Ultra-pure water nuclease free	Thermofisher scientific
Crystal Violet Powder	In-House
High-capacity cDNA reverse transcription kit	Biosciences
Lactate Colorimetric Assay (MAK064)	Sigma-Aldrich
HEPES	Sigma-Aldrich
Sodium Chloride (NaCl)	Sigma-Aldrich
Digitonin	Sigma-Aldrich

Table 2.3 List of Non-TC Reagents & Chemicals

2.1.4 ELISA Reagents

Reagent	Supplier
ELISA Kits: • TNF-α mouse uncoated ELISA kit • IL-6 mouse uncoated ELISA kit • IFN-β mouse uncoated ELISA Kit • CXCL-10/IP-10 mouse uncoated ELISA Kit • IL1-Rα mouse uncoated ELISA Kit	Thermofisher scientific R&D Systems
Tween-20	Thermofisher scientific
10x PBS	Made inhouse
TMB Solution	Thermofisher scientific
Stop Solution (H₂SO₄)	Made inhouse

Bovine Serum Albumin (BSA)	LifeSciences
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Table 2.4 List of ELISA Reagents

2.1.5 Common Lab equipment

Equipment	Supplier
Microscope	Leica
Haemocytometer	Hawksley
Benchtop centrifuge	Biosciences
Plate reader	BioTek
Seahorse XFe96 well analyser machine	Agilent Technologies
Water Bath	Fischer Scientific
40uM nylon mesh cell strainer	Biolegend
70uM cell strainer	Fischer Scientific
Cell scraper (25cm x 20nn blade)	VWR
Falcon tubes (15 ml, 50 ml)	Greiner
Sterile tissue culture plates (6, 12, 24, 48, 96)	Thermofisher scientific
Sterile tissue culture flasks (T25, T75, T175)	Thermofisher scientific
Pipette tips (p10, p200, p1000)	Greiner
Petri dishes (15 mm, 60 mm)	Greiner
Syringes (2ml, 5 ml, 20 ml)	Braun
Eppendorf tubes (0.5 ml, 1.5 ml)	Thermofisher scientific
Serological pipettes (5 ml, 10 ml, 25 ml)	Greiner
Pipette sets (p2, p10, p20, p200, p100)	Gilsen
Aerosol barrier pipette tips (10µl, 20µl, 200µl, 1000µl)	Thermofisher scientific
Transfer pipettes	VWR
FACs tubes (with and without lids)	Scientific Laboratory Supplies
High-binding 96 well ELISA plates	ThermoFisher
MicroAmp 384-well reaction plate	Applied Biosystems

0.2 µm, 70µm and 100µm filters	Thermofisher scientific
Needles (19 g, 23 g, 27 g)	BD Biosciences
Seahorse cell culture plates	Agilent Technologies
Seahorse cartridges	Agilent Technologies
Multi-channel pipettes	Gilsen
Nanodrop 2000 UV spectrophotometer	Thermofisher Scientific
LSR Fortessa	BD Biosciences
Canto cytometer	BD Biosciences
7900HT Fast Real-Time PCR system	Fisher Scientific
MicroAmp optical adhesive film	Applied Biosystems
FlowJo v10 software	BD Biosciences
Prism 9.3.0	Prism Labs
R Studio version 2022.12.0	Posit PBC
R version 4.2.2	Posit PBC

Table 2.5 List of common lab equipment used

2.1.6 List of Cell Lines

Cell Line	Origin	Supplier
hTLR2	Human embryonic kidney cells (HEK293) co-transfected with human TLR2 and SEAP genes, engineered to express high levels of human TLR2	Invivogen
hTLR4	HEK293 cells co-transfected with human TLR4 and SEAP genes, engineered to express high levels of human TLR4	Invivogen
hDectin-1a	HEK293 cells engineered to express high levels of human Dectin-1a isoform and genes involved in dectin1/NF- κB/SEAP pathway	Invivogen
hDectin-1b	HEK293 cells engineered to express high levels of human Dectin-1b isoform and	Invivogen

	genes involved in Dectin1/NF- κ B/SEAP pathway	
RAW-Blue macrophages	Murine RAW 264.7 macrophages with chromosomal integration of an SEAP reporter construct	Invivogen

Table 2.6 List of cell lines used

2.1.7 Mice

2.1.7.1 Ethics and animal license

C57BL/6J mice were generated and maintained at the Comparative Medicine Unit, Trinity College Dublin. Knock-out mice were gifted to use by The O'Neill Lab and The Lavelle Lab (TCD). Mice were bred and maintained under specific pathogen-free conditions with *ad libitum* access to food and water. Mice were used at the age of 8-12 weeks. All experiments were carried out under the approval of the Health Products Regulatory Authority, Ireland and Trinity College Dublin Animal Research Ethics Committee.

2.1.8 Cell lines

Reporter cell lines, as listed in table 2.6, were purchased from Invitrogen and were handled according to the manufacturer's instructions.

2.1.9 Cell culture media

2.1.9.1 BMDM media

Complete DMEM (cDMEM) cell culture medium was prepared by supplementing DMEM with 10% FBS and 1% P/S. Cells were stimulated with varying percentages of LCM depending on the training time course.

2.1.9.2 Cell Line media

Complete DMEM (cDMEM) cell culture medium was prepared by supplementing DMEM with 10% FBS (or 10% HiFBS), 1% P/S and 250x Normocin. Media was further supplemented with selective antibiotics including 500x Zeocin for RAW-blue macrophages, 250x HEK-blue selection for TLR-2 and TLR-4 reporter cells and 250x HEK-blue CLR selection for hDectin-1a and hDectin-1b cell lines.

2.1.10 Cell culture stimulants / inhibitors

Reagent	Vehicle	Stock Concentration	Desired Concentration
2DG	Seahorse Media	1M	10mM-20mM
Antimycin-A	Ethanol	5mM	4 μ M
FCCP	Ethanol	1mM	1 μ M
Oligomycin	Ethanol	2mM	2 μ M
Rotenone	DMSO	1mM	0.1 μ M
Rapamycin	DMSO	1mM	10nM - 20nM
Cytochalasin D	DMSO	2mM	10 μ M - 20 μ M
DMM	Ethanol	5M	5mM – 10mM
CB839	DMSO	10mM	1 μ M – 2 μ M
MTA	DMSO	100mM	1mM – 2mM
CPH	DMSO	100mM	50 μ M
Fhin1	DMSO	10mM	10 μ M
Apenin A5	DMSO	5mM	10 μ M
G3-YSD	Lipofectamine/ptamin	1mg/mL	1 μ g/mL
R848	cDMEM/PBS	1mg/mL	5ng/mL
ODN 2395	cDMEM/PBS	500uM	1 μ M – 10 μ M
LPS-EB Ultrapure	cDMEM/PBS	1mg/mL	1ng/mL 100ng/mL -
Poly (I:C)	cDMEM/PBS	1mg/mL	10 μ g/mL
Transfected Poly (I:C)	Lipofectamine/ptamin	1mg/mL	1 μ g/mL
Zymosan/Zymosan depleted	cDMEM/PBS	1mg/mL	10 μ g/mL 100 μ g/mL –
dWGP	cDMEM/PBS	25mg/mL	100 μ g/mL
sWGP	cDMEM/PBS	5mg/mL	100 μ g/mL
PAM3CSK4	cDMEM/PBS	1mg/mL	10 μ g/mL

Table 2.7 List and concentrations of stimulants and inhibitors used in cell culture

2.1.11 Buffers

Buffer	Composition
Freezing medium	10% DMSO in FBS
Thawing medium	cDMEM
2% FACs buffer	50mL: 1mL FBS + 49mL PBS
1X Perm Buffer	50mL: 5mL 10X perm buffer + 45mL dH ₂ O

Seahorse Medium	DMEM without phenol red + 10mM glucose + 2mM L-glutamine, warmed to 37°C, pH 7.4.
Wash Buffer	0.005% Tween-20 in 1X PBS, pH 7.2 – 7.4
10X PBS	800g NaCl + 20g KCl + 144g Na ₂ HPO ₄ monobasic + 24g KH ₂ PO ₄ dissolved in 8L of dH ₂ O & made up to a final volume of 10L with dH ₂ O, pH 7.2 – 7.4
12X H₂SO₄	64mL 98% stock + 36mL dH ₂ O

Table 2.8 List and composition of buffers used

2.1.12 Flow cytometry antibodies / stains

2.1.12.1 Mouse antibodies

Target	Colour	Clone	Supplier
CD3	APC-Cy7 BV605 APC	17A2 17A2 17A2	Biolegend
CD11b	PE APC APC-Cy7 PerCP	M1/70 M1/70 M1/70 M1/70	Biolegend
NK1.1	BV421	PK136	Biolegend
cKit	APC APC-Cy7	2B8 2B8	Biolegend
Sca-1	PE-Cy7	D7	Biolegend
FIt3/CD135	PE BV421	A2F10.1 A2F10.1	Biolegend
CD150	PE eFluor450	mShad150 mShad150	Invitrogen eBiosciences
CD34	FITC PE Dazzle 594	RAM34 MEC14.7	Biolegend
CD48	PerCPef710	HM48-1	Invitrogen
CD45	SparkBlue 550 APC Fire 810 FITC	30-F11 30-F11 30-F11	Biolegend
F4/80	PeCy7	BM8	BD
Ly6C	PE-CF594 BV605	AL-21 HK1.4	BD Biolegend
Ly6G	BV785	1A8	Biolegend

	R718	RB6-8C5	BD
Ter119	BV605 APC-Cy7	TER-119 TER-119	BD Biolegend
Ly6C/G	BV605 APC-Cy7	RB6-8C5 RB6-8C5	BD Biolegend
B220	BV605 BV510 BV570 APC-Cy7	RA3-6B2 RA3-6B2 RA3-6B2 RA3-6B2	BD Biolegend Biolegend Biolegend
SiglecF	APC		Biolegend
IFN α	FITC		
CCR2	BV711		Biolegend
H3K27ac	PE AF488	EP16602 EP16602	Abcam
H3K4me3	AF647	EPR20551-225	Abcam

Table 2.9 List of mouse flow cytometry antibodies used

2.1.12.2 Flow cytometry stains

Stain	Stock concentration	Desired concentration	Supplier
Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM)	100uM	100nM	Invitrogen
MitoTracker TM Green FM (MTG)	1mM	1uM	Invitrogen
Zombie Aqua Fixable Viability Dye	1000X solution in DMSO	Dilute 1 : 1000	Biolegend

Table 2.10 List of flow cytometry stains

2.1.12.3 Click Chemistry reagents

Reagent	Vehicle	Stock concentration	Desired concentration
O-Propargyl Puromycin	DMSO	1mM	20 μ M
Copper(II) sulfate pentahydrate	dH ₂ O	100mM	1mM

(+)-sodium L-ascorbate	dH ₂ O	1M	10mM
Tris-hydroxypropyltriazolylmethylamine (THPTA)	dH ₂ O	100mM	1mM
Aminoguanidine hydrochloride	dH ₂ O	1M	160mM
Azide fluorophore	DMSO	1mM	2.5 μ M

Table 2.11 List of Click chemistry reagents

2.2 Methods

2.2.1 Reporter cell line work

Cells were first thawed and recovered in 15mL pre-warmed media and centrifuged at 300 rcf for 5 minutes. The pellet was resuspended in 1mL media and transferred to a T-25 tissue treated flask. Media was replaced every two days until cells reached 80-90% confluence, according to the manufacturer's instructions, and cells were then transferred to a T-175 once they had recovered from liquid nitrogen freezing after 3 passages. Media was then replaced every three days and cells were passaged once they reached a confluency of 70-80%.

Upon cell recovery cells were washed with PBS, scraped to detach and were centrifuged at 300 relative centrifugal force (rcf) for 5 minutes. The pellet was then resuspended in 1mL media containing HiFBS and counted to give a final cell density depending on manufacturer's protocol. Cells were then plated in a 96-well plate and left to adhere for 1-2 hours prior to stimulation.

2.2.2 β -glucan preparation

2.2.2.1 Dispersible whole glucan particle (WGP) preparation

WGP was weighed and resuspended in sterile dH₂O giving a final concentration of 25mg/mL and was stored overnight on a rocker at room temperature. The following day the suspension was sonicated in an ice bath using a 5/32" micro sonicator tip. The sonicated WGP was then centrifuged at 1,000 rcf for 10 minutes at room temperature and supernatant was discarded. Pellet was resuspended in 0.2M NaOH and incubated at room temperature for 20 minutes. Following incubation the suspension was then centrifuged at 1,000 rcf for 10 minutes at room temperature and resuspended in sterile dH₂O. This step was repeated three times in order to remove NaOH and the suspension was then further washed two more times with sterile endotoxin free PBS. Preparation was stored at 4°C.

2.2.2.2 Soluble whole glucan particle (sWGP) preparation

sWGP was weighed and resuspended in warm (45-50°C) dH₂O giving a final concentration of 5mg/mL. Preparation was stored at 4°C.

2.2.3 Murine bone marrow derived macrophage (BMDM) cell culture

2.2.3.1 Initial murine bone marrow culture

Following CO₂ asphyxiation, mice ages 8-12 weeks were pinned down and sprayed with ethanol. The lower abdomen was opened using a forceps and scissors, with an incision made down each leg to expose the hips and legs. Without breaking the bone, the femur and tibia were amputated and placed in DMEM media. In a tissue culture hood, the muscle was removed from the bone using a forceps and scissors and then cleaned using an ethanol-soaked tissue. Extremities of the bone were

removed and the bone marrow flushed out using a 25G needle into 5 mL of sterile DMEM. Using a P1000 the bone marrow was pipetted up and down to break up larger clumps of marrow and then centrifuged for 5 minutes at 400 rcf. The supernatant was aspirated and the pellet resuspended in 1 mL of red cell lysis buffer (Sigma) for 2 minutes. 9 mL of DMEM was added to neutralise the lysis buffer and cells were centrifuged at 400 rcf for 5 minutes. Cell number was calculated using Trypan Blue staining and a haematocytometer. 20 mL of cDMEM (cDMEM + 20% L929-conditioned medium) containing 1×10^6 cells/mL was prepared and passed through a 70 μ M cell strainer before being plated on a 15 cm³ culture dish. Media on the plate was replaced every two to three days and on day 6, the cells were detached using cold PBS, counted and seeded at various densities in DMEM, 10% FBS, 10% L929-conditioned medium, one day prior to the experiment. Cells were allowed to differentiate over a 1 week period and the purity of BMDMs was measured using flow cytometry (Figure 2.1).

2.2.3.3 In-vitro induction of trained immunity & tolerance in BMDMs

For the in-vitro induction of trained immunity and tolerance BMDMs were allowed to mature over 6 days as described above and were replated at 1×10^5 cells per mL in 96-well plates or 5×10^5 cells per mL in 24-well plates. Cells were then either left untreated or were stimulated with various concentrations of primary stimulants ranging from 1ug/mL, 10ug/mL to 100ug/mL for training or 1ng/mL, 10ng/mL to 100ng/mL (Table 2.11). Cells were then washed with PBS 24hrs later and fresh media was added supplemented with 10% LCM. Cells were allowed to rest for 6 days feeding on day 3 supplemented with 7% LCM.

On day 6 media was replaced with fresh media prior to secondary stimulation supplemented with 5% LCM. Cells were then stimulated with various concentrations of secondary stimulants (Table 2.11).

For certain experiments where cell number may majorly effect the experiment (Viral infection, western blots & metabolomics) a modified version of the training assay was used. Chiefly, BMDMs were seeded at a density of 2×10^6 cells/mL in 10cm³

petri dishes, 1 dish for untrained and 1 dish for trained per mouse. The standard training protocol was then followed until the day of the experiment. On the day of the experiment cells were lifted as described above, counted and plated depending on the experimental needs.

For viral infection studies BMDMs were trained as described above and counted 2hrs prior to infection. Two viruses were used to infect cells: *Herpes Simplex Virus 1* (HSV1) and *Vesicular stomatitis virus* (VSV) which were kindly gifted to us by The Bowie Lab and The O'Farrelly Lab, TCD. In short viral titres were calculated prior to infection and an MOI of 10 and MOI of 1 were used respectively. MOI was calculated based on the following calculation: No. of cells / Viral titre (/μL) * volume of media needed to cover cells per well (μL). Cells were infected with virus in serum free media for 1hr at 37°C, with gentle agitation of the plate every 15 minutes. After 1hr media containing virus was removed and fresh media containing FBS, P/S and L929 conditioned media was added on for 24hrs. PCR or ELISA was used to quantify viral infection and responses to virus respectively.

Primary Stims	Concentration	Secondary Stims	Concentration
dWGP	100ug/ML	LPS	10ng/mL
sWGP	100ug/ML	R848	5ng/mL
Zymosan	100ug/ML	Poly (I:C)	10ug/mL
LPS	100ng/mL	CPG	1uM
-	-	tPoly (I:C)	1ug/mL
-	-	G3-YSD	1ug/mL
-	-	3'-sh-RNA	100ng/mL
-	-	PAM(3)CSK(4)	10ug/mL
-	-	DMXAA	10uM

Table 2.12 List and concentrations of primary & secondary stimulants used

2.2.4 Flow cytometry analysis

Supernatant was first removed from cells and washed with 1mL ice cold PBS. 1mL of ice cold PBS was then passed to the cells and they were placed on ice for 5 minutes. Cells were then harvested into labelled FACs tubes by blasting cells with ice cold PBS using a p1000. Cells were then spun by centrifugation for 4 mins at 1400 rcf. The supernatants were removed, and cell pellets were disrupted (hereafter

implied when cells are washed) and stained for analysis using the following protocol. The following control cell samples were prepared: Unstained (for each treatment), live/dead, TMRM and Mitotracker Green (MTG). Single-stained bead controls were prepared for each antibody used.

2.2.4.1 TMRM & Mitotracker Green (MTG) co-staining

For quantification of mitochondrial mass and membrane potential, cells were co-stained with MTG and TMRM dyes. An MTG / TMRM master mix was prepared in warm DMEM medium (Table 2.5). Cells were transferred to labelled FACs tubes, washed with 1ml PBS, and re-suspended in 200 μ l of the MTG/TMRM cocktail. Cells were stained for 30 min at 37°C. Cells were then washed X1 with PBS for further staining. Cells stained with TMRM and MTG were not fixed and acquired live on the LSR Fortessa cytometer.

2.2.4.2 Staining of bone marrow progenitors

Bones were first washed and prepared as described previously (2.2.3.1). Prior to use the MACs miltenyi magnet was first placed in the -20°C freezer. Cells were stained as described below. Post staining supernatant was removed and the pellet resuspended in 125 μ L cold PBS and 25 μ L of CD117 (or cKit+) MACs miltenyi magnetic beads and allowed to bind for 20 minutes. After 20 minutes 5mL of ice cold PBS was added and spun at 450g for 5 minutes. Supernatant was removed and pellet was resuspended in 1mL of PBS. During incubation columns were set up and activated by inserting MACs magnet insert LS columns and adding 2mL of ice cold PBS to the column. Following incubation, a 1mL syringe was used to slowly add 1mL of the cell suspension to the MACs columns. This was repeated with the collected cells to increase yield. Collect positive fraction by removing the column from the magnet and adding 5mL PBS. Cells were centrifuged at 450g for 5 minutes and resuspended in 1mL flow tubes. 10 μ L of count beads were added and flow tubes were centrifuged again at 450g for 5 minutes. Cells were resuspended in 200 μ L of PBSs buffer.

2.2.4.3 Isolation and staining of circulating blood immune cells

Mice were culled as previously described and the animal was sterilized using 70% ethanol. An incision was made along the sternum of the animal and the ribcage was exposed. Blood was drawn from the right atrium of the heart using an EDTA coated syringe and blood was stored in EDTA coated tubes. 100-300 μ L of blood was added to 2mL of RBC lysis buffer for 10 minutes in a 15mL falcon. Cells were washed with 10mL of BS and spun at 400g for 10 minutes. If cells still contained red blood cells, pellet was resuspended in a further 1mL of RBC lysis buffer for 10 minutes followed by addition of 10mL PBS and spun again at 400g for 10 minutes. Cell pellet was that resuspended in 200 μ L PBS and added to FACs tubes for further staining.

2.2.4.4 Isolation & staining of murine lung innate immune cells

Mice were culled as previously described and the animal was sterilized using 70% ethanol. An incision was made along the sternum of the animal and the ribcage was exposed. The ribcage was removed by severing the diaphragm and carefully cutting along the sternum. Once removed, the abdominal aorta and the caudal vena cava were cut and 2mL of ice cold PBS was injected gently into the left ventricle to perfuse the lungs. Following perfusion, the superior, middle and inferior lobes of the lungs were harvested from mice and chopped using a scalpel. The lungs were digested with collagenase-D and DNase I for 1hr at 37C, while constantly rotating to facilitate tissue breakdown. Digested tissue was then passed through a 70 μ M cell strainer to obtain a single cell suspension. Cells were centrifuged at 1400 rpm for 4 minutes to obtain a cell pellet and supernatant was tipped off. Cells were resuspended in 2mL RBC lysis buffer for 5 minutes to lysis red blood cells. After 5 minutes, 10mL of cRPMI was added to neutralise the lysis buffer and the cells were again spun as previously described.

To detect intracellular cytokine production ex-vivo, cells were plated at 2×10^6 cells/well in a 12 well plate. Cells were stimulated ex-vivo with 10 μ g/mL of poly I:C for a period of 8 hours. 100 μ L of media was taken off at 4hrs and frozen for ELISA and 100 μ L of brefeldin A was added for the final 4hrs to reach a final concentration

of 5ug/mL. Cells were then stained according to the below protocol. Gating strategy shown in Fig.

2.2.4.5 Preparing and staining cells for Click Chemistry

Cells were seeded into 96 well plates in 37°C Hanks' Balanced Salt Solution (HBSS). Cells were incubated +/- oligomycin (1 μ M) or 2DG (10mM) for 15 minutes. After, pre-warmed alkyne-puromycin (O-propargyl-puromycin, OPP) was added (20 μ M). Cells were incubated for 30 minutes at 37°C, washed and stained for flow analysis as described below. After full staining for flow, cells were washed twice in PBS and the click chemistry reaction was performed. The click reaction master mix was made up in PBS and reaction was performed in the exact order as follows: 1mM copper (II) sulfate pentahydrate, 10mM sodium L-ascorbate, 1mM tris-hydroxypropyltriazolylmethylamine (THPTA), 160 mM aminoguanidine hydrochloride and 2.5 μ M AZDye 647 Azide. Cells were kept in the dark for 1 hour at room temperature, washed twice with PBS and then resuspended in FACS buffer.

2.2.4.6 Live cell staining

To ensure gating on live cells only, a live/dead stain was prepared in PBS (Table 2.10). Cells were re-suspended in 50 μ l of the live/dead master mix and incubated in the dark at room temperature (RT) for 20 min. For single-stain and unstained controls, 50 μ l of PBS was added. Cells were washed with 1ml PBS for further staining.

2.2.4.7 Extracellular staining and cell fixing

An extracellular fluorochrome-labelled antibody cocktail with Fc block was prepared in FACS buffer (Table 2.8). Cells were re-suspended in 50 μ l of the master mix and incubated in the dark at RT for 30 min. For unstained controls, 50 μ l of FACS buffer was added. Cells were washed with 1ml FACS buffer for further staining or re-suspended in 150 μ l of FACS buffer prior to acquisition on a FACS Canto, FACS Fortessa or the Cytex Aurora. For experiments involving intracellular staining, 100 μ l of IC fixation buffer was added to each tube and cells were fixed for 30 min at 4°C.

2.2.4.8 Intracellular staining

After cellular fixation, cells were washed X2 with 1ml 1X perm buffer (Table 2.8). An intracellular fluorochrome-labelled antibody cocktail was prepared in 1X perm buffer. Cells were incubated with this master mix at a final volume of 50µl per tube for 25 min. For unstained controls, 50µl of 1X perm buffer was added. Cells were washed with 1X FACS buffer for further staining or re-suspended in 150µl of FACS buffer prior to acquisition on a FACS Canto, LSR Fortessa or Cytex Aurora cytometers.

2.2.5 Metabolic phenotyping

2.2.5.1 Metabolic analysis: Seahorse XF analyser

The extra-cellular flux analyser, Seahorse XFe96 analyser, was used to investigate the metabolic profile of murine BMDMs. An adapted version of the XF cell mitochondrial stress test was used to measure key parameters of both mitochondrial and non-mitochondrial function through the oxygen consumption rate (OCR) as well as analysis of the extracellular acidification rate (ECAR) of the media to investigate glycolytic flux.

Cells were plated in the seahorse plate at 100,000 cells per well for 24 hour or 72 hour stimulation assays. Cells were stimulated with various stimulants as previously described (Table 2.11) for 24 hour. For 72 hour measurements cells were washed with PBS and 200uL of fresh cDMEM/7%LCM was added to each well 24hrs after initial stimulation.

2.2.5.2 Hydration of cartridge

On the day prior to measurement the calibration cartridge was hydrated with 200uL of XF Seahorse calibration media and was placed in a non-CO₂ incubator overnight at 37°C.

2.2.5.3 Preparation of cells

On the day of the assay, cells were washed X2 with seahorse medium (Table 2.8) to remove any pH buffers or excess medium that the cells had been cultured in. Each well was then topped up with 180uL of seahorse medium and the plate was placed into a non-CO₂ incubator at 37°C for 20 min before the beginning of the Seahorse run. While the plate was incubating, working stocks of the metabolic inhibitors – oligomycin, fluoro-carbonyl cyanide phenylhydrazine (FCCP), rotenone, antimycin-A and 2DG – were prepared in Seahorse medium, as seen in Table 2.12. 20µl or 22µl of inhibitor was added to the designated ports. The loaded cartridge and calibration plate were loaded into the Seahorse XFe96 analyser and calibrated for 20 min. Following completion of calibration the utility plate was removed and the cell culture plate was placed into the machine for analysis of cellular metabolism in real time.

Compound	Stock conc	Final conc	Vehicle (Seahorse media) (uL)	Inhibitor (uL)
Oligomycin	2mM	2uM	2970	30
FCCP	1mM	1uM	2969.7	30.3
Rot/Anti-A	1mM/5mM	0.1uM/4uM	2967.24	3.63/29.13
2DG	1M	30mM	1818.9	1181.1

Table 2.13 List and concentration of inhibitors used in Seahorse analysis

2.2.5.4 Metabolic analysis: Extracellular lactate assay quantification

In order to measure extracellular L(+)-lactate in supernatants the Lactate Assay Kit (MAK064) was used. This colorimetric assay utilises the enzymatically induced colour change which is proportional to the levels of lactate in the supernatant. Importantly, all supernatant was used fresh to avoid lactate degradation during any freeze thaw cycles. A master mix was prepared and a standard curve was generated with a top standard of 100 picomole/well. Samples were prepared at a range of dilutions in order to find a concentration that fell within the standard curve, with BMDM supernatant from 12-well plates generally being diluted 1:100 in assay buffer. Samples were ran in oneplate and master mix was added on top. The plate

was wrapped in tinfoil to isolate from light was placed on a rocker at room temperature for 30 minutes. The plate was then measured at 570nm on a spectrophotometer and the standard curve was used to determine the concentration within the samples.

2.2.5.5 Metabolic analysis: Cytosolic extraction using digitonin fractionation

BMDMs were plated in 12 well plates and post treatment were removed as previously described. Cells were pelleted in RNase free epis by spinning them at 4C for 5 minutes at 500g. Supernatant was gently disposed of and pellets were resuspended in 400uL of extraction buffer (150mM NaCl, 50mM HEPES pH 7.4, and 25ug/mL digitonin). Samples were then placed into a rotating mixer at 4C for 10 minutes and centrifuged for 5 minutes at 4C at 2,000g. The supernatant was separated from the pellet and the sample split. RNA was isolated from half of the supernatant (which contained the cytosolic fraction) using a standard RNA prep kit as previously described. The other half was concentrated using strataclean resin and analysed via western blot. The pellet contained membrane bound organelles and were lysed with Ripa Buffer for western blot analysis or lysis buffer for RNA analysis. The list of antibody and sequence targets for mitochondrial RNA is shown in box. Separation and purification of cytosolic fraction from the organelle fraction is shown below (**Fig 1.1**)

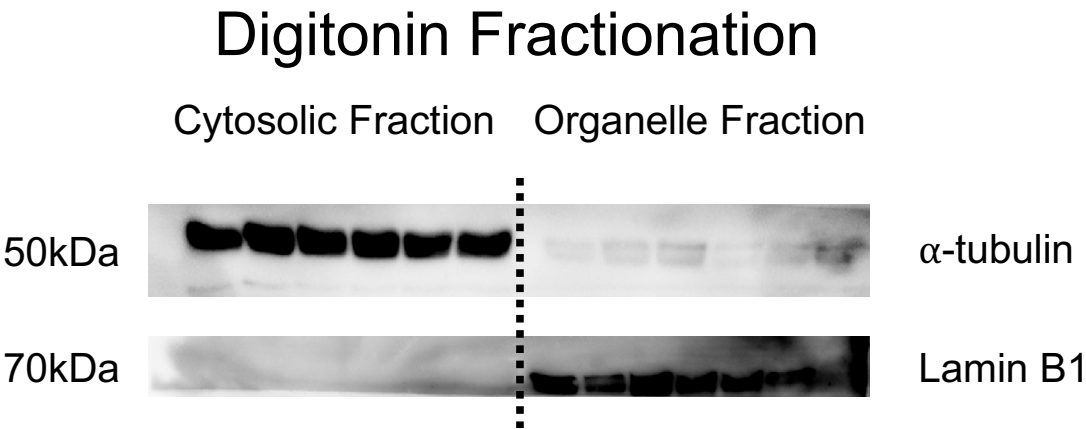


Figure 2.1 Western blot showing separation of the cytosolic and organelle fraction via digitonin fractionation

2.2.5.6 Metabolic analysis: Isotope tracing and metabolomics

Untrained and trained cells were prepared as described above. Chiefly, cells were replated on the day of the experiment at a well concentration of 1×10^6 cells per well. Cells were allowed to adhere, in a CO₂ incubator at 37°C, for 2 hours prior to the start of the experiment. After two hours, cells were washed with pre-warmed PBS and 1mL of pre-pHed tracer media was added to the cells, as shown in table. 1mL of tracer media was added to a well containing no cells as a control for metabolite uptake. Cells were kept in tracer media for a 24hr period in a CO₂ incubator at 37°C.

On the day of metabolite extraction all reagents and buffers were made up as shown in table. Cell plate was gently agitated to ensure complete mix of tracer media across cells. 1mL of tracer media was removed from each well and added to prelabelled Eppendorf tubes. Hence forth, all steps were carried out over ice. Samples were centrifuged at 300g at 4°C for 5 mins and supernatant was collected into newly labelled Eppendorf if significant debris was present. All tubes were stored in the -80°C freezer until extraction.

After collecting necessary media samples, wells were further aspirated, and any remaining spent medium was removed from each well. 1mL 0.9% saline solution was added to each well and aspirated (2X) as above to rinse. All steps were now carried out on ice under a fume cupboard. 250µL of pre-cooled MeOH was added to each well and plate was moved around to ensure coverage of all cells. Plate was left on ice for approximately 1 minute. 100µL of milliQ water containing norvaline was added to each well and again plate was moved to mix. Each well was scraped using cell scrapers or the tip of P1000 pipette, where all liquid was first pipetted up, then a new tip was used to scrape cells off plate and washed with the media. Aspirated media was then added to 250µL of pre-cooled chloroform and Eppendorf's were capped. Samples were then taken to the cold room where they were vortexed for 5 minutes. Samples were then centrifuged for 5 min at max speed (>12000 g) at 4°C. Samples were then taken back to the fume hood where the upper layer containing polar metabolites was removed and added to fresh Eppendorfs.

Samples were then dehydrated using a pressure dehydrator for 1hr and 20 minutes before being stored at -80°C freezer until extraction

Samples were shipped to collaborators (Wallace Lab UCD) where Gas Chromatography-Mass Spectrometry (GC/MS) was performed.

2.2.6 RNA analysis

To prevent the risk of RNA degradation, all of the following steps were performed on ice unless otherwise stated. The centrifuge was first pre-cooled to 4°C and all tubes used were free from RNases/DNases. All equipment was first sprayed down with ethanol and a lab coat was worn at all times. All pipette tips were also sterile and filtered.

2.2.6.1 RNA isolation from cells

Cell culture plates were put on ice and supernatant was removed and kept at -20°C for future cytokine measurements. 350uL of RNA lysis buffer was then added and cells were gently scraped and added to prelabelled Eppendorf tubes left on ice. These samples were then left at -80°C or until the purification step. In order to isolate total RNA the PureLink RNA Mini Kit (Thermofisher) protocol was adapted.

2.2.6.2 Quantification of RNA quality and concentration

In order to check the concentration and quality of the extracted RNA the Nanodrop 2000 UV spectrophotometer (Thermofisher Scientific) was used. The machine was first cleaned and blanked using RNase-free dH₂O and 1uL of each sample was run in order to obtain both the concentration, the 260/280 ratio and the 260/230 ratio. A 260 / 280 and 260 / 230 ratio of ~ 2 indicates a pure RNA yield.

2.2.6.3 cDNA synthesis

Prior to reverse transcription, RNA was first cleansed of contaminating DNA via a DNase step using amplification grade DNase I from thermofisher, according to

manufacturer's instructions. In brief, samples were first normalized to a set value of total RNA (1ug, unless samples had a lower yield) by adding RNase-free dH₂O and bringing samples to a final volume of 8uL. 2uL of DNase buffer/DNase I were added to each sample for 15 minutes at RT. DNase was inactivated with the addition of 1uL of 25mM EDTA solution to the reaction mix. Samples were heated at 65°C for 10 minutes and were then ready for reverse transcription. Here, samples were brought to a final volume of 20uL by adding reverse transcription mix, the ingredients for which are shown in Table 2.13. Following this, samples were then placed into a centrifuge and spun at 500 rcf for 20 seconds. These were then placed into the thermocycler and run on the cycle outlines, which are shown in Table 2.14. For short term storage samples were stored at -20°C and for long term storage samples were stored at -80°C until samples were ready for use in real-time PCR.

Component	Volume per reaction (uL)
10X Reverse Transcription Buffer	2
25X 100mM dNTP	0.8
10X Random Primers	2
RNase free water	3.2
Reverse Transcriptase	1
Total	9

Table 2.14 List of reagents for reverse transcription mix for mRNA

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (minutes)	10	120	5	∞

Table 2.15 Thermocycler program for reverse transcription of mRNA to cDNA

2.2.6.4 Quantitative real time PCR (qPCR) Taqman

In order to analyse the relative quantities of mRNA the pre-designed Taqman real-time PCR gene expression assays that were FAM-labelled were used. Using 1ug of the total RNA, cDNA was diluted by 1:10 with RNase-free dH₂O. The dilution factor was adjusted in cases where samples had a lower yield. All samples were run in triplicate per probe (list of probes shown in Table X) and 2uL of each sample was

added to a well of the MicroAmp 384-well reaction plate (Applied Biosystems). The RT-PCR master mix was made as shown in Table 2.15 and 8uL of this was added to each well. HPRT or 18S was used as the housekeeper gene for normalisation of mRNA. To seal each plate, MicroAmp optical adhesive film (Applied Biosystems) was used. Once sealed the plate was spun at 400 rcf for 1 minute and was run using the Applied Biosystems 7900HT Fast Real-Time PCR system. The protocol for this is shown below in Table 2.16. In order to analysis the RT-PCR data $\Delta\Delta$ Cycle Threshold ($\Delta\Delta Ct$) value method was utilised. The Ct value was normalised to each of the corresponding endogenous controls in order to generate the ΔCt value. This was then used to obtain the $\Delta\Delta Ct$ value for each sample by subtracting ΔCt value from each control sample from the ΔCt value of the equivalent test sample. In order to generate a value for the fold change in the expression of the target gene relative to the control of the same sample the formula $2^{(-\Delta\Delta Ct)}$ was used which enabled the normalisation of the $\Delta\Delta Ct$.

Component	Volume per reaction (uL)
2X Taqman FAST advanced master mix	5
RNase free water	2.5
20X Taqman gene expression assay probe	0.5
Total	8

Table 2.16 List of reagents for real-time q-PCR

Step	1 - Hold	2 - Hold	3 - Cycle	4 - Cycle
Temperature (°C)	50	95	95	60
Time	2 (Min)	10 (Mins)	15 (Secs)	1 (Min)

Table 2.17 Real-Time qPCR program

2.2.6.4 Quantitative real time PCR (qPCR) SyberGreen

The protocol for SyberGreen was the same as for Taqman. However, there was a difference in how the probes were made up as shown in table 2.17. Prior to use SyberGreen reverse and forward sequences were mixed to give a 10X stock

concentration. This was dependant on the starting concentration of the stock sequences. A list of sequences used is shown in Table 2.18.

Component	Volume per reaction (uL)
2X SyberGreen master mix	5
RNase free water	2.2
10X SyberGreen reverse & forward gene	0.8
Total	8

Table 2.18 List of reagents used for SyberGreen PCR

Gene of interest	Forward Sequence	Reverse Sequence
<i>Rps18</i>	GCGAGTACTCAACACCAACA	CCTCAACACCACATGAGCATA
<i>Isg15</i>	GCCAGAAGCAGACTCCTTAAT	ACACCAGGAAATCGTTACCC
<i>Usp18</i>	TCAGTGCCTGCAGAAATACA	TCCGTGATCTGGTCCTTAGT
<i>Irg1</i>	AGAAGCCCTGCCAAGGAGTCCAAA	CCAGAGCTTCTCGGCACTTTGT CG
<i>Irf1</i>	AGGCCAAGAGGAAGTCATGTG	CTGTGTAGCTGCTGTGGTCA
<i>Irf5</i>	GTGTACTTCCCTGTCTCTTTAG	GGCTTCAGTGGGTCAAC
<i>D-Loop</i>	AATCTACCATCCTCCGTGAAACC	TCAGTTTAGCTACCCCCAAGTTT AA
VSV N	CCTGATGACATTGAGTATACATCTC TT	GGATCCTACTGCATAAGCGTAC A
<i>HSV gB</i>	GCAGTTTACGTACAACCACATACA GC	AGCTTGCGGGCCTCGTT

Table 2.19 List of genes and sequences used for SyberGreen PCR

2.2.7 Protein analysis

2.2.7.1 Cytokine analysis measured by Enzyme-linked Immunosorbent Assays (ELISA)

Cytokine quantification was determined using Invitrogen's (Thermo Fisher) uncoated ELISA kits and R&D systems uncoated ELISA kits. High-binding 96 well ELISA plates (ThermoFisher) were used by adding 50uL of coating antibody diluted in coating buffer to each well and was left to incubate over night at 4°C. ELISA plates

were then washed three times using wash buffer (Table 2.8). Following each wash, plates were allowed to dry upside down on tissue and were blotted by banging them on the tissue to remove excess wash buffer. In order to block remaining binding sites 150uL of ELISA diluent was added to each well and were left sealed on a rocker at room temperature for 1 hour.

Diluent was removed and each plate was washed as before. Standards were then generated through serial dilution and a blank was also generated through ELISA diluent alone. Samples and standards were then added to each plate in duplicate, with each sample being diluted, in assay diluent, depending on the cytokine. This dilution had previously been determined so as to fall within the standard curve. The plates were sealed and left on a rocker at 4°C over night. The following day plates were then washed as previously described and 50uL of detection antibody was added to each well. Plates were sealed and placed on a rocker at room temperature for 1 hour. Plates were then washed again as previously described and 50uL of streptavidin-horseradish peroxidase conjugate diluted in ELISA diluent was added to each well. Plates were sealed with tinfoil to prevent light exposure and were placed on the rocker for 30 minutes at room temperature. Plates were then washed seven times and 50uL of TMB substrate was added to each well. This results in a colorimetric reaction and the reaction was stopped when the middle standard began to change blue. The reaction was stopped through the addition of 25uL of H₂SO₄. The plates were then measured at 450nm using a microplate reader. Values were saved to Excel and a standard curve was generated in order to determine the cytokine concentration.

2.2.7.2 Protein analysis: Cell harvesting for Western Blot

Cells were removed as previously described and were lysed in 1X sample loading buffer containing 20mM DTT, phosphatase and protease inhibitors. Cell lysates were sonicated for 30 seconds to shear the DNA and subsequently boiled at 99°C for 5 min to denature the proteins. Cell lysates were stored at – 80°C until the day of the experiment and boiled once more before use.

2.2.7.3 SDS-PAGE

Resolving and stacking polyacrylamide gels were prepared as per the recipe (Table 2.19 and Table 2.20). The proteins in the cell lysates were resolved, based on their molecular weight, through the gel in 1X running buffer (Table 2.21) at 80 V.

Component	Volume (mL)
H ₂ O	5.9
30% acrylamide mix	5
1.5M Tris (pH 8.8)	3.8
10% SDS	0.15
10% Ammonium persulfate	0.15
TEMED	0.006

Table 2.20 List of List of components for 15mL 10% resolving gel for SDS-PAGE

Component	Volume (mL)
H ₂ O	3.4
30% acrylamide mix	0.83
1M Tris (pH 6.8)	0.63
10% SDS	0.05
10% Ammonium persulfate	0.05
TEMED	0.005

Table 2.21 List of List of components for 5mL 5% stacking gel for SDS-PAGE

Component	Weight (g)
Tris	30.3
Glycine	144
SDS	10

Table 2.22 List of List of components to make 1L of 10X SDA-PAGE running buffer

2.2.7.4 Protein transfer

A wet transfer was used to electrotransfer the resolved proteins onto a PVDF membrane, activated in methanol. A gel-membrane sandwich was assembled, with each component soaked in 1X transfer buffer (Table 2.22) + 10% methanol and placed into the transfer cassette. The cassettes were loaded into the transfer apparatus and submerged in 1X transfer buffer +10% methanol. An ice block was

positioned in the unit to prevent over-heating. Proteins were transferred onto the membrane over 2 hr at 45 V.

Component	Weight (g)
Tris	30.28
Glycine	150.14

Table 2.23 List of List of components to make 1L of 10X SDA-PAGE transfer buffer

2.2.7.5 Membrane blocking, antibody incubation and imaging

To prevent non-specific antibody binding, membranes were incubated with blocking buffer (3% BSA in Tris buffer) for 1 hr at RT with moderate agitation. Membranes were washed X3 with 1X TBST (hereafter implied) for 5 min at RT. Membranes were incubated with primary antibodies diluted in primary antibody dilution buffer overnight at 4°C with gentle agitation. Membranes were washed and incubated with HRP-conjugated secondary antibodies diluted in secondary antibody dilution buffer for 1hr at RT. Membranes were washed and incubated with ECL substrate. Images were developed on a Biorad Gel Doc imaging system, based on the luminescence produced by HRP-ECL chemical reaction.

2.2.8 Mouse in-vivo work

As mentioned previously mice were kept according to HPRA ethical standards. Mice were fed in-house regular diet (RD) or WGP supplemental diet (WD) which was enriched with WGP 0.05% W/W. Mice were fed diets for either 1 or 4 weeks. In some experiments (mentioned in figure legends) mice were returned to regular diets for 1 week in order to test for trained responses in-vivo.

In collaboration with The Lavelle Lab, mice were anaesthetised using 3% isoflurane with 1.5% O₂ for 5 minutes prior to immunisation. Formulations were made up to administer 1mg/Kg pIC intranasally per mouse, not exceeding 25µL. Mice were placed in an inclined recovery position when returned to their cage and monitored for 30 minutes.

After 6hrs, mice were culled as previously described and bronchoalveolar lavage (BAL) fluid was extracted. To do this the trachea was first exposed, and two silk suture threads were placed around the trachea. A one tie suture tight at the top of trachea near the mouth was used to pull make the mouth to fully expose the trachea and the other was tied loosely nearer the lung. Using a 22G needle, the catheter was gently inserted into the lung and the second silk suture thread was tied to hold

the catheter in place. 700 μ L of ice-cold PBS (W/O calcium and magnesium and containing 0.5M EDTA) was injected into the lung. After 3 seconds this was removed and placed into falcon containing protease and phosphatase inhibitors. The volume removed from each lung was noted in order to normalise cytokine to volumes after ELISA analysis.

2.2.9 RNA sequencing

2.2.9.1 Data processing and analysis

RNA sequencing data and gene expression data was analysed out of house by Novogene and both analysed and raw data was provided to us. In some cases RAW data was reanalysed in-house using R studio 4.2.2 processing software. DeSeq2 analysis was used to normalise and examine differential expressed genes with a false discovery rate (FDR) cut off at a P-value <0.05.

2.2.10 Statistical analysis

GraphPad Prism 9.3.0 was used for statistical analysis. For all experiments, a 95% confidence interval was used and a p-value ≤ 0.05 was considered statistically significant. A D'Agostino-Pearson omnibus normality test was first performed to test if the data was normally distributed (Gaussian distribution). If the n number was too small to perform a D'Agostino-Pearson omnibus normality test, Shapiro-Wilk test was performed. If data was normally distributed, parametric testing was performed. If data was not normally distributed, non-parametric testing was performed. When comparing 2 groups, an unpaired or paired two-tailed student's t-test was used when appropriate. When comparing more than 2 groups, an ordinary one-way ANOVA with Dunnett or Šidák test was used. When comparing data with two variables, a two-way ANOVA with Šidák test was used. For correlation analysis, simple linear regression was used. The number of Biological or experimental replicates used for statistical analysis is indicated in each legend. In all figures, N.S = Not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

2.2.11 Gating Strategies

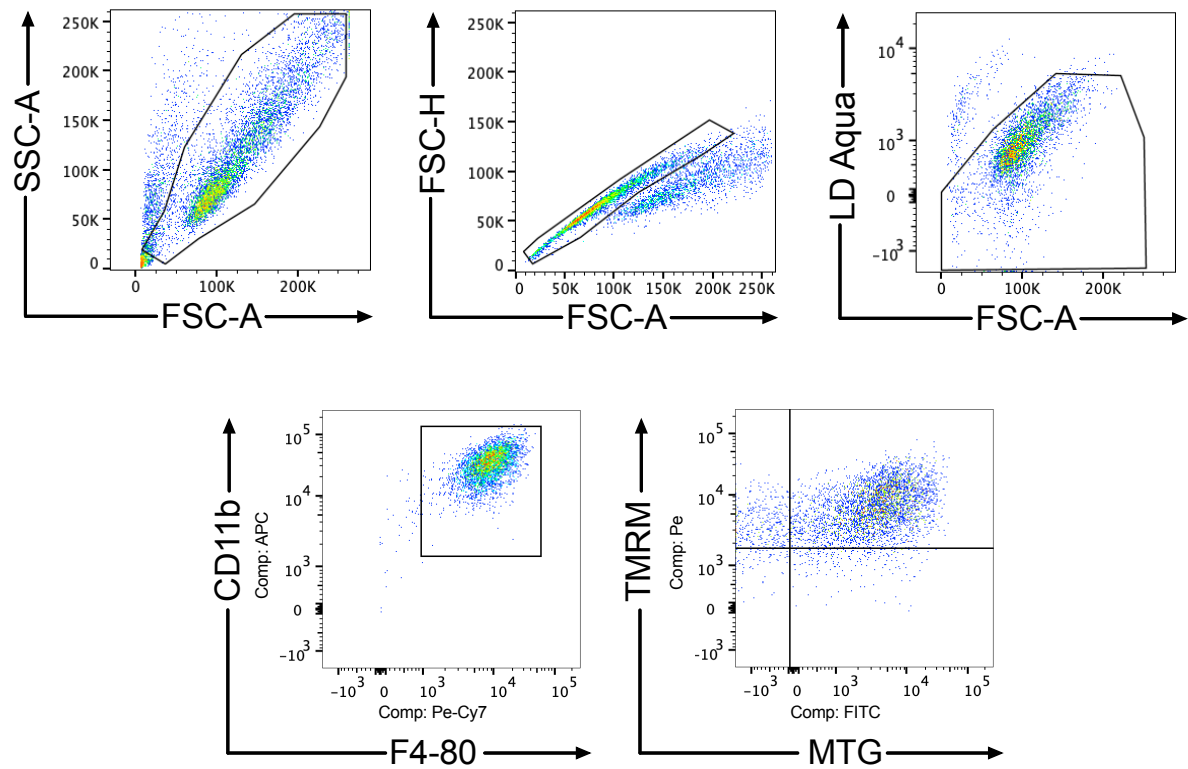


Figure 2.2 Gating strategy to identify changes in mitochondrial polarisation in BMDMs

BMDMs were cultured as previously described and were stained for flow cytometry as described in 2.2.4.1 and 2.2.4.6 to 2.2.4.7. Single leukocytes were selected based on forward and side scatter. Dead cells were excluded based on negative staining of Zombie Aqua Live/Dead stain. BMDMs were gated on cells that expressed both CD11b and F4-80. Cells were then gated on based on their uptake of MTG and TMRM staining.

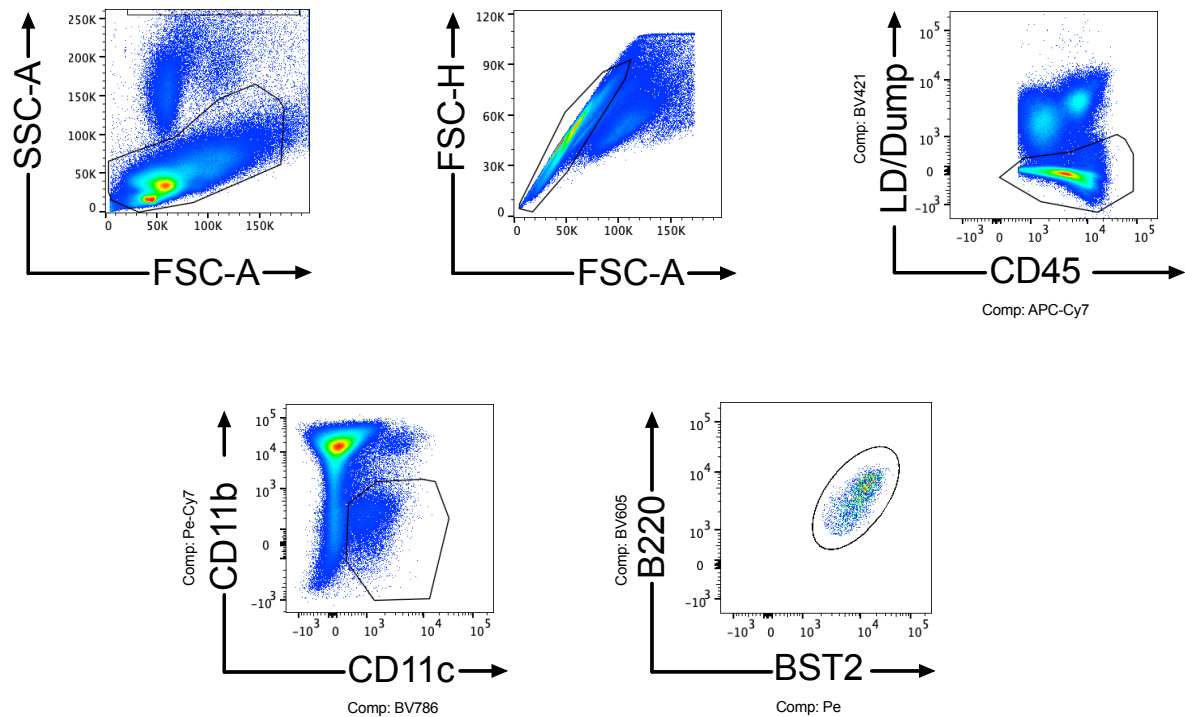


Figure 2.3 Gating strategy for isolation of pDCs in the bone marrow

Bone-marrow cells were harvested as previously described and were stained for flow cytometry as described in 2.2.4.2 and 2.2.4.6 to 2.2.4.7. The mature cell fraction was taken after isolation and stained as followed. Single leukocytes were selected based on forward and side scatter. Dead cells, as well as those positive for CD3, NK1.1 and CD19 were excluded, based on staining of Zombie Aqua Live/Dead stain and LIN-. Only cells that were dump – and CD45+ were selected. Dendritic cells were gated as CD11b-CD11c+. Finally, pDCs were gated as being B220+BST2+.

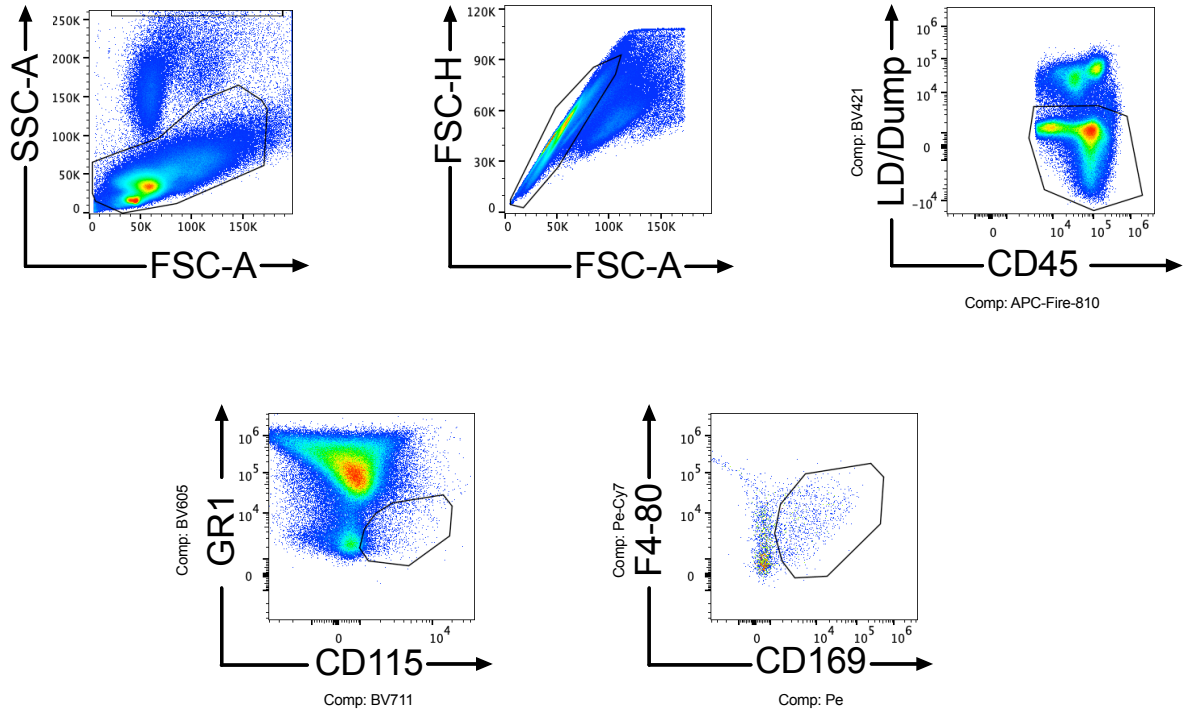


Figure 2.4 Gating strategy for isolation of CD169+ macrophages in the bone marrow

Bone-marrow cells were harvested as previously described and were stained for flow cytometry as described in 2.2.4.2 and 2.2.4.6 to 2.2.4.7. The mature cell fraction was taken after isolation and stained as followed. Single leukocytes were selected based on forward and side scatter. Dead cells, as well as those positive for CD3, NK1.1 and CD19 were excluded based on staining of Zombie Aqua Live/Dead stain and LIN-. Only cells that were dump – and CD45+ were selected. Macrophages were gated as GR1-CD115^{Inter}. Finally, CD169+ macrophages were gated as being F4-80+CD169+.

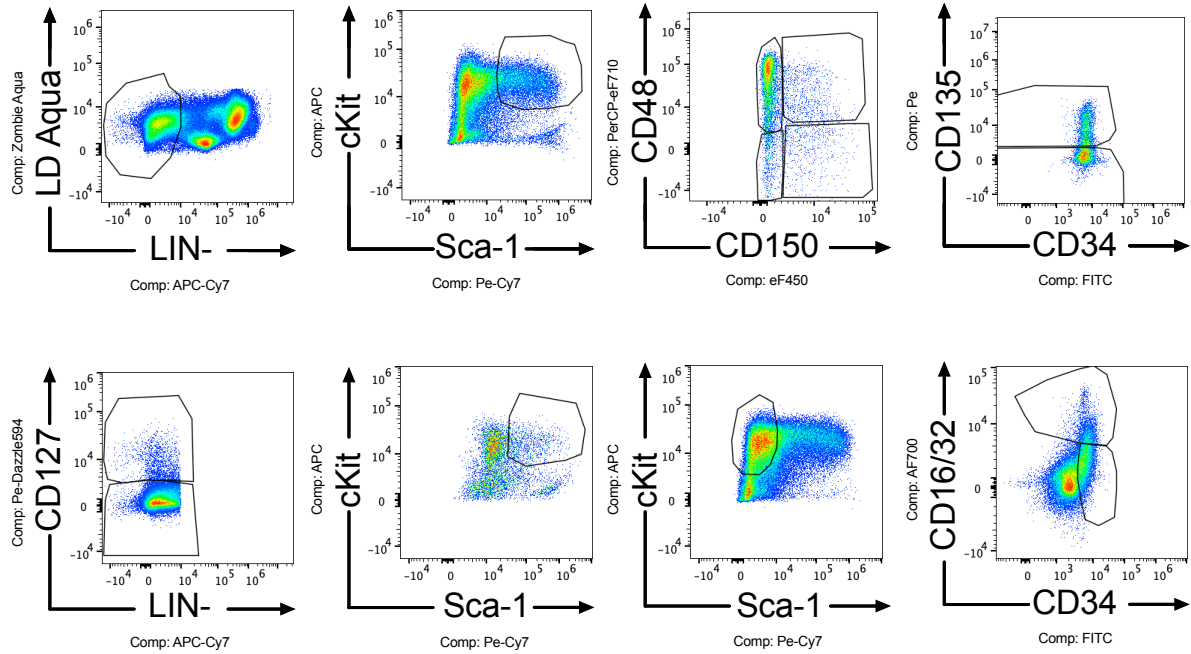


Figure 2.5 Gating strategy for isolation of bone marrow stem and progenitor cells

Bone-marrow cells were harvested as previously described and were stained for flow cytometry as described in 2.2.4.2 and 2.2.4.6 to 2.2.4.7. The cKIT enriched fraction was taken after isolation and stained as followed. Single leukocytes were selected based on forward and side scatter. Dead cells, as well as those positive for LIN+ cocktail were excluded based on staining of Zombie Aqua Live/Dead stain and LIN+. Only cells that were LIN- were selected. Total LKS+ cells were gated as positive for cKIT and Sca-1. Within the LKS+ fraction, MPPs were CD48+CD150-, LT-HSCs were CD48+CD150+, ST-HSCs were CD48-CD150+. Within the MPP population MPP3 were described as CD135+CD34+ and MPP4 were CD135-CD34+. LIN- cells were further analysed based on their expression of CD127. CLPs were classified as CD127+cKIT+Sca-1+. CMPs were classified as CD127-cKIT+Sca-1-CD16/32-CD34+ where as GMPs were CD127-cKIT+Sca-1-CD16/32+CD34+.

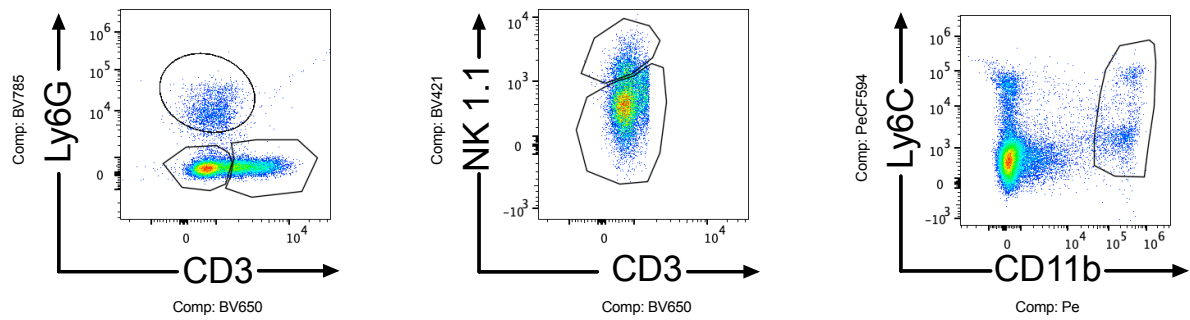


Figure 2.6 Gating strategy for isolation of circulating immune cell populations

Circulating blood cells were harvested as previously described and were stained for flow cytometry as described in 2.2.4.3 and 2.2.4.6 to 2.2.4.7. Single live cells were gated as previously described. Circulating T cells were gated out due to their expression of CD3. Circulating neutrophils were gated out based on their expression of Ly6G. Cells that were CD3-Ly6G⁻ were further gated based on their expression of NK1.1. NK cells were NK1.1⁺. Cells negative for NK1.1 were further gated based on their expression of Ly6C and CD11b. Monocytes were Ly6C⁺CD11b⁺. Ly6C^{High} monocytes are the murine equivalent of classical monocytes and Ly6C^{Low} monocytes are the murine equivalent of non-classical monocytes.

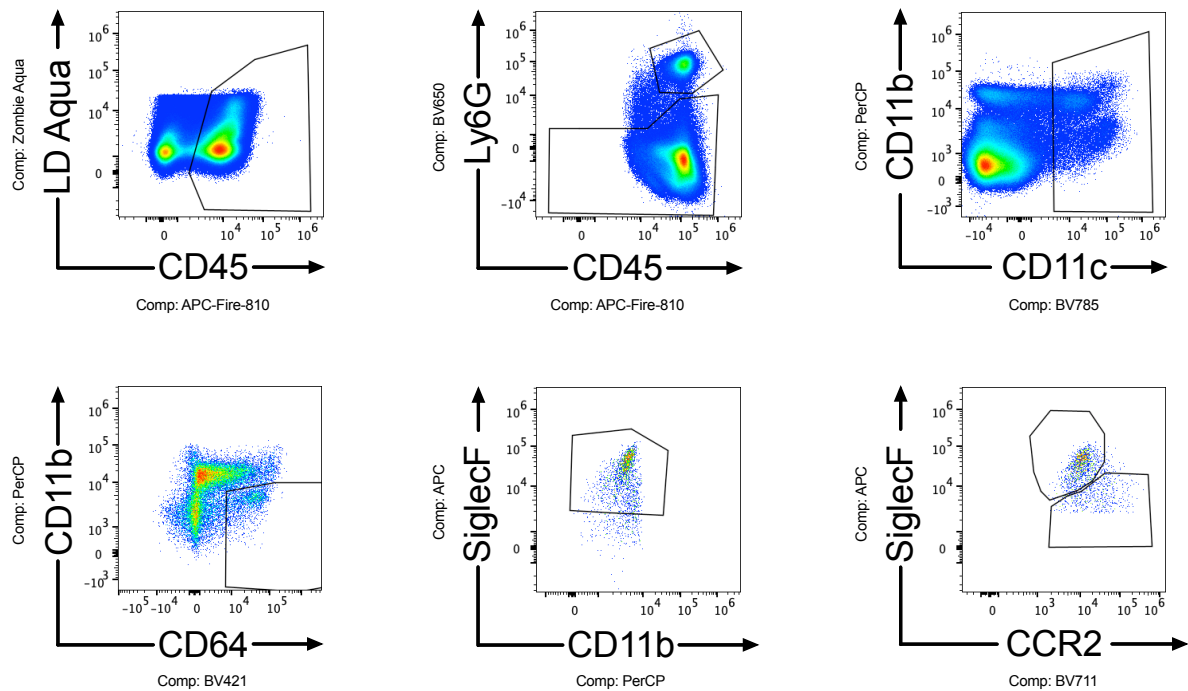


Figure 2.7 Gating strategy for isolation of lung alveolar macrophages

Lung cells were harvested as previously described and were stained for flow cytometry as described in 2.2.4.4 and 2.2.4.6 to 2.2.4.7. Single cells were gated as previously described. Live CD45⁺ cells were gated on to first get the live immune cell populations in the lung. Neutrophils were gated out based on their expression of Ly6G. Macrophage and dendritic cell populations were gated based on their expression of CD11b and CD11c. Alveolar macrophages were gated as CD11b^{Low/-} CD11c⁺ CD64⁺ SiglecF⁺. Within the alveolar macrophage population Foetal-yolk sac monocyte derived alveolar macrophages (FeMoAMs) which are tissue resident cells were gated based on being SiglecF^{High} CCR2^{Low/-}, whereas bone marrow monocyte derived alveolar macrophages (BMoAMs) were gated based on being SiglecF^{Low} CCR2⁺.

Chapter 3. WGP β -glucan drives classical trained immunity via immunometabolic reprogramming

3. 1 Introduction

Trained immunity has been shown to be induced by a wide range of ligands, including both endogenous molecules like oxidized low-density lipoproteins (OxLDLs) and exogenous/foreign/non-self-molecules, including *Bacillus calmette-guérin* (BCG) vaccine and β -glucans^{100, 137, 194}. In this chapter we focused on trained immunity induced by the yeast derived β -glucan, whole glucan particle (WGP). β -glucans have been shown to activate the C-type lectin receptor (CLR) Dectin-1 through its recognition of the β -1,3/ β -1,6 glycosidic bonds⁹⁸. Particulate β -glucans have been shown to activate NF κ B through a SYK-CARD9-BCL10 dependent manner in macrophages to produce pro-inflammatory cytokine responses, whereas soluble β -glucans do not¹⁹⁵.

In the context of trained immunity, the recognition of fungal β -glucan by Dectin-1 has been shown to drive immunometabolic reprogramming downstream of Dectin-1 activation, with a switch from oxidative metabolism to aerobic glycolysis via a mTOR-HIF1 α dependent pathway¹⁹⁶. Importantly, this early work into β -glucan induced trained immunity was induced by a β -glucan derived from the opportunistic fungus *Ca*. More recently, it has been shown that differing concentration of this β -glucan can differentially affect the metabolic phenotype, with higher concentrations driving classical aerobic glycolysis and lower concentrations driving sustained oxidative phosphorylation as well as glycolysis in monocytes¹²⁰.

Our lab is interested in studying whether food grade β -glucans, such as *Saccharomyces cerevisiae* derived Baker's Yeast, can drive a trained phenotype^{99, 197}. The ability of non-pathogenic and food grade β -glucans to drive trained immunity highlights an attractive area to design food supplements to be used in the clinic to drive trained immunity in a controlled manner¹⁹⁸. Since the *S. cerevisiae*-derived WGP is a well described Dectin-1 activator¹⁹⁹ I therefore hypothesized that this β -glucan would drive similar training responses to *Ca* β -glucans. In addition, I also hypothesized that this training effect would be mediated by epigenetic and immunometabolic pathways that occur downstream of Dectin-1 activation.

3.2 Results

3.2.1 β -glucans activate the C-type lectin receptor Dectin-1 depending on their level of solubility

β -glucans signal through the CLR, Dectin-1, which occurs in two isoforms that differ in their β -glucan binding capacity, allowing us to test different levels of solubility in β -glucans (**Figure 3.01**). I therefore first compared the ability of our different β -glucans to activate the Dectin-1 receptor using hDectin1-HEK293 NF κ B-linked SEAP reporter cells. I used the hDectin1**a**-HEK293 cells to test the ability of different concentrations of soluble β -glucans to activate the receptor, as well as hDectin1**b**-HEK293 cells to test non-soluble β -glucans (**Figure 3.02A-B**). I first confirmed that the TLR-4 agonist LPS did not activate the reporter cells. Whole glucan particle (WGP) as well as (crude) zymosan were both capable of significantly activating both isoforms of the Dectin-1 receptor at their top concentrations respectively. However, soluble WGP (sWGP) was only able to activate the Dectin-1a isoform, confirming its soluble nature.

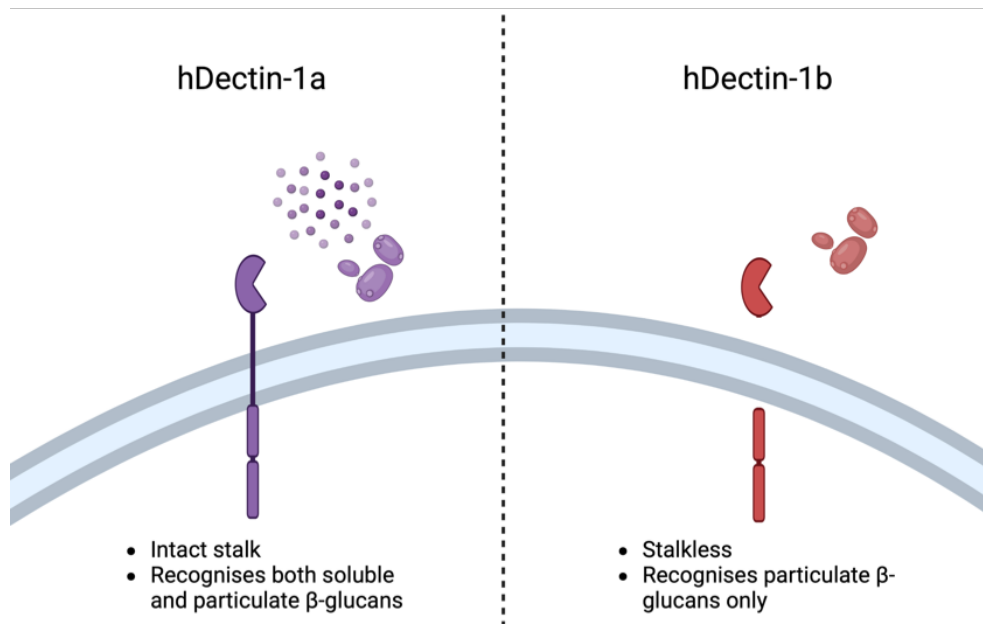


Figure 3.1 Graphical representation of both isoforms of the Dectin-1 receptor

Human dectin-1 is split into two isoforms (Dectin-1a & Dectin-1b) for the purpose of measuring different types of β -glucans. Dectin-1a has a fully intact stalk region and can recognise both soluble and particulate β -glucans. Dectin-1b lacks the stalk region and can only recognise particulate β -glucans.

3.2.2 β -glucans drive a wide range of innate immune memory phenotypes

Since I saw that different types of β -glucans can induce different levels of Dectin-1 activation I decided to test their ability to induce innate immune memory. To do this I treated BMDMs with various concentrations of β -glucans or LPS as a tolerance control, according to the labs common training assay (**Figure 3.03**). Upon restimulation with LPS, I measured increased production of TNF in a manner dependent on the concentration of WGP used to train the cells (**Figure 3.04**). I saw a significant increase relative to the untrained control with the top dose of WGP. However, sWGP was not capable of inducing training, potentially due to its poor ability to bind Dectin-1 in-vitro, as shown by my previous results. In contrast, Zymosan and LPS both induced innate immune tolerance with significantly reduced production of TNF in response to LPS rechallenge.

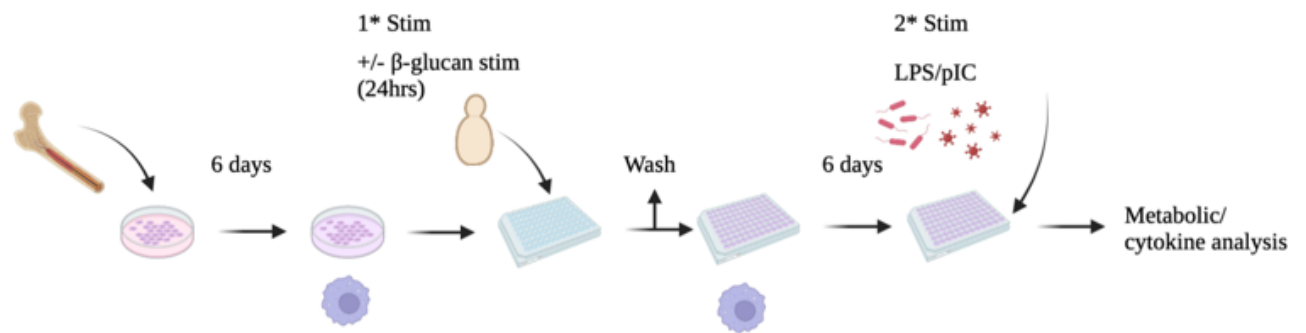


Figure 3.3 Graphical representation of inducing trained immunity in-vitro

Bone marrow derived macrophages (BMDMs) are plated and cultured with or without the training stimulus for a period of 24hrs. Following this period, cells are washed with warm PBS and media is replenished containing 7% L929 conditioned media (LCM). On day 3 post training media is removed and replenished with fresh media containing 7%LCM. On day 6 post training cells are restimulated with various challenges.

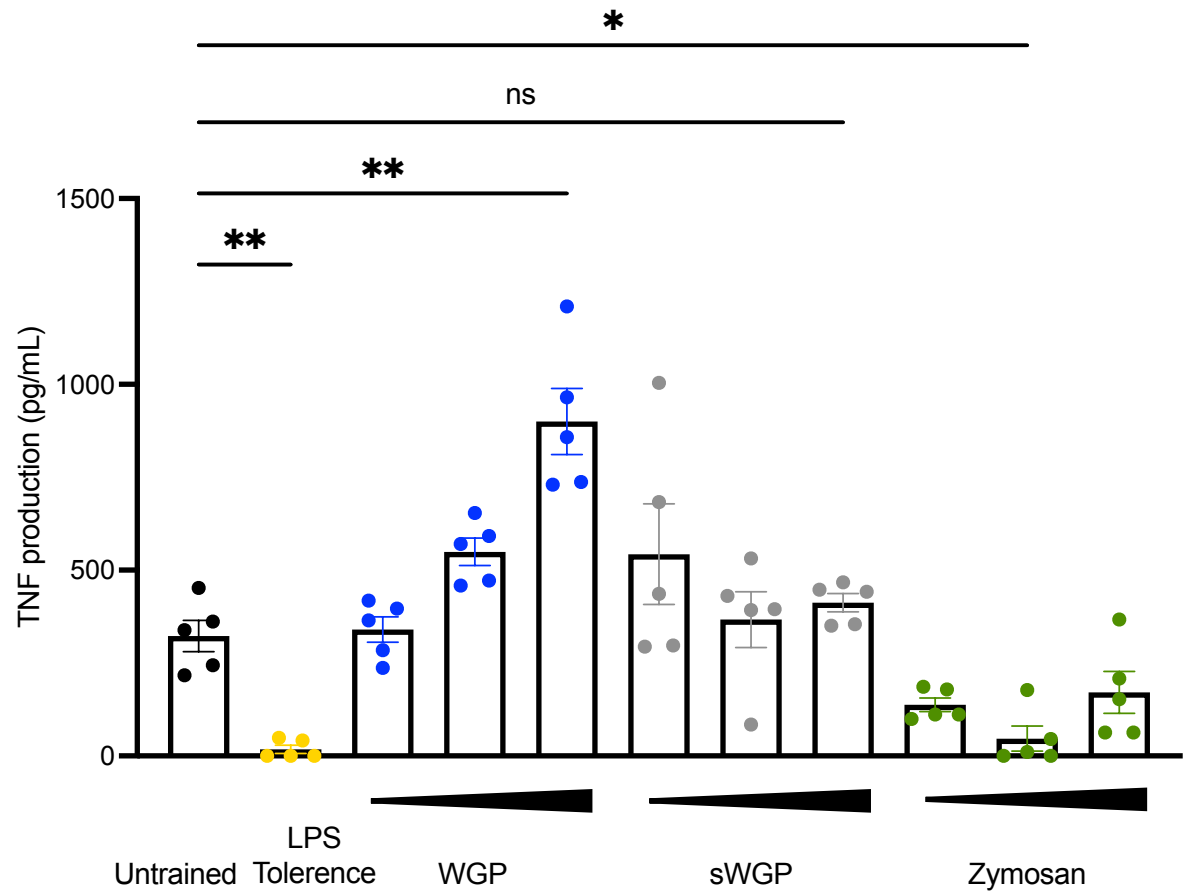


Figure 3.4 β -glucans induce various innate immune memory phenotypes post in-vitro culture

BMDMs were treated for 24hrs with various concentrations of β -glucans or LPS as a tolerance control (1 μ g/mL, 10 μ g/mL & 100 μ g/mL or 100ng/mL respectively). Cells were then allowed to rest for a period of 6 days before being challenged with LPS (10ng/mL) for a period of 4hrs. Supernatant was removed from cells and TNF was measured via ELISA. Each dot is a representative of an individual experiment (Biological N = 5). *P< 0.05; **P<0.01; ns = not significant (RM one-way ANOVA with Dunnett's multiple comparison test).

3.2.3 β -glucan preparation affects the innate immune memory phenotype that BMDMs will display during rechallenge

WGP and Zymosan are both β -glucans derived from *S. cerevisiae* but differ in their abilities to drive training or tolerance, respectively as observed in **Fig 3.04**. Therefore, I decided to investigate this further. Upon stimulation of BMDM with the various β -glucans, Zymosan could induce a significant increase in the pro-inflammatory cytokines TNF and IL-6, similar to that of LPS stimulation (**Figure 3.05A-B**). However, neither WGP nor sWGP induced significant levels of either TNF or IL-6 production. This was further confirmed using NF κ B reporting RAW-Blue macrophages, which have endogenous expression of most PRR receptors expressed by primary murine macrophages. Here, I saw that again Zymosan and LPS treatment both induced similar NF κ B-SEAP linked activity (**Figure 3.06**). However, again neither WGP nor sWGP could significantly induce this activity.

Since I saw these difference, I decided to investigate whether WGP or Zymosan were driving activation of other PRRs other than Dectin-1. It has been recently shown that activation of other TLRs can have an impact on the training effects of β -glucans, especially TLR-2 and TLR-4^{99, 200}. Therefore, I decided to test whether Zymosan or WGP had any ligands that might activate these receptors. To do this I stimulated hTLR-2 and hTLR-4-HEK293 NF κ B-linked SEAP reporter cells with the β -glucan panel, as well as the TLR-2 agonist PAM₍₃₎CSK₍₄₎ (PAM) and the TLR-4 agonist LPS as respective controls (**Figure 3.07A-B**). PAM was able to induce a significant increase in TLR-2 activation. Interestingly, Zymosan was also able to induce a dose dependent significant increase of TLR-2 activation. However, neither WGP nor sWGP induced any TLR-2 activation. Similar to these results, when I stimulated hTLR-4 reporters I saw a significant increase of TLR-4 activation post LPS stimulation, as well as a dose response in activation with Zymosan. However, again neither WGP nor sWGP induced TLR-4 activation.

To further quantify the observed results, I correlated a wide panel of β -glucans ability to drive NF κ B activation in RAW-Blue macrophages with their ability to drive either training or tolerance (**Figure 3.08**). Consistent with my previous results I saw a

direct correlation between NF κ B activation and the ability of β -glucans to drive tolerance. In contrast there was no correlation between NF κ B activation and trained immunity, highlighting the importance of regulated transient NF κ B response in cells to drive a long-term trained phenotype.

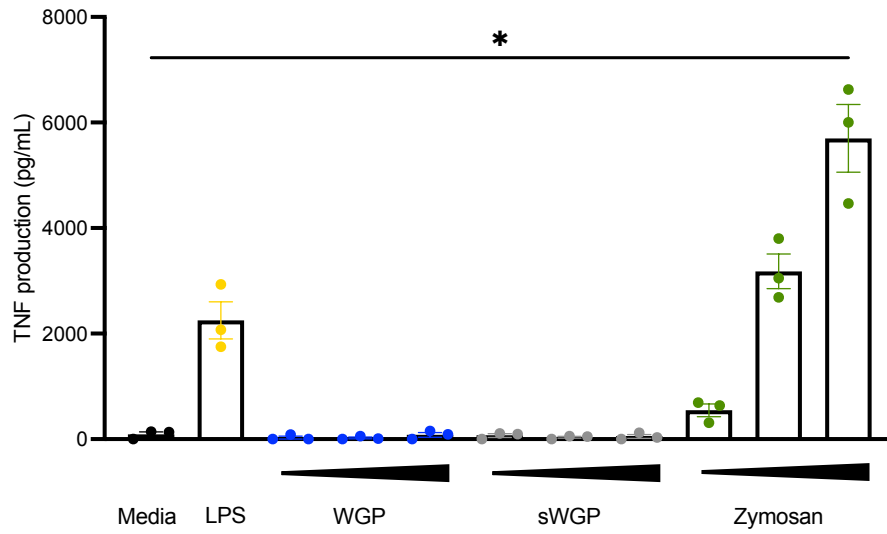
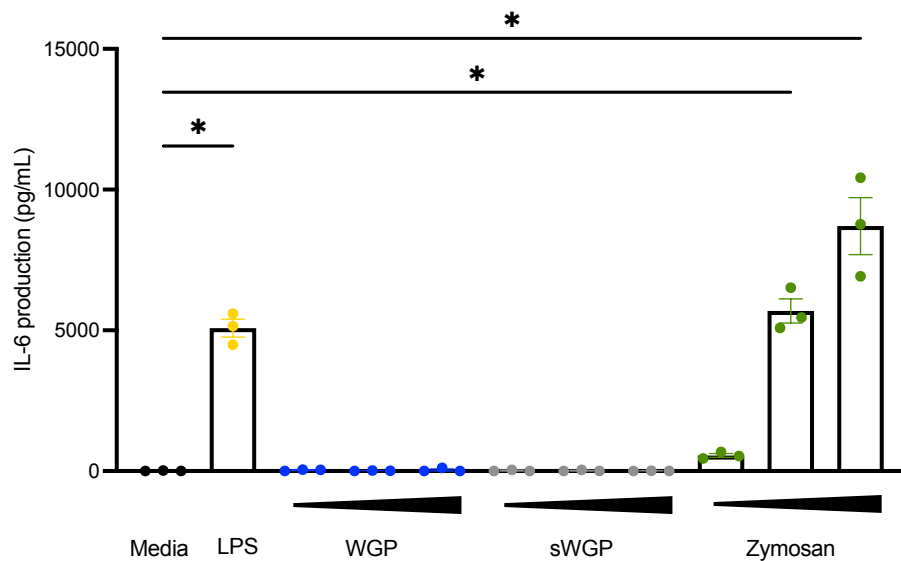
A**B**

Figure 3.5 Disparate β -glucans preparations induce different (acute) cytokine responses

BMDMs were treated with various concentrations of β -glucans or LPS as a positive control (1 μ g/mL, 10 μ g/mL & 100 μ g/mL or 100ng/mL respectively) for a period of 24hrs. Supernatant was removed from cells and TNF (A) and IL-6 (B) was measured via ELISA. Each dot is a representative of an individual experiment (Biological N = 3). *P< 0.05; (RM one-way ANOVA with Dunnett's multiple comparison test).

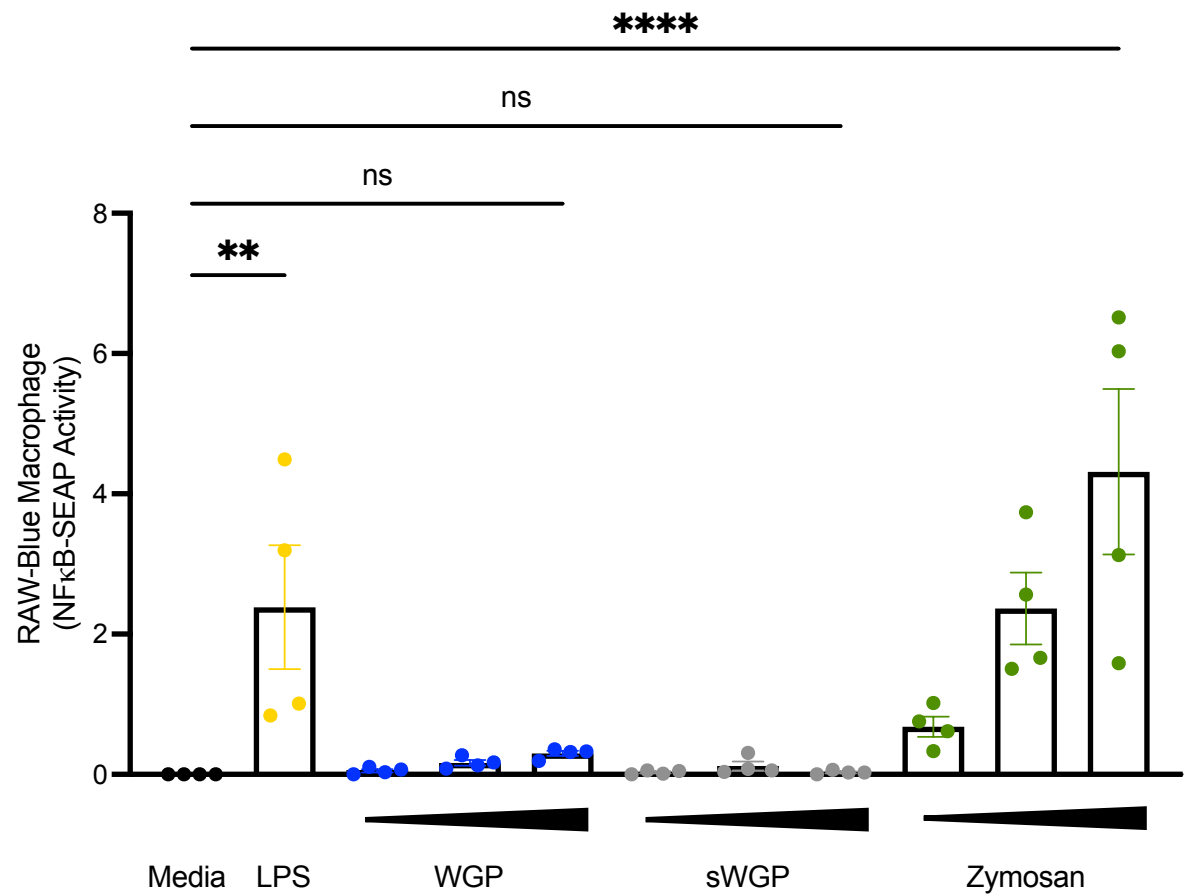


Figure 3.6 β -glucans can robustly activate macrophages depending on their preparation

RAW-Blue macrophages were treated with various concentrations of β -glucans or LPS as a positive control for a period of 24hrs. Supernatant was removed from cells and NF κ B-SEAP linked activity was measured. Each dot is a representative of an individual experiment (Biological N = 4). **P<0.01, ****P<0.0001; ns = not significant (Ordinary one-way ANOVA with Dunnett's multiple comparison test).

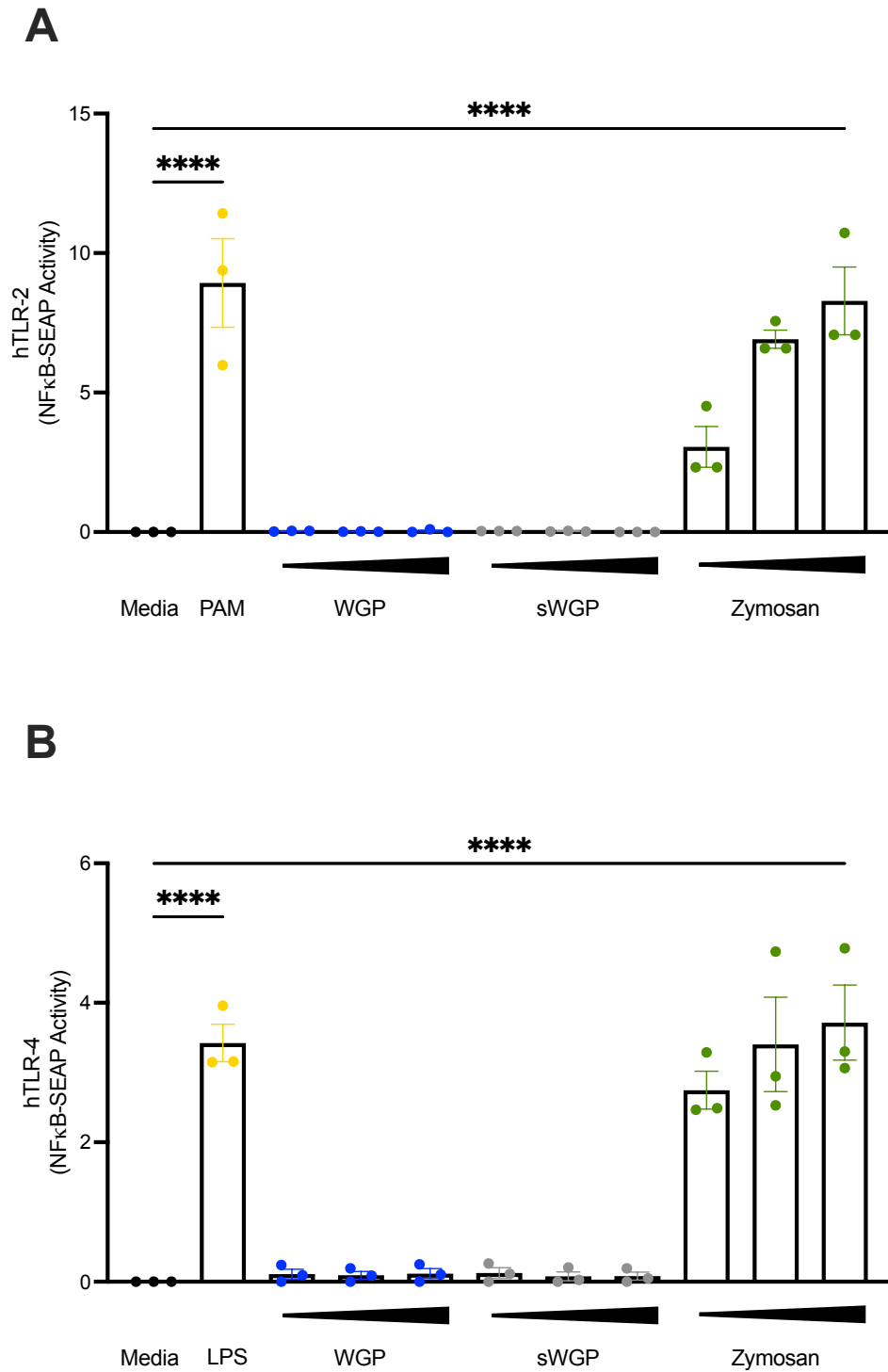


Figure 3.7 Zymosan β -glucan can signal through TLR-2 and TLR-4

HEK-293 overexpressing TLR-2 (A) or TLR-4 (B) were stimulated with a range of β -glucans, sWGP, WGP & zymosan, as well as LPS and media controls (-) for 24hrs with increasing concentrations (1 μ g/mL, 10 μ g/mL & 100 μ g/mL). Following 24hrs, relative NF κ B-linked SEAP activity was measured at OD-655. Receptor activation is relative to NF κ B-linked SEAP activity. Each dot is a representative of an individual experiment (A. Biological N = 3, B. Biological N = 3). ****P<0.0001; ns = not significant (RM one-way ANOVA with Dunnett's multiple comparison test).

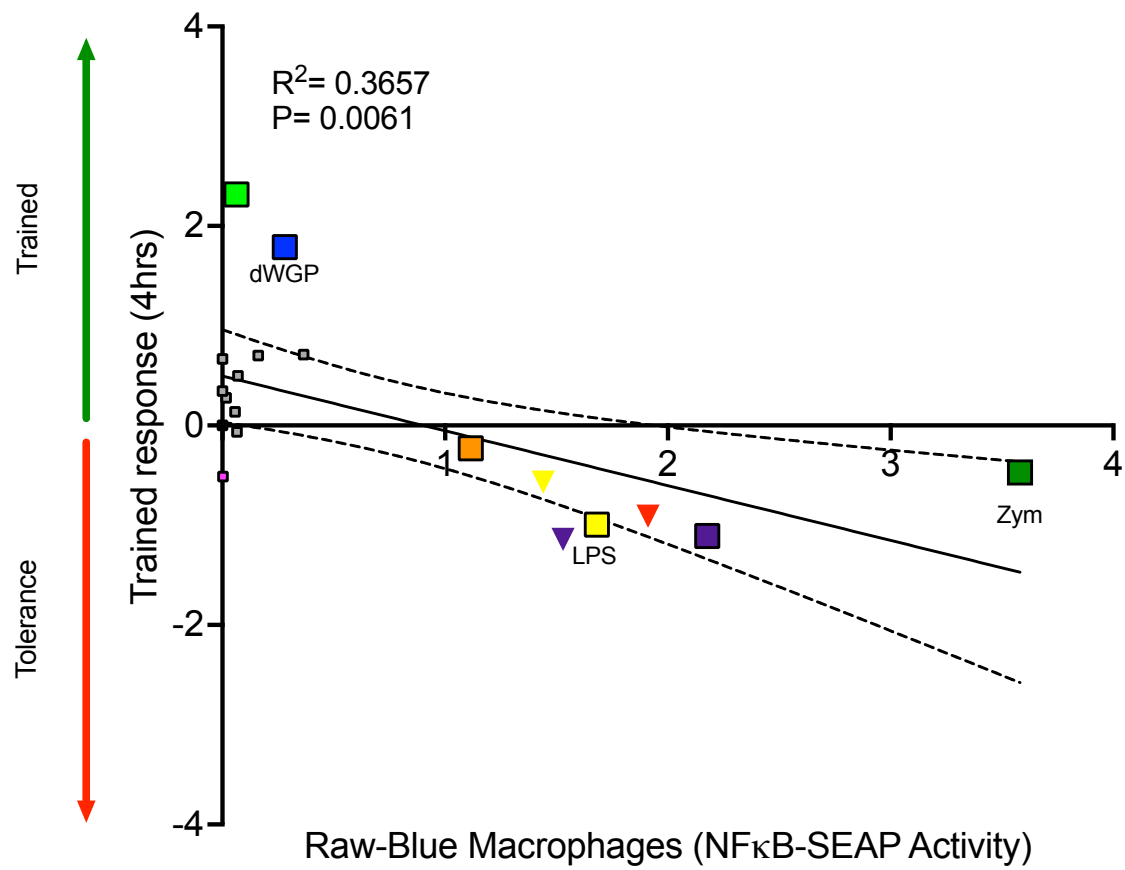


Figure 3.8 Correlation between macrophage activation during primary stimulation and innate immune memory phenotype

Correlation plot between different phenotypes of innate immune memory and NF κ B activation during the initial response. Briefly, RAW-Blue macrophages and BMDMs were stimulated for 24hrs with various β -glucans (100 μ g/mL WGP, 100 μ g/mL zymosan, 100 μ g/mL curdlan [Purple square], 10 μ g/mL curdlan [purple triangle], 100 μ g/mL pustulan [red square] or 10 μ g/mL pustulan [red triangle]) or 10 ng/ml LPS (yellow triangle) or 100ng/mL LPS (yellow square). NF κ B activation was measured in the RAW-Blues by SEAP activity assay and BMDMs were allowed to rest for a period of 6 days before a 4hr restimulation of LPS. Exact P-value shown.

3.2.4 WGP induces rapid, broad, non-specific trained immunity in murine BMDMs

Since WGP induced the most significant training out of the different β -glucans at the top concentration of 100 μ g/mL I decided to use this for the rest of my thesis. Since trained immunity is described as an innate immune response with enhanced kinetics, I hypothesized that WGP-trained macrophages would significantly upregulate cytokine production at an enhanced rate to untrained cells with a return to untrained levels at later timepoints. To test this hypothesis untrained or trained BMDMs were stimulated with LPS for 3, 6, 12 and 24hrs. TNF production was measured via ELISA to quantify the cytokine production. WGP significantly enhanced cytokine production rapidly relative to untrained cells at early timepoints (3hrs and 6hrs), however, enhanced TNF was maintained at 12hrs and 24hrs (**Figure 3.09A**). Importantly, TNF levels decreased 24h post-LPS in both trained and untrained cells, highlighting that Trained Immune responses resolve similar to untrained responses, i.e. – are not maintained in a permanent pro-inflammatory state.

However, enhanced kinetics is not the only key feature of trained immunity. Trained cells are also said to induce protection through a broad-spectrum immune response. Therefore, I decided to test this hypothesis by again stimulating untrained or trained cells with LPS for 8hrs and quantifying a number of different inflammatory cytokines via ELISA (**Figure 3.10A-D**). As I have previously shown trained BMDMs significantly upregulate pro-inflammatory TNF in response to LPS (**Figure 3.10A**). However, I also decided to measure the pleiotropic inflammatory cytokine IL-6 after 8hrs of LPS stimulation. Here I saw that trained BMDMs also significantly upregulated IL-6 production in response to LPS (**Figure 3.10B**). However, a balanced response is key to protect against infections and maintain homeostasis. Therefore, I measured the anti-inflammatory cytokines IL-10 and IL-1Ra (**Figure 3.10C-D**). In contrast to the TNF and IL-6 results, trained BMDMs did not significantly alter their production of IL-10 in response to 8hrs LPS stimulation. However, I saw that trained cells had significantly upregulated IL-1Ra production in response to LPS. Interestingly, basal IL-1Ra production was also significantly

upregulated suggesting that trained cells maintain an anti-inflammatory phenotype basally.

Since increases in cell number may affect cytokine output, I also measured the mRNA expression of *Tnf* via qPCR (**Figure 3.11A**). *Tnf* gene expression was significantly increased in LPS stimulated trained BMDMs as compared with *Tnf* gene expression in untrained BMDMs, highlighting that cytokine increases are likely due to increase mRNA transcription. During qPCR preparation I normalised the abundance of RNA within each sample and utilise housekeeping genes to normalise any changes in gene expression, therefore, all results seen are independent of cell number changes.

Finally, since trained immunity is described as a non-specific memory phenotype displayed by myeloid cells, I decided to test different TLR agonists as re-stimuli after β -glucan training. I used three different agonists for this. The TLR-4 agonist LPS was used to replicate bacterial infections, the TLR-2/Dectin1 agonist Zymosan was used to replicate fungal infections, and the TLR1/2 agonist PAM was used to replicate both fungal and bacterial infections (**Figure 3.12A-C**).

As I have previously shown after 4hrs stimulation with LPS, WGP trained BMDMs significantly upregulated TNF production compared with the untrained control. No differences or significant increases in TNF production was seen in any of the unstimulated controls (**Figure 3.12A**). Similar to this, I also saw that WGP training significantly upregulated TNF production 4hrs post stimulation with Zymosan compared with the untrained control BMDMs (**Figure 3.12B**). Finally, I saw that WGP training also significantly upregulated TNF production in response to 4hr stimulation with PAM (**Figure 3.12C**). Taken together this data highlights that's WGP trained BMDMs show a trained phenotype with enhanced kinetics, broad cytokine responses and non-specificity.

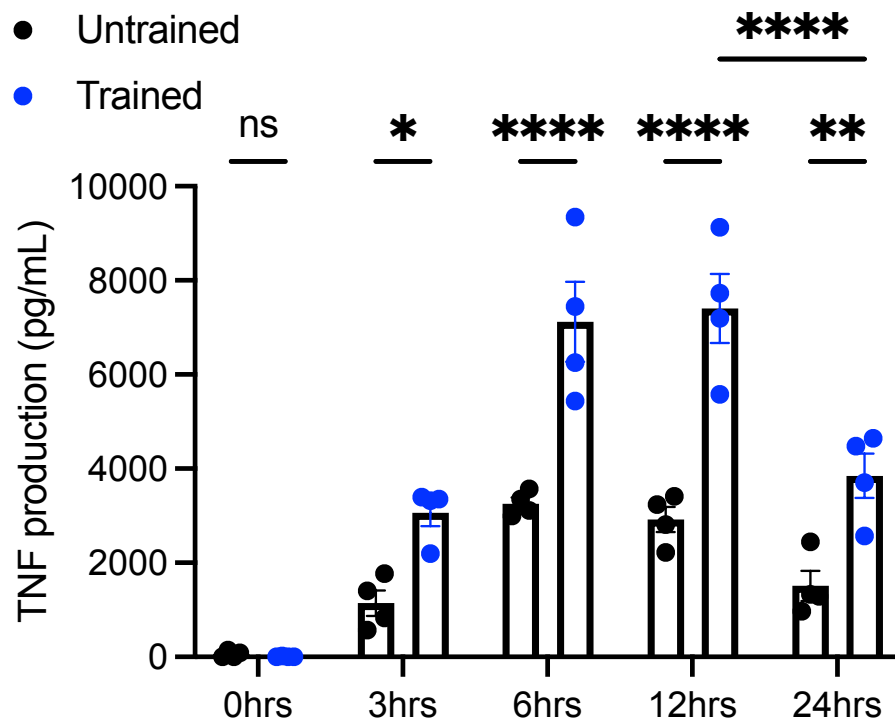


Figure 3.9 Trained response time course showing response to LPS stimulation over a 24hr period

Secondary challenge time course showing changes in TNF production over time in response to 10ng/mL LPS. BMDMs were trained with 100 μ g/mL WGP β -glucan for a period of 24hrs and left to rest for 6 days before a secondary challenge with 10ng/mL LPS. Supernatant was removed from BMDMs at indicated time points and TNF was measured via ELISA. Each dot is a representative of an individual experiment (Biological N = 4). *P< 0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns = not significant (Two-way ANOVA with Šídák's multiple comparisons test).

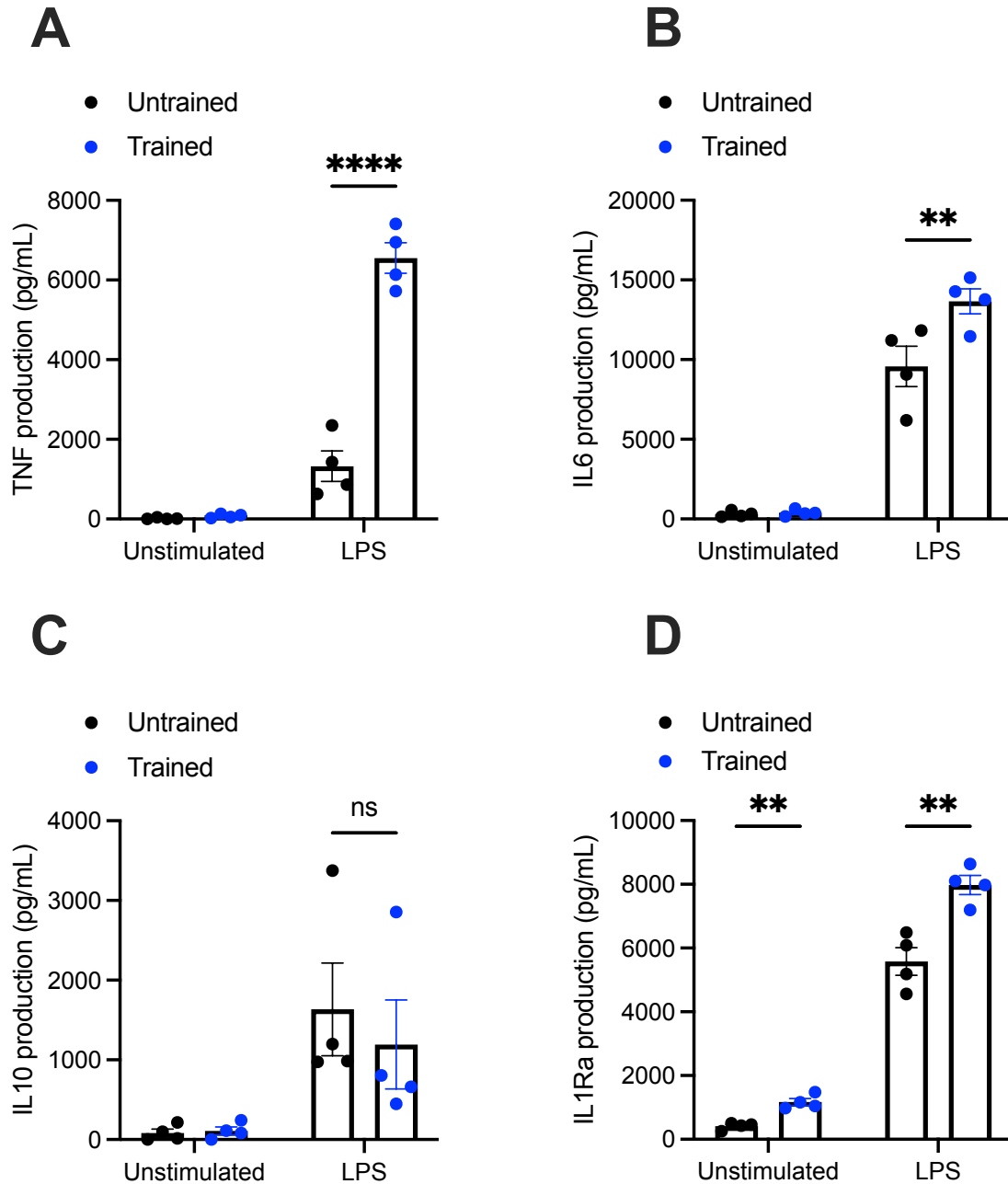


Figure 3.10 Trained response to LPS secondary challenge showing broad range cytokine production 8hrs post LPS stimulation

Different cytokine responses in untrained vs trained BMDMs in response to 10ng/mL LPS. BMDMs were treated with 100 μ g/mL WGP β -glucan for a period of 24hrs and left to rest for 6 days before a secondary challenge with 10ng/mL LPS. Supernatant was removed from BMDMs 8hrs post LPS stimulation and TNF (A), IL-6 (B), IL-10 (C) and IL-1Ra (D) were measured via ELISA. Each dot is a representative of an individual experiment (Biological N = 4). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns = not significant (Two-way ANOVA with Šídák's multiple comparisons test).

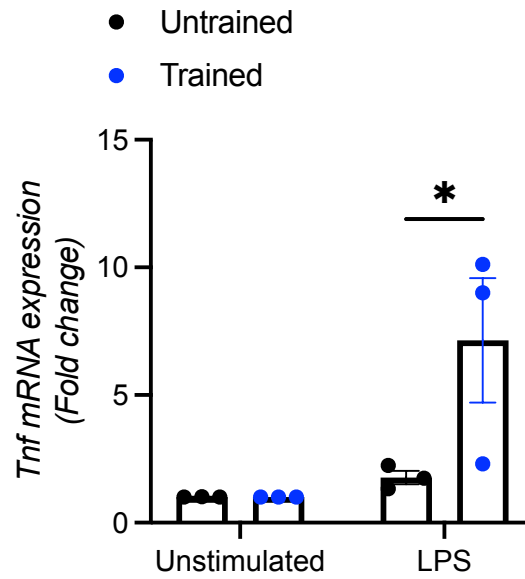


Figure 3.11 mRNA Trained response to LPS secondary challenge showing enhanced TNF mRNA expression 4hrs post LPS stimulation

mRNA expression was measured from untrained vs trained BMDMs in response to 10ng/mL LPS. BMDMs were treated with 100 μ g/mL WGP β -glucan for a period of 24hrs and left to rest for 6 days before a secondary challenge with 10ng/mL LPS. RNA was extracted from BMDMs 4hrs post LPS stimulation and TNF gene expression was measured via qPCR. Each dot is a representative of an individual experiment (Biological N = 3). *P < 0.05; ns = not significant (Two-way ANOVA with Šídák's multiple comparisons test).

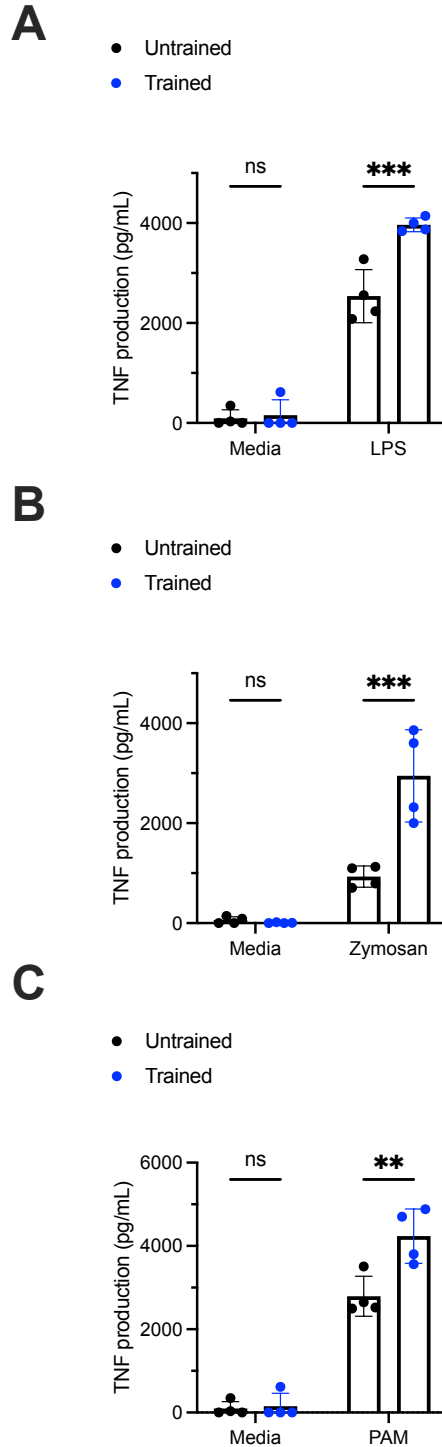


Figure 3.12 Trained response to different TLR agonist secondary challenges showing non-specificity of WGP trained immunity

TNF production was measured in untrained vs trained BMDMs in response to 10ng/mL LPS (A), 10ng/mL Zymosan (B) and 10ug/mL PAM (C). BMDMs were treated with 100μg/mL WGP β-glucan for a period of 24hrs and left to rest for 6 days before a secondary challenge. Supernatant was removed from BMDMs 8hrs post stimulation and TNF protein was measured via ELISA. Each dot is a representative of an individual experiment (Biological N = 4). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns = not significant (Two-way ANOVA with Šídák's multiple comparisons test).

3.2.5 WGP mediated trained immunity is dependent on epigenetic modifications in murine BMDMs

The hallmark enhanced responsiveness of trained immunity is dependent on epigenetic modifications that occur during the training process. Two well described epigenetic modifications include H3K27ac and H3K4me3, which have been described to enhance the expression of certain pro-inflammatory genes. It was important therefore to measure these changes. To do this I utilised the Labs standard training protocol, however, I modified this to include various inhibitor treatments, either 5'-methylthioadenosine (MTA) a well described methyltransferase inhibitor or cyproheptadine (CPH) which I have shown to inhibit both methylation as well as acetylation (**Figure 3.13**), but has been shown in the literature to target the lysine methyltransferase SET7/9²⁰¹.

To measure these changes, I performed intranuclear staining with antibodies specific for each chromatin modification, first analysing total H3K27 acetylation at two specific time points; 24hrs and 72hrs post training (**Figure 3.14A**). Interestingly, I saw that 24hrs after WGP there was no significant increase in total H3K27 acetylation of BMDMs (**Figure 3.14B**). However, I was able to show that CPH significantly reduced the levels of H3K27ac compared with both the media control and WGP stimulated BMDMs. However, in contrast to this there was a trend toward increased H3K27 acetylation 72hrs post WGP stimulation although this was non-significant due to variation observed (**Figure 3.14C**). Again, CPH treatment significantly reduced this effect when compared with WGP stimulated BMDMs. There were no significant differences between the inhibitor group and the media control.

I then measured total H3K4 tri-methylation at the same time points post training (**Figure 3.15A**). Again, I saw that 24hrs after WGP there was no significant increase in total H3K4me3 of BMDMs treated with WGP (**Figure 3.15B**). There was a slight reduction in cells pretreated with CPH, although this reduction was not significant. However, in contrast to this there was a significant increase in H3K4me3 72hrs post WGP stimulation (**Figure 3.17C**) with CPH significantly reducing the increase in H3K4me3 at this time-point. There was no significant difference between the media

treated and the WGP plus CPH treated groups showing that CPH specifically abolishes the epigenetic changes induced in WGP treated BMDMs.

Since I saw the most pronounced changes with histone methylation, I decided to specifically target these changes using the methylation inhibitor MTA during the first 24hrs of WGP training, as well as at 72hrs post WGP training when acetylation was significantly upregulated (**Figure 3.16A-B**). Having seen that there was no significant increase in methylation during the first 24hrs of WGP I did not expect to see any significant changes when BMDMs were pretreated with MTA for the first 24hrs of WGP mediated trained immunity. As expected, pretreatment of MTA at this timepoint did not significantly reduce WGP mediated trained immunity by day 6, with an increase in TNF still seen when exposed to LPS for a period of 8hrs (**Figure 3.16A**). However, when MTA was added for 24hrs at day 3 post-training, when methylation began to increase, there was a significant decrease in the training effect seen after LPS stimulation on day 6 in BMDM treated with MTA (**Figure 3.16B**). Taken together this data confirms the importance of epigenetic changes mediated by WGP stimulation to induce trained immunity and highlights that these modifications may take time to manifest, which can affect strategies to target or block these for research or treatments.

CPH has been described in the literature to target the H3K4me1 lysine methyltransferase Set7 encoded by the gene *Setd7*¹²⁰. Therefore, I decided to investigate whether this gene is upregulated by WGP and if it is, at what timepoint it is upregulated. I first investigated whether the expression of *Setd7* was upregulated 24hrs post WGP stimulation (**Figure 3.17A**). At this timepoint there was a slight increase however this did not reach statistical significance and was variable across multiple experiments. However, by 72hrs there was a significant increase in the expression of this gene (**Figure 3.17B**). This data suggests that WGP mediated epigenetic changes do not occur immediately after β -glucan treatment (24hrs) but takes until the 72hr timepoint for changes to become apparent.

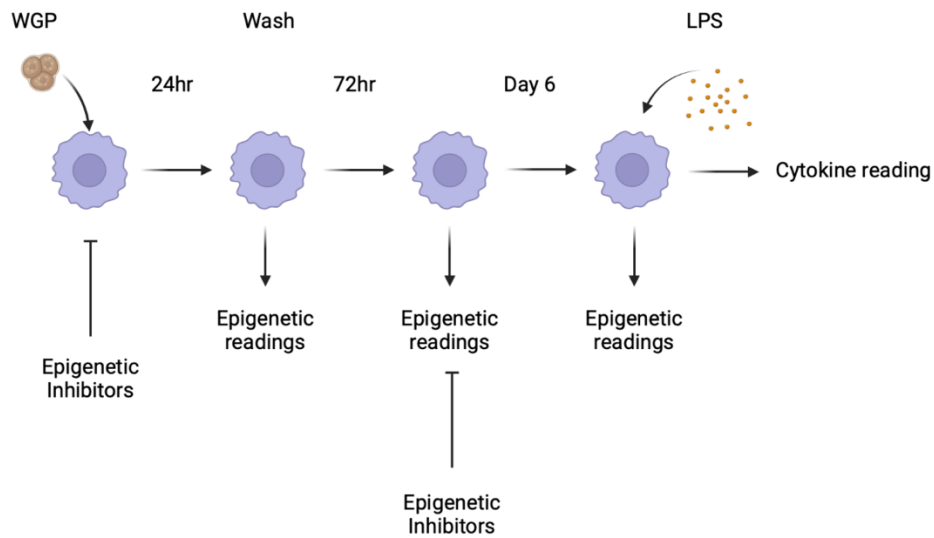


Figure 3.13 Graphical representation of protocol for defining the epigenetic profile of WGP mediated trained immunity

100 μ g/mL of WGP was used to train BMDMs for a 24hr period. Epigenetic inhibitors were added 1hr prior to WGP in some experiments and left in for the full 24hr training period. Bulk H3K27ac and H3K4me3 modifications was measured by flow cytometry 24hrs, 72hrs and 6 days post WGP training. Epigenetic inhibitors were also added to BMDMs 72hrs post training for a 24hr period and washed off. For restimulation experiments 10ng/mL LPS was added to BMDMs for a period of 8hrs and supernatant was taken for TNF measurement via ELISA.

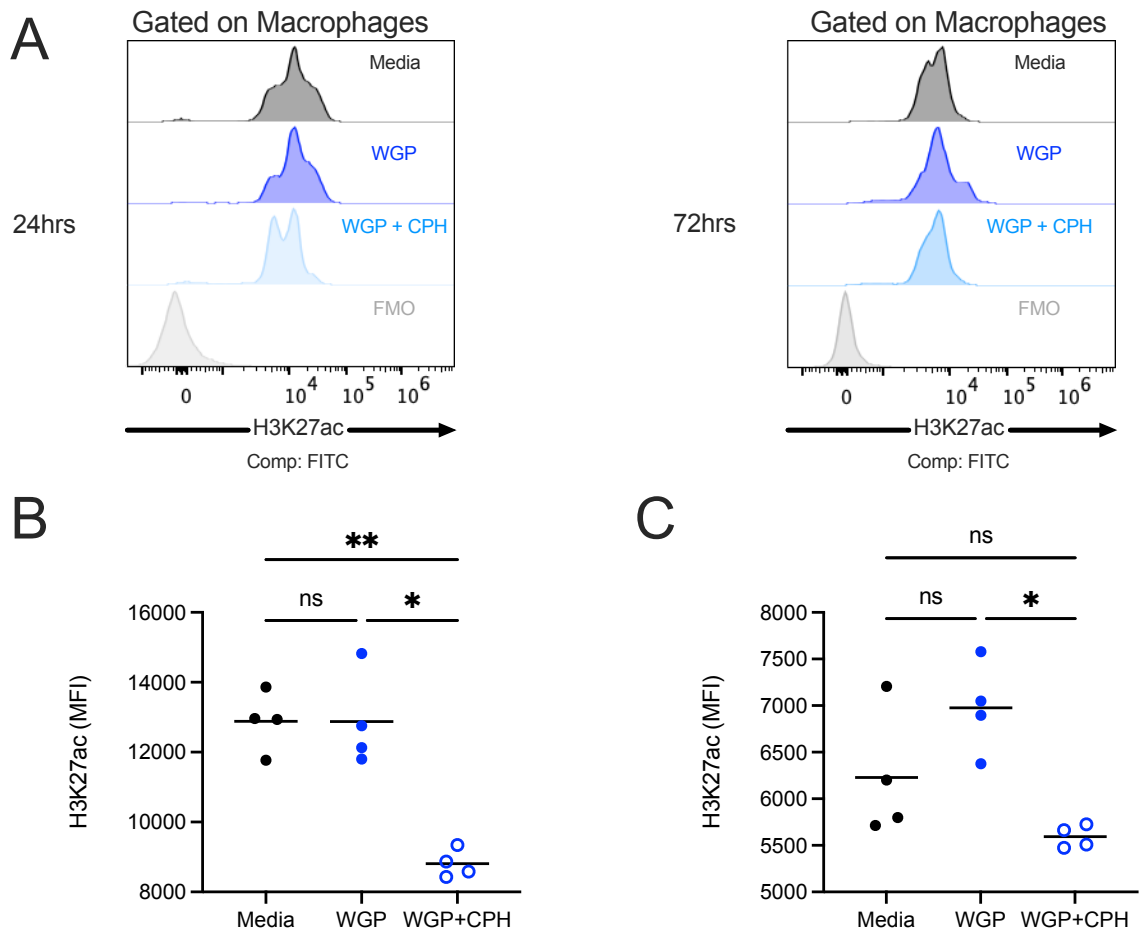


Figure 3.14 WGP enhances total H3K27ac expression 72hrs but not 24hrs post WGP stimulation

Total H3K27ac was measured using flow cytometry 24hrs and 72hrs post WGP stimulation as shown in the flow plots in (A). Cells were pretreated for 1hr with 50 μ M of cyproheptadine (CPH) or a vehicle control followed by treatment with 100 μ g/mL WGP β -glucan for a period of 24hrs (B) and 72h (C). BMDMs were scrapped on ice for FACS analysis 24hrs post stimulation (B) or washed and allowed to rest for 72hrs before being analysed via FACS (C). Expression of H3K27ac is shown as mean fluorescent intensity (MFI). (Biological N = 4). *P< 0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns = not significant. Individual replicates with mean bar shown. (RM One-way ANOVA with Šídák's multiple comparisons test).

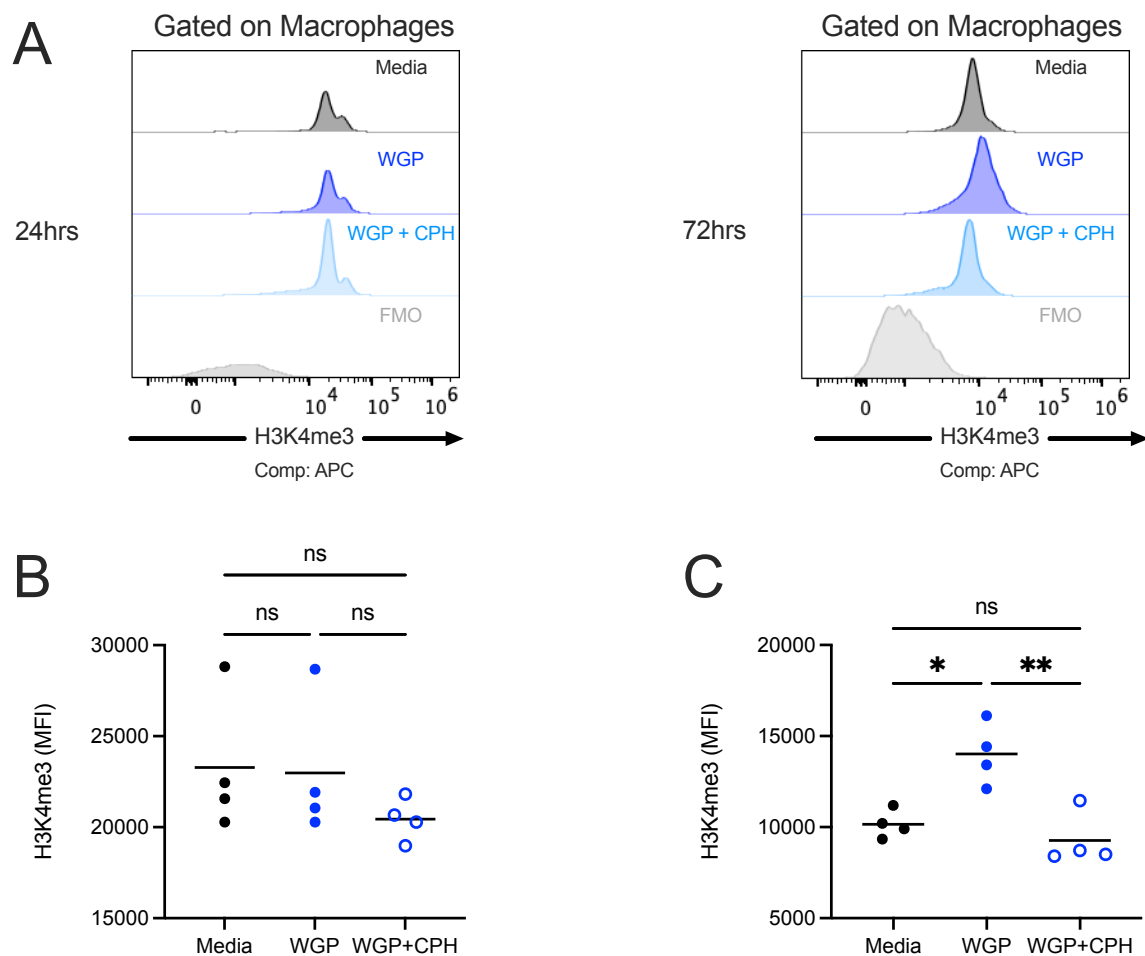


Figure 3.15 WGP enhances total H3K4me3 expression 72hrs but not 24hrs post WGP stimulation

Total H3K4me3 was measured using flow cytometry 24hrs and 72hrs post WGP stimulation as shown in (A). Cells were pretreated for 1hr with 50 μ M of cyproheptadine (CPH) or a vehicle control followed by treatment with 100 μ g/mL WGP β -glucan for a period of 24hrs (B) and 72h (C). BMDMs were scrapped on ice for FACS analysis 24hrs post stimulation (B) or washed and allowed to rest for 72hrs before being analysed via FACS (C). Expression of H3K27ac is shown as mean fluorescent intensity (MFI). (Biological N = 4). ns = not significant, *P< 0.05; **P<0.01; Individual replicates with mean bar shown. (RM One-way ANOVA with Šídák's multiple comparisons test).

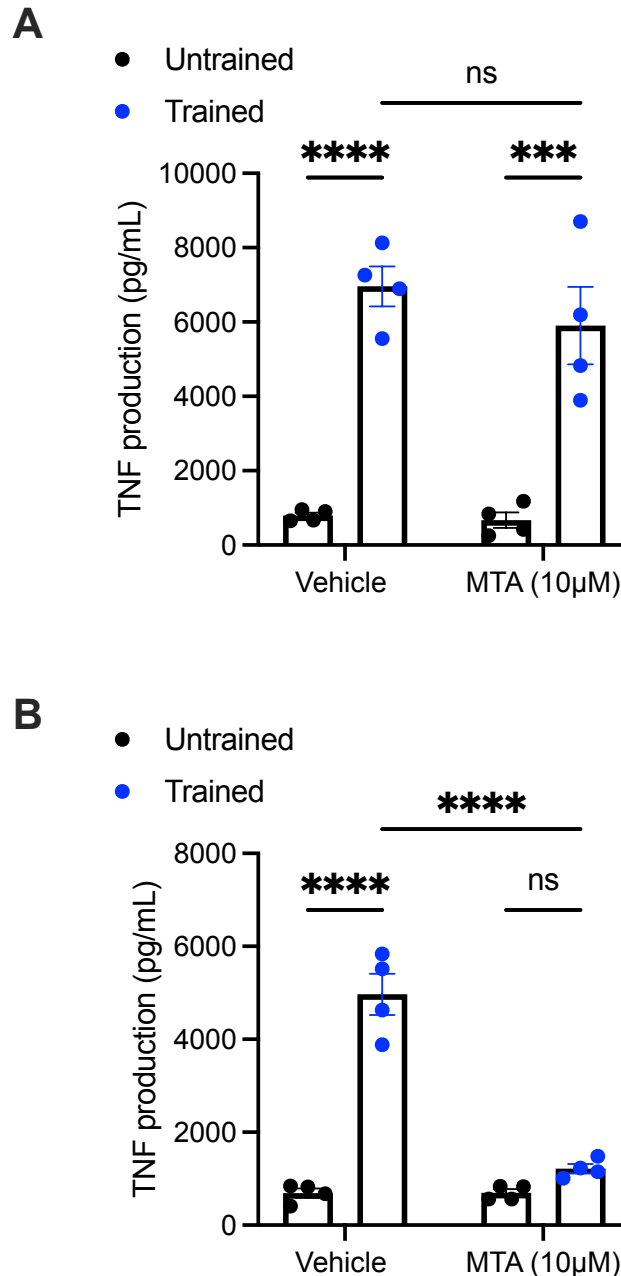


Figure 3.16 Trained responses are dependent on epigenetic modifications that occur 72hrs post WGP stimulation

5'-methylthioadenosine (MTA) was used to inhibit epigenetic modifications induced by WGP at 24hrs (A) or 72hrs (B) post WGP-training. (A) Cells were pretreated for 1hr with 10 μ M of MTA or a vehicle control followed by treatment with 100 μ g/mL WGP β -glucan for a period of 24hrs. BMDMs were allowed to rest for 6 days followed by 8hr stimulation with LPS. Supernatant was taken at this time point and TNF production was measured via ELISA. (B) Cells were treated with 10 μ M of MTA or a vehicle control 72hrs after 100 μ g/mL WGP for a period of 24hrs. BMDMs were allowed to rest for 3 days followed by 8hr stimulation with LPS. Supernatant was taken at this time point and TNF production was measured via ELISA. Each dot is a representative of an individual experiment

(Biological N = 4). *P< 0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns = not significant (Two-way ANOVA with Šídák's multiple comparisons test).

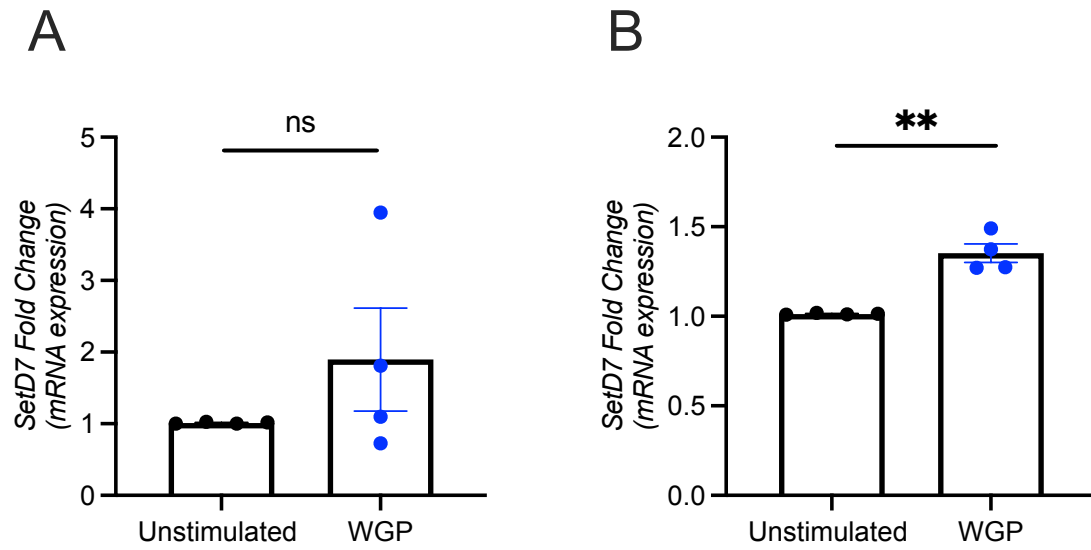


Figure 3.17 WGP enhances expression of the epigenetic enzyme SETD7 72hrs but not 24hrs after WGP stimulation

BMDMs were treatment with 100 μ g/mL WGP β -glucan for a period of 24hrs. Following 24hrs of stimulation RNA was either extracted (A) or BMDMs were washed and allowed to rest for 2 days (B). RNA was extracted from cells by this timepoint and qPCR was used to measure expression of *Setd7*. (Biological N = 4). **P=0.005; ns = not significant (Paired T-test).

3.2.6 WGP stimulation enhances early glycolytic genes and glycolysis

Epigenetic changes are a hallmark of β -glucan induced trained immunity. However, since I saw differences in inhibition between CPH and MTA I hypothesized that CPH may target both epigenetics and some other pathway. Metabolism is a major source of anabolic and catabolic metabolites that can induce the molecules necessary to drive these epigenetic modifications¹³⁶. Therefore, I wanted to test whether CPH may impact immunometabolic reprogramming. Glycolysis was the first metabolic pathway described to be a major driver of β -glucan induced trained immunity, therefore I decided to investigate how WGP can modulate this pathway and whether it played a role in mediating WGP trained immunity (**Figure 3.18**).

Since glycolysis is often an acute response to stimuli I first decided to measure early (8hrs) gene transcripts for certain parts of the glycolytic pathway, *Slc2a1* (Glut1), *Hk2*, and *Ldha* (**Figure 3.19A-C**). LPS was used as a positive control and enhanced the expression of all three genes. Similarly, WGP stimulation also upregulated the transcription of all genes, with notable significant increases in *Slc2a1* and *Hk2*, although to a lesser extent when compared with LPS.

I next looked at the expression of these genes again at a later 24hr time point (**Figure 3.19D-F**). Again, LPS acted as a positive control and significantly upregulated the expression of all three genes. However, unlike earlier timepoints WGP only significantly upregulated the expression of *Hk2* although again to a lower extent when compared with LPS. This suggests that WGP stimulation induces the expression of glycolytic genes at a rapid but restrained rate.

Having seen these changes at the mRNA transcript level I next wanted to measure whether these changes lead to a functional change in cellular glycolysis 24hrs post stimulation. I utilised extracellular flux analysis (Seahorse) and lactate assay to measure changes in extracellular acidification rate and lactate respectively (**Figure 3.20A-C & Figure 3.21**). Both LPS and WGP were able to significantly increase the

basal glycolytic rate of BMDMs as shown by both the trace and ECAR analysis (**Figure 3.20A-B**). However, only WGP was able to significantly increase the glycolytic capacity of BMDMs (**Figure 3.20C**). These results were further confirmed using the lactate assay with both LPS and WGP inducing increased lactate production and LPS producing higher levels to WGP (**Figure 3.21**).

Finally, I wanted to test whether these changes could be impacted by CPH inhibition. Therefore, I pretreated cells for 1hr with the epigenetic inhibitor CPH followed by a 24hr stimulation with WGP. Consistent with an up-regulation of Slc2a1 mRNA with short term WGP stimulation (8hrs) I saw a significant increase in GLUT1 protein expression in BMDMs treated with WGP after 24hrs (**Figure 3.22**). This was significantly reduced in the presence of the CPH inhibitor suggesting that either early epigenetic changes are required for glycolytic reprogramming in WGP treated BMDMs or that CPH can affect metabolic reprogramming in BMDMs.

Taken together this data indicates that increased expression of glycolytic genes drives increased glycolysis in WGP and LPS treated BMDMs. Although, LPS induces higher rates of glycolytic gene expression and lactate, WGP has overall higher glycolytic capacity potential indicating that not all glucose is being utilised for glycolysis unlike LPS treated cells. This is coupled with a link between glycolysis and epigenetic reprogramming in WGP treated BMDMs.

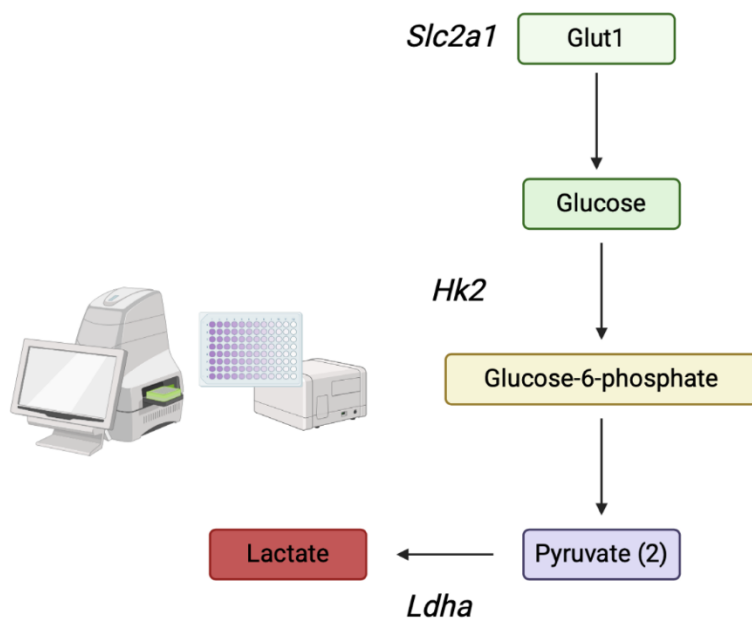


Figure 3.18 Graphical illustration of the glycolytic pathway, genes of interest and metabolic assays

Illustration shows a simplified version of glycolysis showing the key genes that were measured for this study. *Slc2a1* encodes for Glut1, which is the receptor for glucose uptake. *Hk2* encodes for the enzyme hexokinase 2, which is vital for converting glucose into Glucose-6-phosphate. *Ldha* encodes for lactate dehydrogenase alpha subunit, which converts pyruvate into lactate or lactic acid which is a key readout of glycolytic capacity. Lactate can be measured by the extracellular flux analyzer Seahorse or a lactate assay.

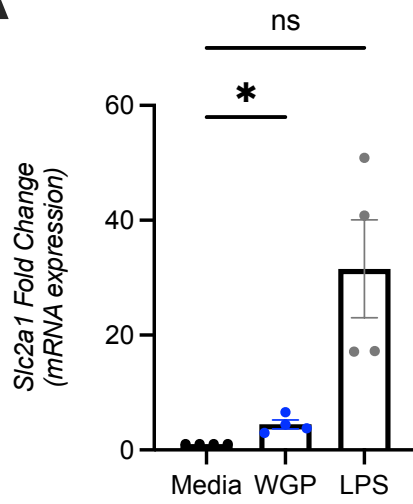
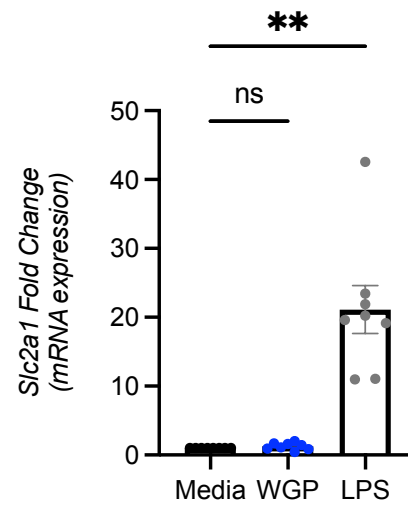
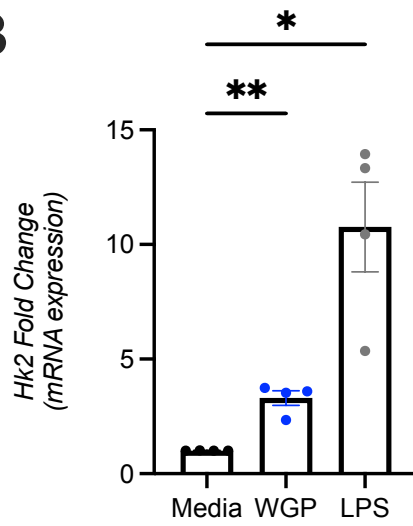
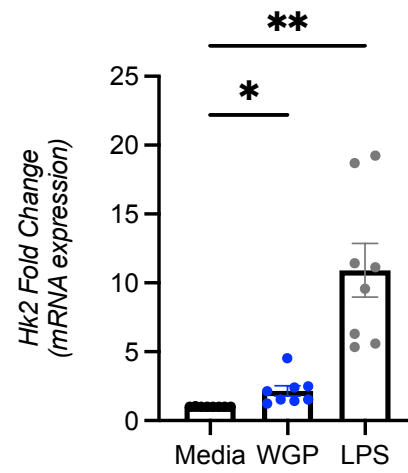
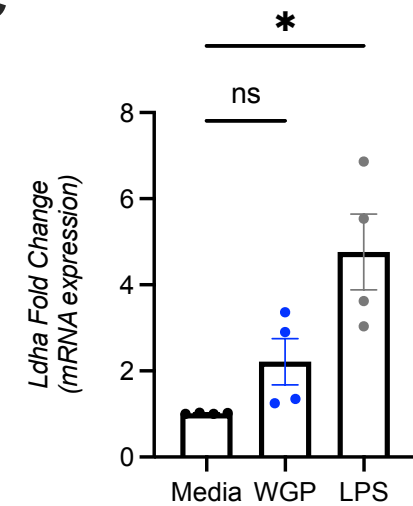
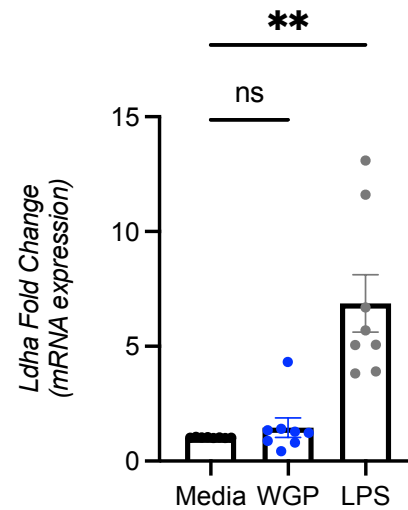
A**D****B****E****C****F**

Figure 3.19 8hr and 24hrs WGP stimulation leads to changes in glycolytic gene expression

BMDMs were treated for 8hr or 24hrs with 100 μ g/mL of WGP β -glucan, 100ng/mL LPS or a vehicle control. Cell lysate was taken and RNA was extracted for qPCR. (A) The *Slc2a1* gene which encodes Glut1 was measured relative to the house keeper *Hprt* after 8hrs. Fold change is based on unstimulated control. (B) The *Hk2* gene which encodes hexokinase 2 was measured relative to the house keeper *Hprt* after 8hrs. Fold change is based on unstimulated control. (C) The *Ldha* gene which encodes lactate dehydrogenase a was measured relative to the house keeper *Hprt* after 8hrs. Fold change is based on unstimulated control. (D) The *Slc2a1* gene which encodes Glut1 was measured relative to the house keeper *Hprt* after 24hrs. Fold change is based on unstimulated control. (E) The *Hk2* gene which encodes hexokinase 2 was measured relative to the house keeper *Hprt* after 24hrs. Fold change is based on unstimulated control. (F) The *Ldha* gene which encodes lactate dehydrogenase a was measured relative to the house keeper *Hprt* after 24hrs. Fold change is based on unstimulated control. Each dot is a representative of an individual experiment (Biological N = 4). *P< 0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns = not significant (RM One-way ANOVA with Dunnett's multiple comparisons test).

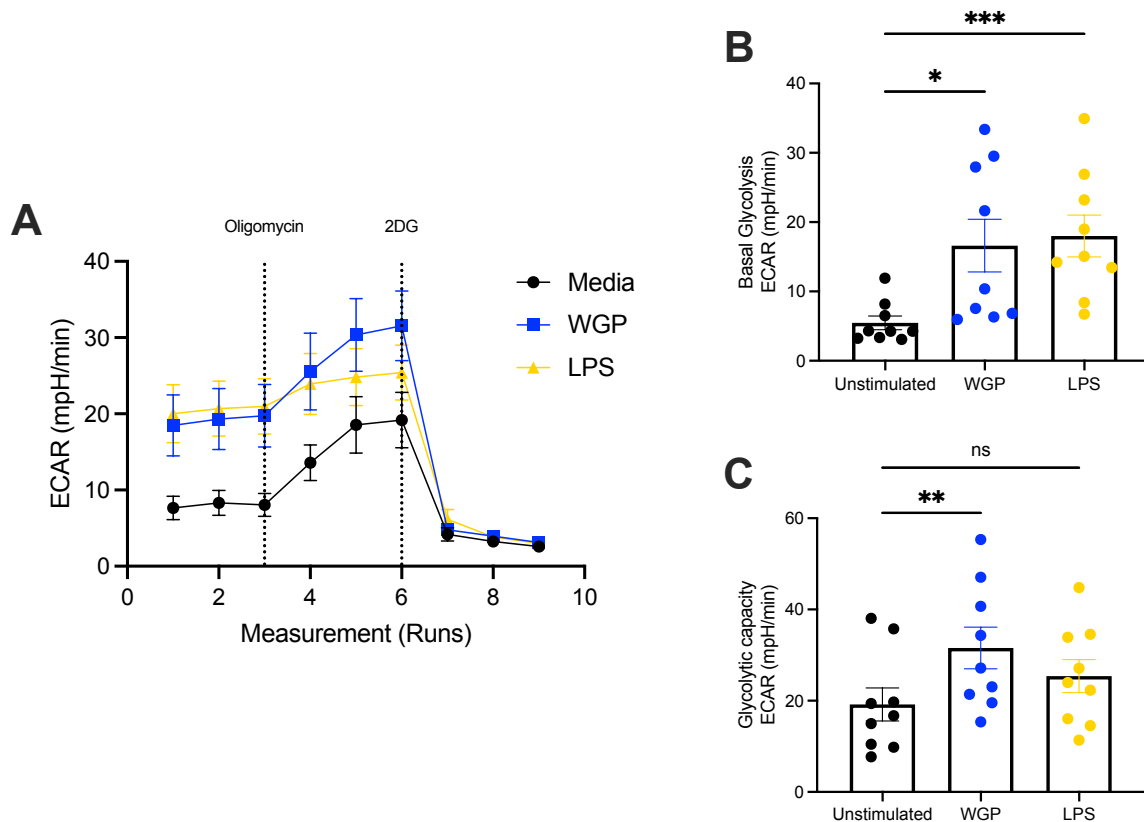


Figure 3.20 24hr WGP stimulation leads to enhanced glycolysis

BMDMs were treated for 24hr with 100 μ g/mL of WGP β -glucan, 100ng/mL LPS or a vehicle control. Metabolic phenotype was measured via extracellular flux analysis (Seahorse). (A) The change in extracellular acidification rate was measured before and after various inhibitors to see changes in overall glycolysis. (B) Basal glycolysis was measured by the difference between the last measurement before addition of oligomycin and the last measurement after 2DG when no glycolysis can occur. (C) The glycolytic capacity is the last measurement after oligomycin injection when all metabolic function is due to glycolysis. Cells were normalised by crystal violet to account for changes in cellular density during experimental procedure. Each dot is a representative of an individual experiment (Biological N = 9). *P< 0.05; **P<0.01;****P<0.0001; ns = not significant (RM One-way ANOVA with Dunnett's multiple comparisons test).

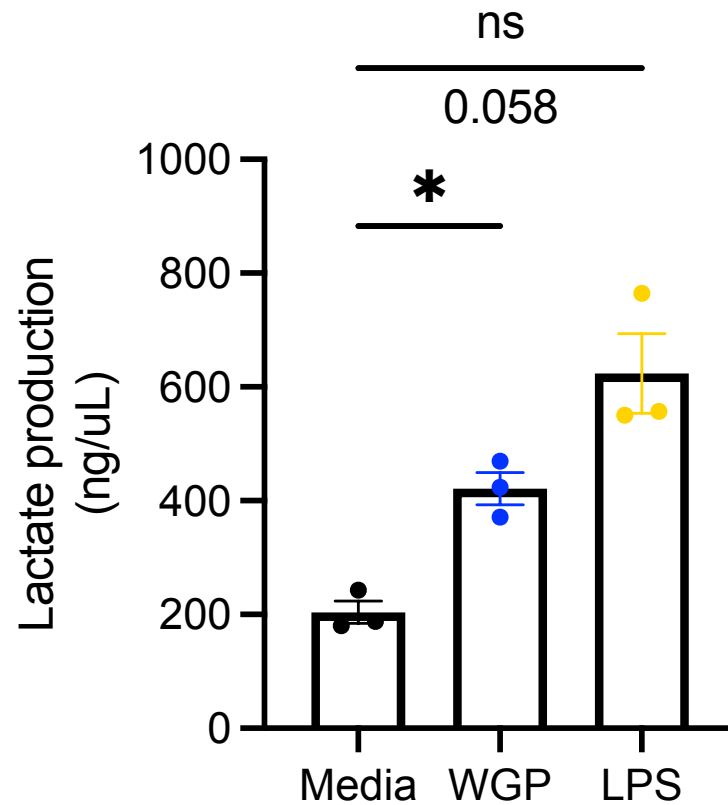


Figure 3.21 24hr WGP stimulation leads to increased lactate production

BMDMs were treated for 24hr with 100 μ g/mL of WGP β -glucan, 100ng/mL LPS or a vehicle control. Lactate production was measured via lactate quantification assay. Each dot is a representative of an individual experiment (Biological N = 3) ns = not significant, *P< 0.05; (RM One-way ANOVA with Dunnett's multiple comparisons test).

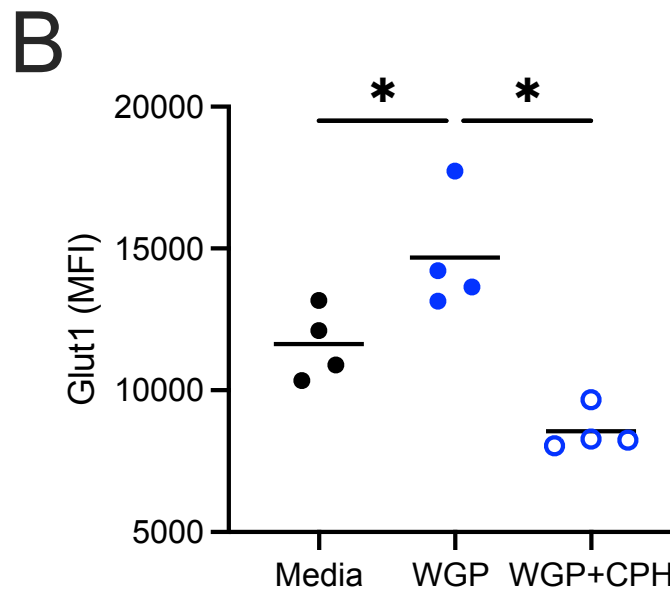
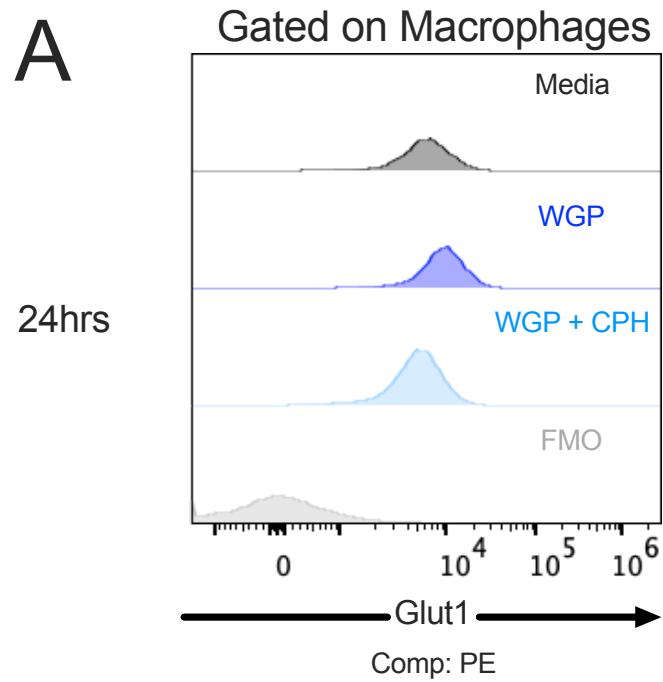


Figure 3.22 WGP glycolytic reprogramming is dependent on epigenetic changes

Total Glut1 was measured using flow cytometry 24hrs post WGP stimulation as shown in (A). Cells were pretreated for 1hr with 50 μ M of cyproheptadine (CPH) or a vehicle control followed by treatment with 100 μ g/mL WGP β -glucan for a period of 24hrs. BMDMs were scrapped on ice for FACS analysis 24hrs post stimulation (B). Expression of Glut1 is shown as mean fluorescent intensity (MFI). (Biological N = 4). *P< 0.05; Individual biological replicates with mean bar shown. (RM One-way ANOVA with Šídák's multiple comparisons test).

3.2.7 WGP stimulation leads to long term glycolytic reprogramming

Since I saw this early induction of glycolysis, I hypothesized that this would be maintained during the training assay and that glycolysis would be the main driver of the trained phenotype. To test this hypothesis, I measured glycolytic genes three days (72hrs) post WGP stimulation (**Figure 3.23A-C**). By 72hrs most of the glycolytic genes had return to baseline expression levels for LPS stimulated BMDMs. In contrast, although not significant, WGP treated cells maintained upregulated expression of all measured glycolytic genes. This data suggests that WGP stimulated BMDMs maintain glycolytic reprogramming over the course of the training protocol whereas LPS stimulated BMDMs upregulate glycolysis and return to baseline by day 3.

Next, I further wanted to quantify this change in metabolic reprogramming and used the extracellular flux analyser to measure changes in the metabolic phenotype of these BMDMs 72hrs post WGP or LPS stimulation. Similar to the gene expression results I saw that LPS treated cells return to baseline levels 72hrs post stimulation with no significant difference in both basal and glycolytic capacity (**Figure 3.24A-C**). In contrast, WGP stimulated BMDMs had significantly enhanced basal glycolysis and glycolytic capacity.

These glycolytic reprogramming results were further shown by measuring lactate in the media from BMDMs treated with either LPS or WGP. Like the Seahorse results I showed that WGP treated cells produced significantly more lactate at 72hrs (**Figure 3.25A**). BMDMs that had been treated with LPS still maintained high levels of lactate in the media which might have accumulated over the 48hr period post PBS washing of the cells and the addition of fresh media.

I finally decided to measure Glut1 expression in the presence or absence of the epigenetic inhibitor CPH (**Figure 3.26A**). Similar to what I saw at earlier timepoints WGP enhanced the expression of Glut1 significantly (**Figure 3.26B**). However, there was a significant reduction in Glut1 expression in the presence of CPH. Taken together this data indicates that WGP stimulated BMDMs sustain a glycolytic

signature over the duration of the training course, whereas LPS stimulated BMDMs undergo rapid but transient glycolytic reprogramming. This is coupled with epigenetic changes which occur at these timepoints indicating a potential need for glucose uptake for epigenetic modifications to occur or that epigenetics is supporting these changes.

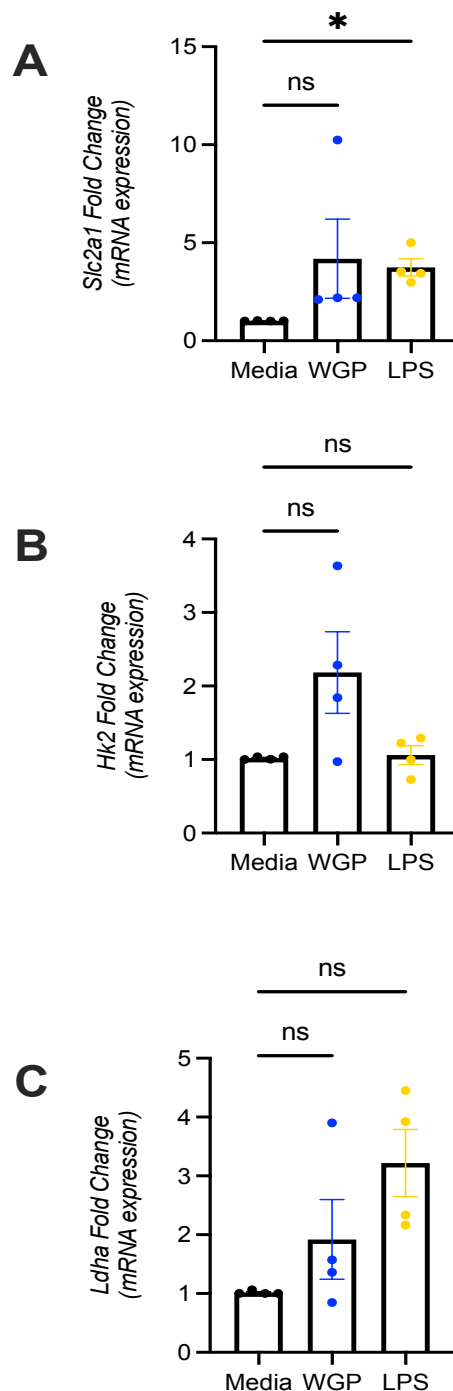


Figure 3.23 WGP stimulation leads to changes in glycolytic gene expression after 72hrs

BMDMs were treated for 24hr with 100 μ g/mL of WGP β -glucan, 100ng/mL LPS or a vehicle control. Cell lysate was taken and RNA was extracted for qPCR. (A) The *Slc2a1* gene which encodes Glut1 was measured relative to the house keeper Hprt. Fold change is based on unstimulated control. (B) The *Hk2* gene which encodes hexokinase 2 was measured relative to the house keeper Hprt. Fold change is based on unstimulated control. (C) The *Ldha* gene which encodes lactate dehydrogenase a was measured relative to the house keeper Hprt. Fold change is based on unstimulated control. Each dot is a representative of an individual experiment (Biological N = 4). *P < 0.05; ns = not significant (RM One-way ANOVA with Dunnett's multiple comparisons test).

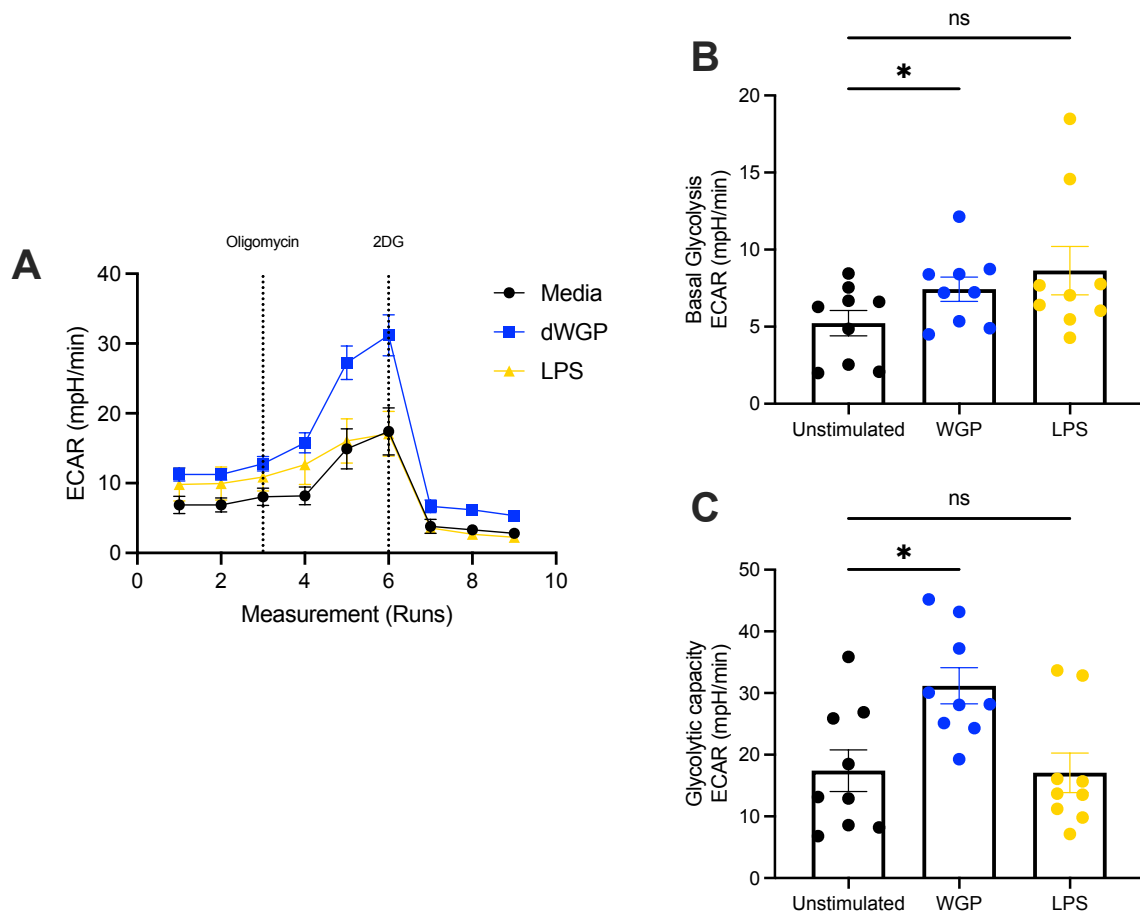


Figure 3.24 WGP stimulation leads to maintained enhanced glycolysis by 72hrs

BMDMs were treated for 24hr with 100 μ g/mL of WGP β -glucan, 100ng/mL LPS or a vehicle control. Metabolic phenotype was measured via extracellular flux analysis (Seahorse). (A) The change in extracellular acidification rate was measured before and after various inhibitors to see changes in overall glycolysis. (B) Basal glycolysis was measured by the difference between the last measurement before addition of oligomycin and the last measurement after 2DG when no glycolysis can occur. (C) The glycolytic capacity is the last measurement after oligomycin injection when all metabolic function is due to glycolysis. Cells were normalised by crystal violet to account for changes in cellular density during experimental procedure. Each dot is a representative of an individual experiment (Biological N = 9). *P < 0.05; ns = not significant (RM One-way ANOVA with Dunnett's multiple comparisons test).

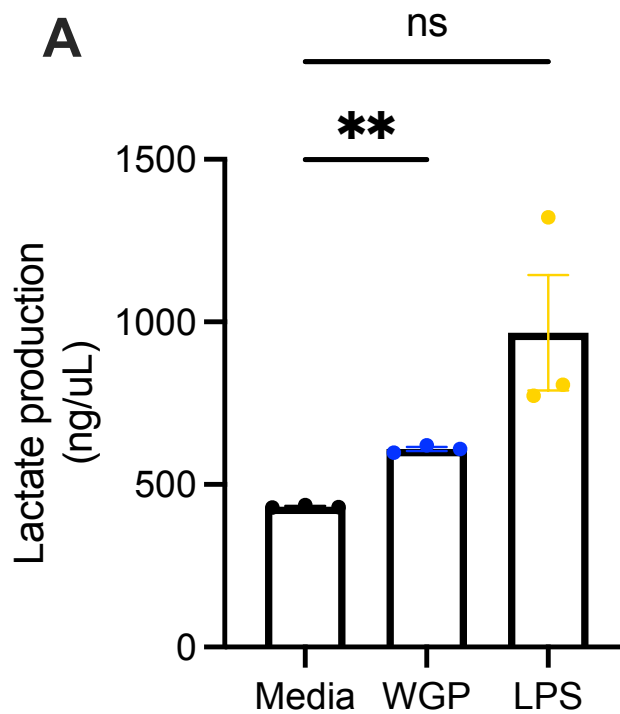


Figure 3.25 WGP stimulation leads to long-term increased lactate production

BMDMs were treated for 24hr with 100 μ g/mL of WGP β -glucan, 100ng/mL LPS or a vehicle control. After 24hrs cells were washed with PBS and fresh media was added to each well. Lactate production was measured via lactate quantification assay at 72hrs. Each dot is a representative of an individual experiment (Biological N = 3). **P<0.01; ns = not significant (RM One-way ANOVA with Dunnett's multiple comparisons test).

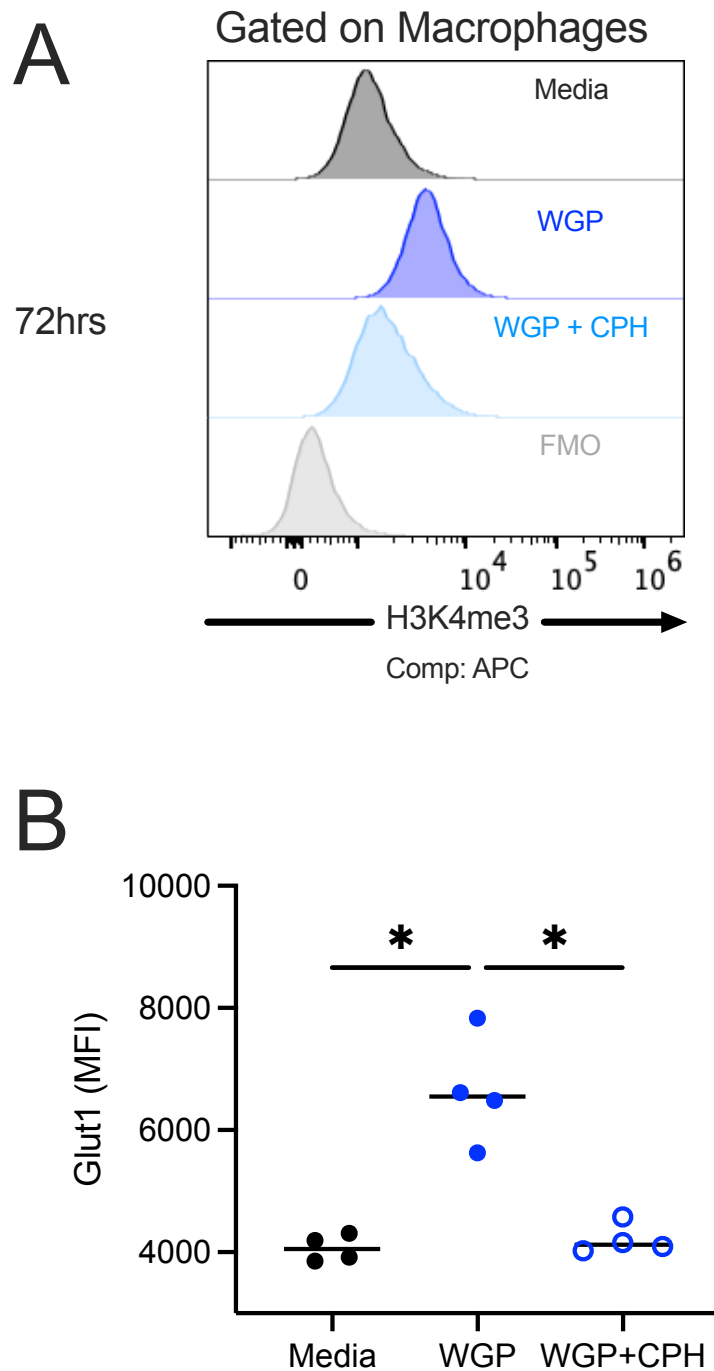


Figure 3.26 Long-term glycolytic reprogramming is inhibited by CPH

Total Glut1 was measured using flow cytometry 72hrs post WGP stimulation as shown in (A). Cells were pretreated for 1hr with 50 μ M of cyproheptadine (CPH) or a vehicle control followed by treatment with 100 μ g/mL WGP β -glucan for a period of 24hrs and washed and allowed to rest for 72hrs before being analysed via FACS. BMDMs were scrapped on ice for FACS analysis 72hrs post stimulation. (B) Expression of Glut1 is shown as mean fluorescent intensity (MFI). (Biological N = 4). *P< 0.05. Error bars shown as means. (RM One-way ANOVA with Šídák's multiple comparisons test).

3.2.8 WGP induced trained immunity is independent of glycolysis and mTORC activation

Having seen that WGP stimulated macrophages upregulate various glycolytic genes and pathways, and since other training mechanisms rely on glycolysis for the training phenotype to occur, I decided to test this using two different inhibitors of aerobic glycolysis. The inhibitor of mammalian target of rapamycin complex I (mTORC1) rapamycin and the inhibitor of hexokinase 2-deoxy-D-glucose (2DG) were used.

First, I investigated the effects of inhibiting mTORC1 activation on glycolysis in WGP treated cells, during the first 24hrs of stimulation. As I have previously shown stimulation with WGP significantly increases the production of lactate in cell media (**Fig 3.27A**). However, in the presence of rapamycin during this initial 24hr period there is no significant increase in lactate production. In fact, pretreatment with rapamycin significantly reduces the amount of lactate produced by WGP stimulated cells when compared with the WGP stimulated vehicle control. This data highlights that mTORC1 activation is important for WGP mediated aerobic glycolysis.

Having seen that rapamycin influences aerobic glycolysis I decided to block WGP stimulated BMDMs from upregulating aerobic glycolysis via 2DG (**Fig 3.28A**) as well as blocking them from driving aerobic glycolysis through mTORC1 activation via rapamycin (**Fig 3.28B**) during the first 24hrs of WGP stimulation. The standard training protocol was then followed, and 1 week later BMDMs were stimulated with LPS for a period of 8hrs. As expected, I saw a significant increase in TNF production from the vehicle control treated WGP-trained BMDMs. Surprisingly, neither 2DG nor rapamycin had any significant effect on WGP mediated trained immunity.

However, since I saw earlier that many of the epigenetic modifications induced by WGP occur 72hrs post the initial stimulation with WGP I hypothesized that potentially the changes in epigenetics coincide with glycolytic reprogramming and targeting this pathway at later times post-training would reveal more changes. Therefore, I stimulated untrained or trained BMDMs for 24hrs with 2DG (**Fig 3.29A**) or rapamycin (**Fig 3.29B**) at day 3 (72hrs) post initial WGP stimulation. Cells were

then stimulated for 8hrs with LPS on day 6. Again, I saw a significant increase in TNF production in WGP trained vehicle control cells. However, just like inhibiting mTOR/glycolysis at 24h post-training, treatment with 2DG or rapamycin at day 3 did not significantly reduce the training effect. In fact, stimulation with rapamycin at this time point significantly increased the training effects seen with WGP trained immunity.

Taken together this data indicates that although WGP enhances glycolytic genes and metabolism the overall training phenotype develops independently of both aerobic glycolysis as well as mTORC1 activation. This suggests that activation of glycolysis might be a bystander effect of activation of cells and that a different metabolic pathway drives the training phenotype I see with WGP.

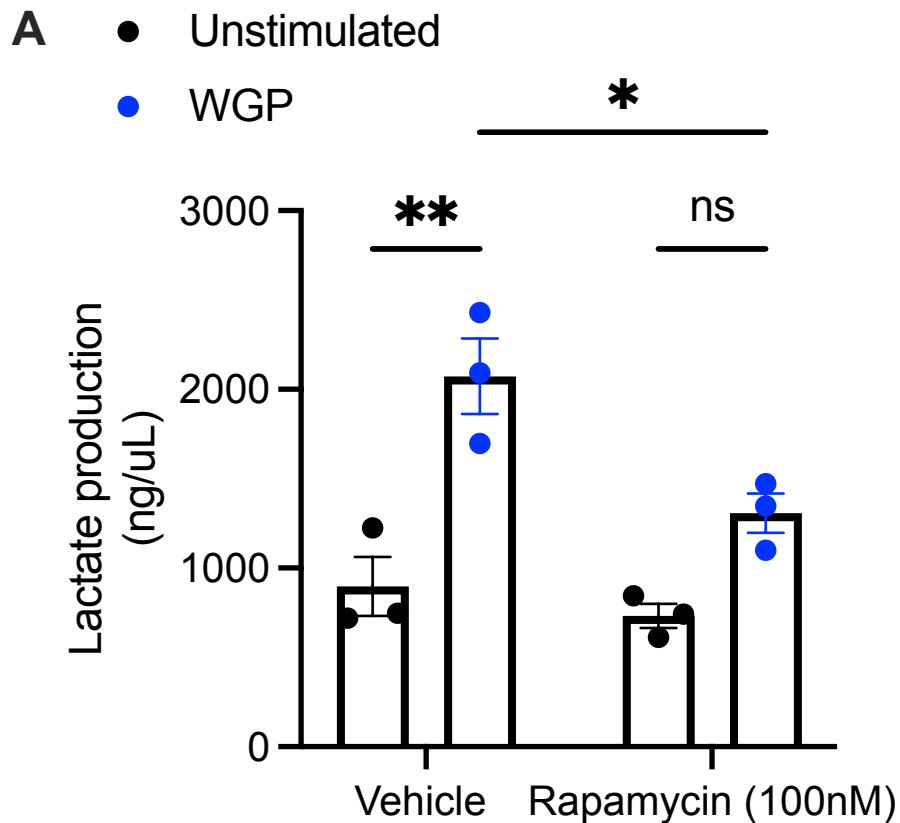


Figure 3.27 mTORC1 activation by WGP drives initial aerobic glycolysis responses

Rapamycin was used to inhibit lactate production induced by WGP at 24hrs (A). (A) BMDMs were pretreated for 1hr with 100nM of rapamycin or a vehicle control followed by treatment with 100 μ g/mL WGP β -glucan for a period of 24hrs. Supernatant was taken at this time point and lactate production was measured via lactate assay kit. Each dot is a representative of an individual experiment (Biological N = 3). *P< 0.05; **P<0.01; ns = not significant (Two-way ANOVA with Šídák's multiple comparisons test).

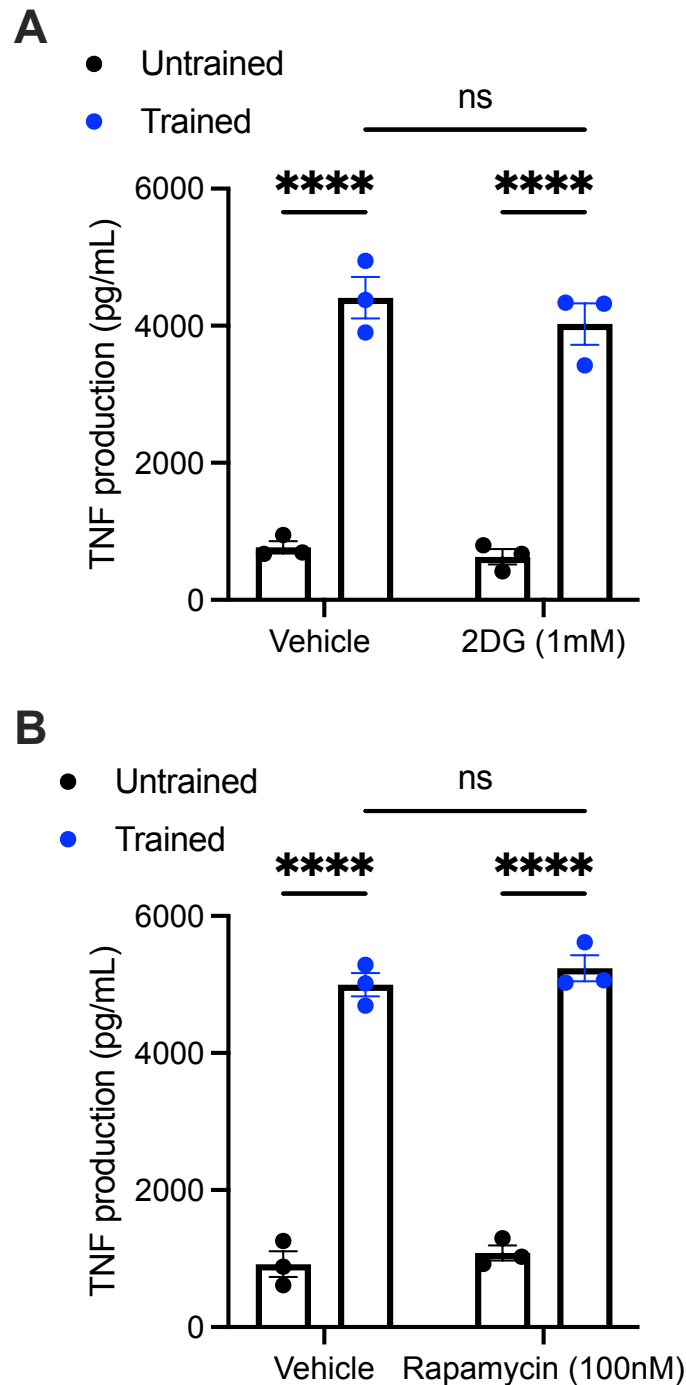


Figure 3.28 Trained responses are independent of early glycolytic reprogramming

2-deoxy-D-glucose (2DG) (A) and rapamycin (B) were used to inhibit aerobic glycolysis induced by WGP at 24hrs. (A) BMDMs were pretreated for 1hr with 1mM of 2DG or a vehicle control followed by treatment with 100 μ g/mL WGP β -glucan for a period of 24hrs. (B) BMDMs were pretreated with 100nM of rapamycin or a vehicle control followed by treatment with 100 μ g/mL WGP β -glucan for a period of 24hrs. BMDMs were allowed to rest for 6 days followed by 8hr stimulation with LPS. Supernatant was taken at this time point and TNF production was measured via ELISA. Each dot is a representative of an individual experiment (Biological N = 3). *P< 0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns = not significant (Two-way ANOVA with Šidák's multiple comparisons test).

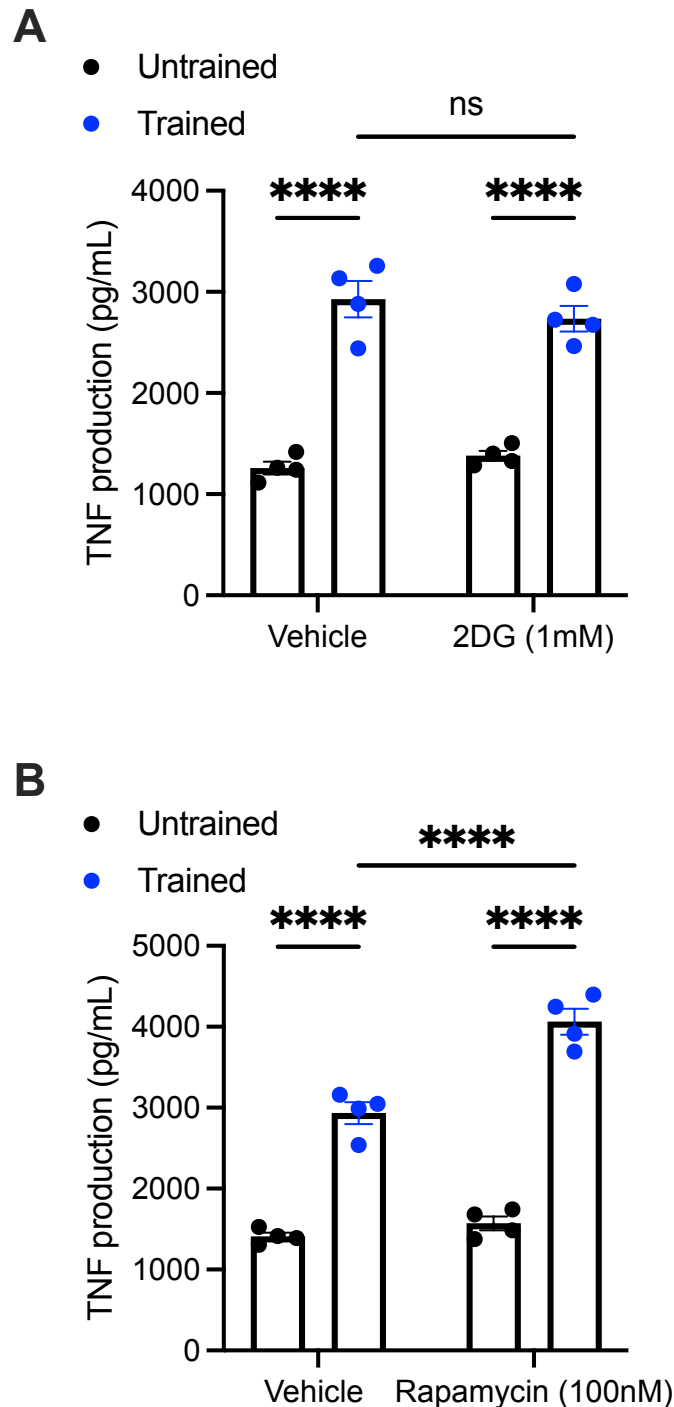


Figure 3.29 Trained responses are independent of early glycolytic reprogramming

2-deoxy-D-glucose (2DG) (A) and rapamycin (B) were used to inhibit aerobic glycolysis induced by WGP at 72hrs. (A) BMDMs were treated for 24hrs with 1mM of 2DG or a vehicle control 72hrs post WGP β -glucan stimulation. (B) Cells were treated with 100nM of rapamycin or a vehicle control at the same timepoint. BMDMs were allowed to rest for 3 days followed by 8hr stimulation with LPS. Supernatant was taken at this time point and TNF production was measured via ELISA. Each dot is a representative of an individual experiment (Biological N = 4). ****P<0.0001; ns = not significant (Two-way ANOVA with Šídák's multiple comparisons test).

3.2.9 WGP stimulation drives short-term TCA cycle reprogramming

Since I saw that WGP drove glycolytic reprogramming, which was not required for the training phenotype, I decided to investigate the role of TCA cycle and oxidative phosphorylation on trained immunity. I first examined the gene expression of TCA cycle genes 24hrs post WGP stimulation (**Figure 3.30A-G**). Although I did not see any significant changes in the expression of these genes, I did note that certain genes began to be upregulated. These included the expression of citrate synthase (Cs) (**Figure 3.30A**), succinate-CoA ligase (*Suclg1*) (**Figure 3.30C**) and malate dehydrogenase2 (*Mdh2*) (**Figure 3.30G**). Although not significant this data, suggests that WGP stimulation does have some effect on TCA cycle enzyme gene expression. Having seen these changes, I decided to investigate whether this would have any impact on oxidative phosphorylation as well as mitochondrial polarisation. Firstly, I measured changes in oxidative phosphorylation (OxPhos) using Seahorse extracellular flux analysis, 24hrs post WGP stimulation (**Figure 3.31A-C**). This allowed me to measure the oxygen consumption rate of the cells, a pseudo readout of TCA and electron transport chain (ETC) activity. I used LPS as a negative control for OxPhos since it has been well documented that LPS decreases OxPhos with an increase in aerobic glycolysis. 24hrs post LPS stimulation there was a significant change in OxPhos highlighted by a reduction in both basal OxPhos as well as the max respiratory rates (**Figure 3.31B-C**). In contrast, WGP stimulated BMDMs maintained their capacity to induce OxPhos, although there were no significant differences (**Figure 3.31B-C**). This data was also further highlighted by the energy plot with LPS shifting towards a glycolytic state (**Figure 3.32**). In contrast to LPS, WGP stimulation pushed the BMDMs into an overall more metabolically active state with both heightened glycolysis and OxPhos as shown by the energy plot (**Figure 3.32**).

Taken together this data highlights that 24hr WGP stimulation drives an overall metabolically active phenotype in murine BMDMs. This is in contrast with LPS stimulation which drives a glycolytic phenotype which may distinguish training phenotypes from that of tolerogenic phenotypes.

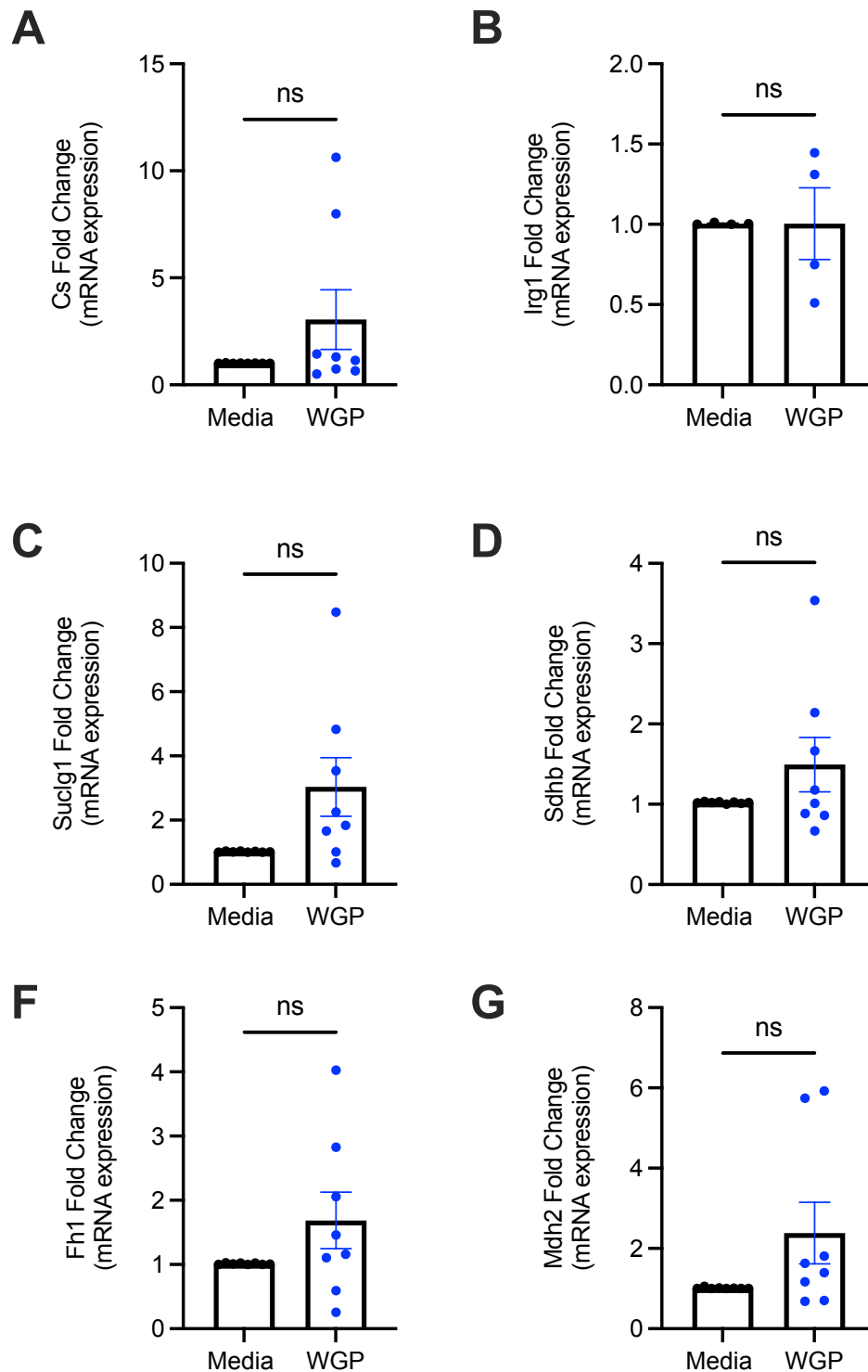


Figure 3.30 24hr WGP stimulation leads to changes in TCA cycle gene expression

BMDMs were treated for 24hr with 100 μ g/mL of WGP β -glucan or a vehicle control. Cell lysate was taken and RNA was extracted for qPCR. (A-G) All genes were measured relative to the house keeper Hprt. Fold change is based on unstimulated control. Each dot is a representative of an individual experiment (Biological N = 8, (B) biological N = 4). ns = not significant (Paired t-test).

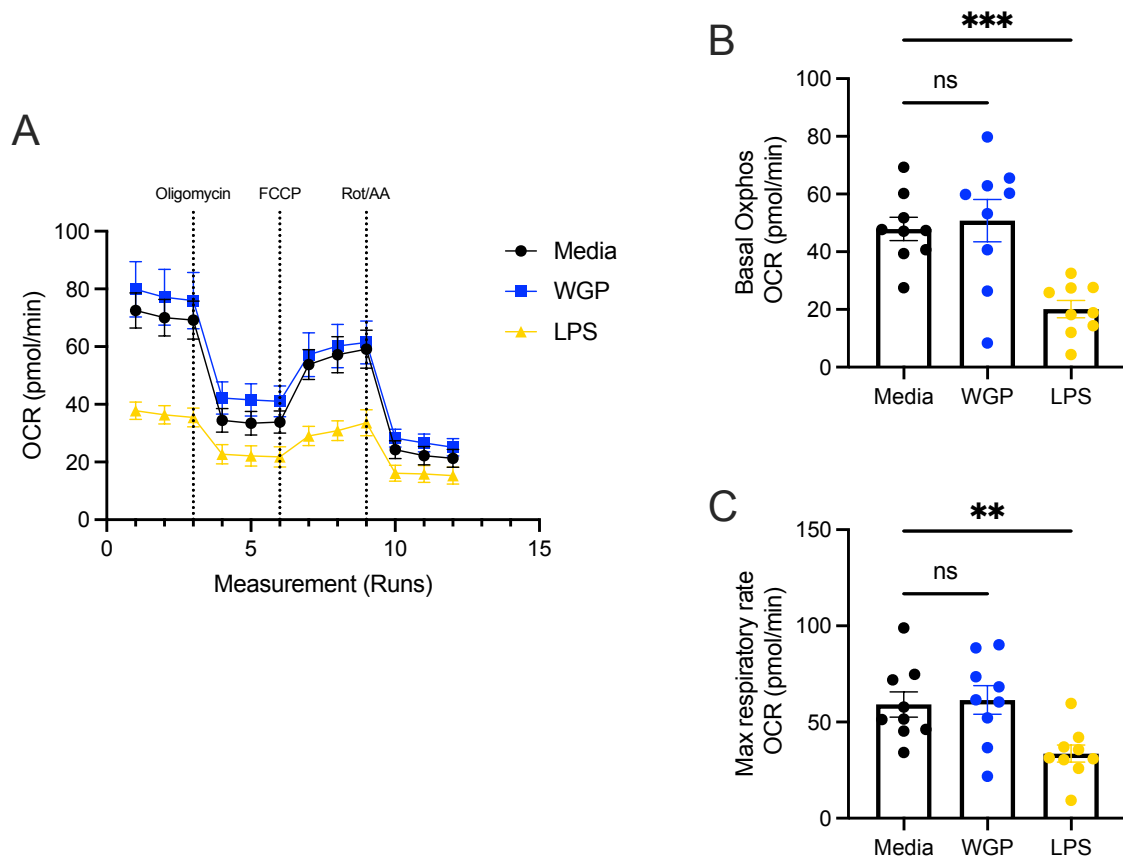


Figure 3.31 24hr WGP stimulation maintains oxidative phosphorylation

BMDMs were treated for 24hr with 100 μ g/mL of WGP β -glucan, 100ng/mL LPS or a vehicle control. Metabolic phenotype was measured via extracellular flux analysis (Seahorse). (A) The change in oxygen consumption rate (OCR) was measured before and after various inhibitors to see changes in overall oxidative phosphorylation. (B) Basal OxPhos was measured by the difference between the last measurement before addition of oligomycin and the last measurement after Rotenone (Rot) and antimycin A (AA) when no OxPhos can occur. (C) The max respiratory rate is the last measurement after the injection of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) when mitochondrial OxPhos is uncoupled. Cells were normalised by crystal violet to account for changes in cellular density during experimental procedure. Each dot is a representative of an individual experiment (Biological N = 9). **P<0.01; ***P<0.001; ns = not significant (RM One-way ANOVA with Dunnett's multiple comparisons test).

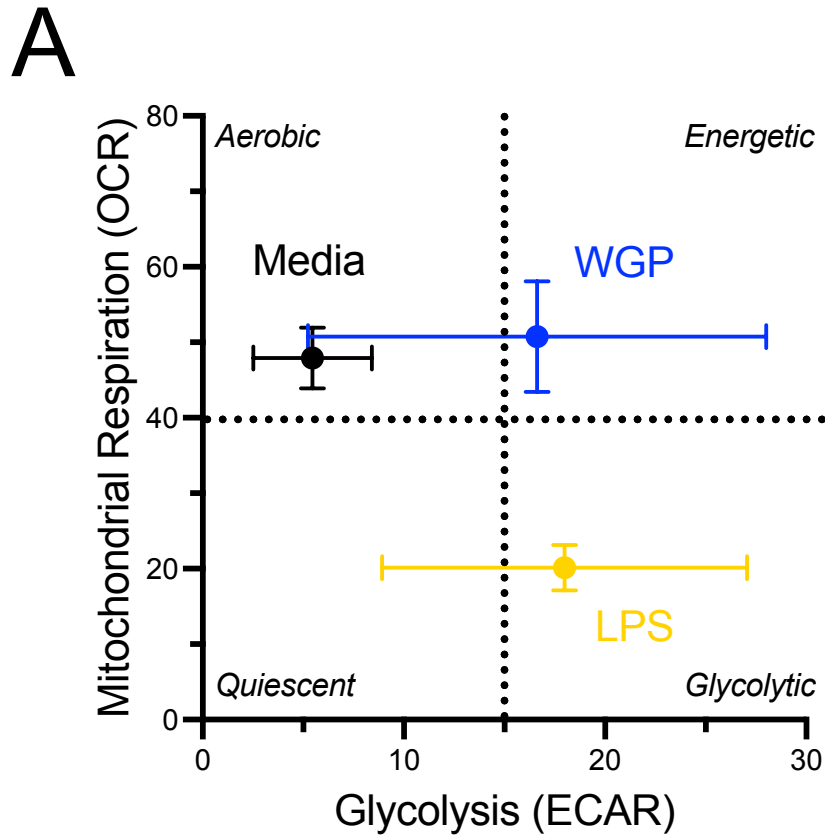


Figure 3.32 Energy plot showing shift in energy profiles 24hrs post WGP stimulation

BMDMs were treated for 24hr with 100 μ g/mL of WGP β -glucan (blue), 100ng/mL LPS (yellow) or a vehicle control (black). Metabolic phenotype was measured via extracellular flux analysis (Seahorse). Data shown is change in energetic profiles due to different treatments 24hrs post stimulation.

3.2.10 WGP stimulation drives long-term TCA cycle reprogramming

Having seen that WGP stimulated macrophages maintain their OxPhos phenotype and TCA cycle gene expression (with non-significant trends toward increased expression) I decided to investigate whether this phenotype persists over the course of the training protocol. Similar to my investigation into glycolysis, I looked at the OxPhos phenotype 72hrs after WGP stimulation. Firstly, I measured changes in TCA cycle gene expression via qPCR at this timepoint (**Figure 3.33**). 72hrs post WGP stimulation I saw a significant increase in the expression of the genes *Cs*, *Irg1*, *Suclg1* and *Mdh2* (**Figure 3.33A, B, C & F**). There was a trend towards an increase in *Sdhb* although this increase did not reach statistical significance (**Figure 3.33D**). Notably however I saw a significant reduction in the expression of *Fh1* (**Figure 3.33E**). Taken together this data indicates that WGP stimulation induces late TCA cycle gene induction in bone marrow derived macrophages.

Having seen changes in TCA cycle genes expression I wanted to see if this would influence the metabolic phenotype of these cells. Using extracellular flux analysis, I measured changes in OxPhos activity 72hrs post WGP stimulation (**Figure 3.34**). At 72hrs post WGP stimulation, BMDMs exhibited heightened OxPhos as indicated by the OCR trace (**Figure 3.34A**). WGP treated BMDMs had a trend towards significant increase in basal OxPhos (p-value = 0.06) and had a significant increase in the max respiratory rate (**Fig 3.34B-C**). In contrast LPS tolerized BMDMs had lowered OxPhos, potentially highlighting differences in training and tolerance. LPS tolerized BMDMs had significantly reduced basal OxPhos, as well as max respiratory capacity. Taken together this data indicates that WGP stimulation of BMDMs induces further metabolic changes at 72hrs, indicating a potential requirement for OxPhos in establishment of TI at this timepoint.

These changes were further quantified using an energy plot to highlight the shifts in the metabolic profile at the different timepoints (**Figure 3.35**). At 72hrs unstimulated BMDMs fell into the quiescent energy profile with overall lower glycolysis and OxPhos. When stimulated with LPS at 72hrs BMDMs adopt an overall glycolytic

phenotype. In contrast BMDMs stimulated with WGP adopt a phenotype between aerobic and energetic (**Figure 3.35A**).

Taken together the above data indicates that there are significant metabolic and mitochondrial changes that occur at the 72hr timepoint which agrees with my previous observations that functional epigenetic changes were not manifesting until this timepoint. this may highlight a link between oxidative metabolism, mitochondrial changes and epigenetic modifications in WGP mediated trained immunity.

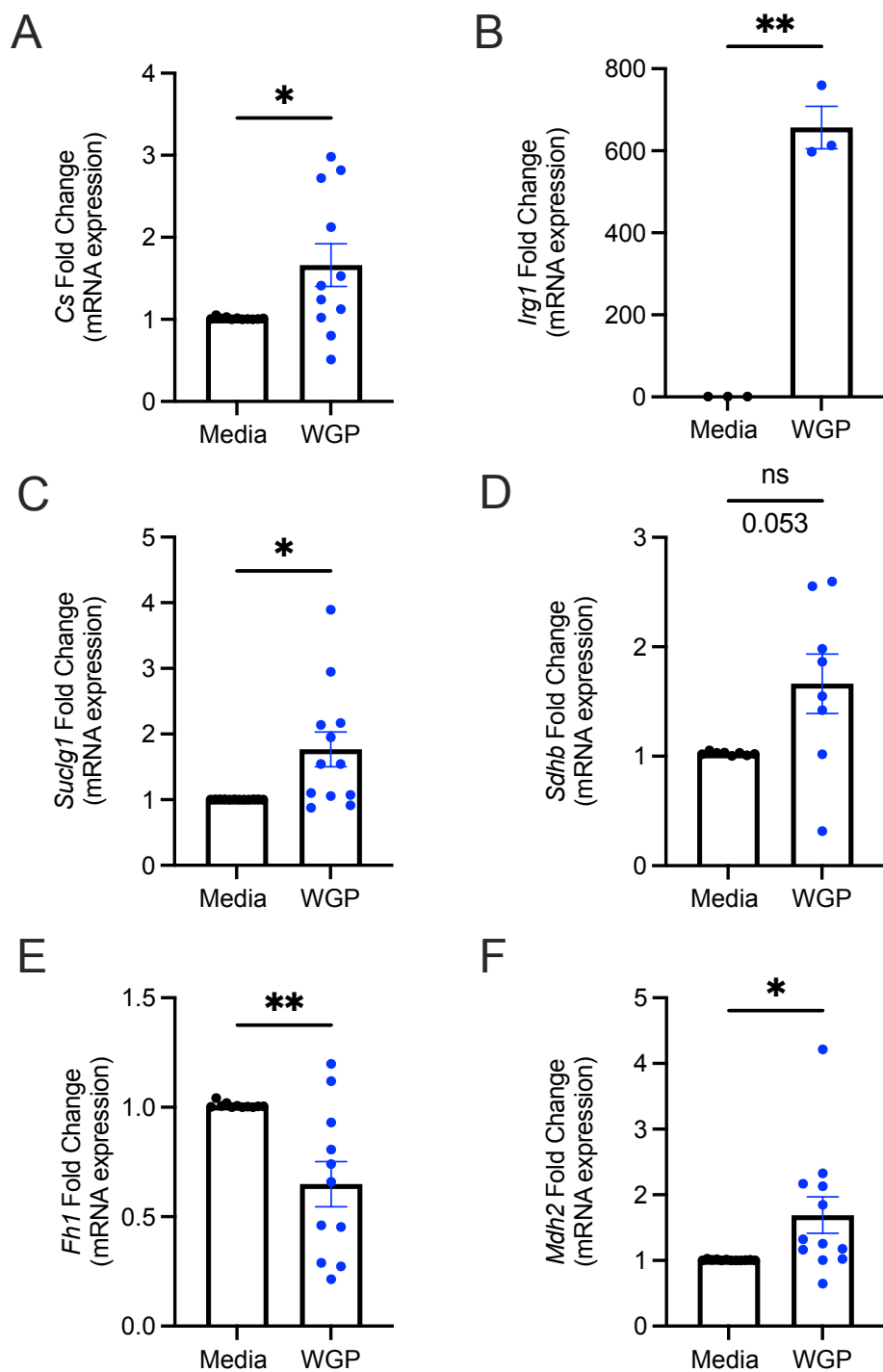


Figure 3.33 72hr WGP stimulation leads to changes in TCA cycle gene expression

BMDMs were treated for 24hr with 100 μ g/mL of WGP β -glucan or a vehicle control. Cells were washed after 24hrs and allowed to rest for 2 days. Cell lysate was taken and RNA was extracted for qPCR. (A-G) All genes were measured relative to the house keeper *Hprt*. Fold change is based on unstimulated control. Each dot is a representative of an individual experiment (Biological N = 11, (B) biological N = 3). * $P < 0.05$; ** $P < 0.01$; ns = not significant (Paired t-test).

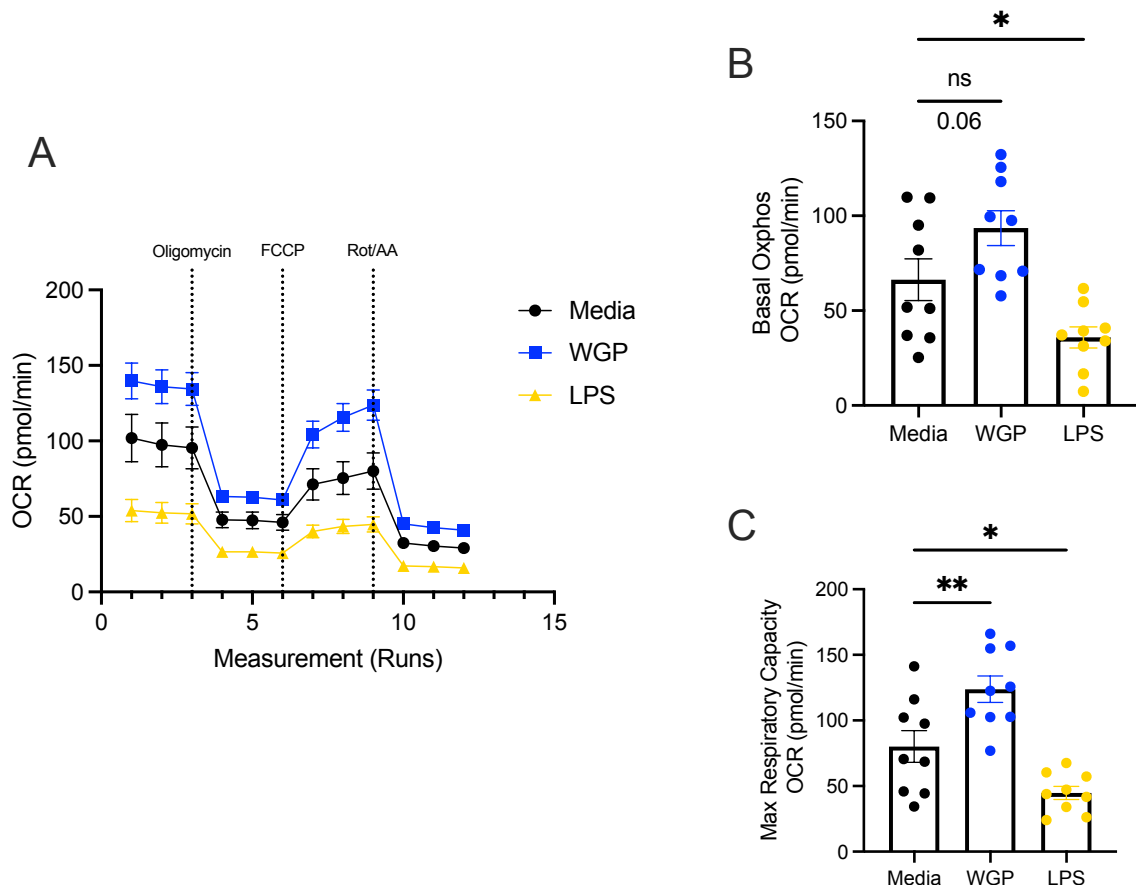


Figure 3.34 WGP stimulation leads to an enhanced Oxidative phosphorylation phenotype at 72hrs

BMDMs were treated for 24hr with 100 μ g/mL of WGP β -glucan, 100ng/mL LPS or a vehicle control. Cells were washed and allowed to rest for 2 days. Metabolic phenotype was measured via extracellular flux analysis (Seahorse). (A) The change in oxygen consumption rate (OCR) was measured before and after various inhibitors to see changes in overall oxidative phosphorylation. (B) Basal OxPhos was measured by the difference between the last measurement before addition of oligomycin and the last measurement after Rotenone (Rot) and antimycin A (AA) when no OxPhos can occur. (C) The max respiratory rate is the last measurement after the injection of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) when mitochondrial OxPhos is uncoupled. Cells were normalised by crystal violet to account for changes in cellular density during experimental procedure. Each dot is a representative of an individual experiment (Biological N = 9). *P< 0.05; **P<0.01; ns = not significant (RM One-way ANOVA with Dunnett's multiple comparisons test).

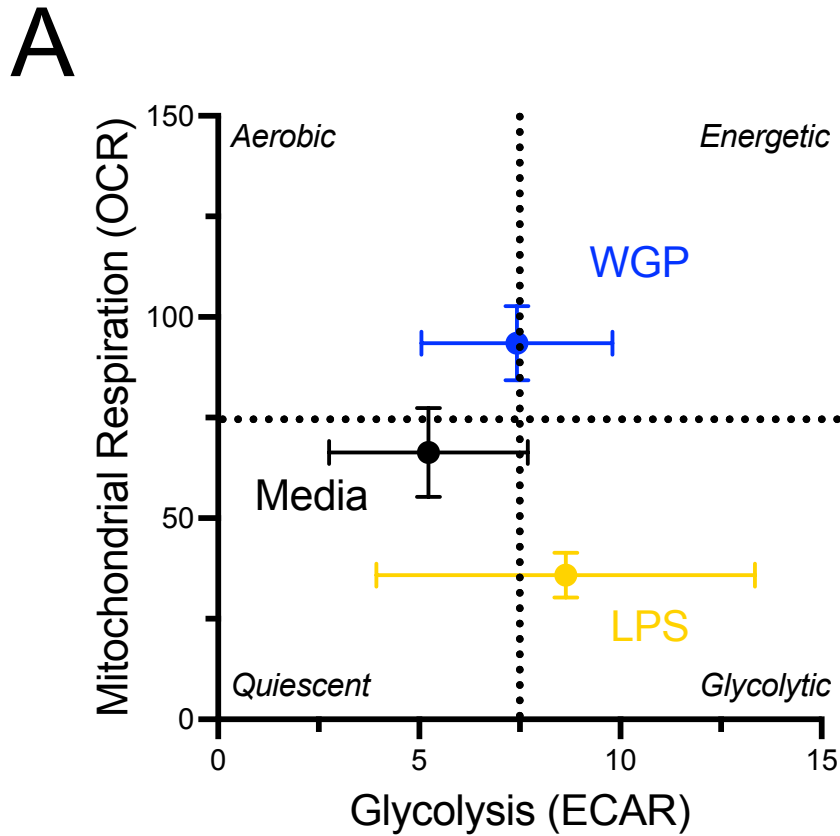


Figure 3.35 Energy plot showing shift in energy profiles 72hrs post WGP stimulation

BMDMs were treated for 24hr with 100 μ g/mL of WGP β -glucan (blue), 100ng/mL LPS (yellow) or a vehicle control (black). After 24hrs cells were washed with PBS and fresh media was applied. Cell metabolic phenotype was measured via extracellular flux analysis (Seahorse) 72hrs post original stimulation. Data shown is change in energetic profiles due to different treatments 72hrs post stimulation (A).

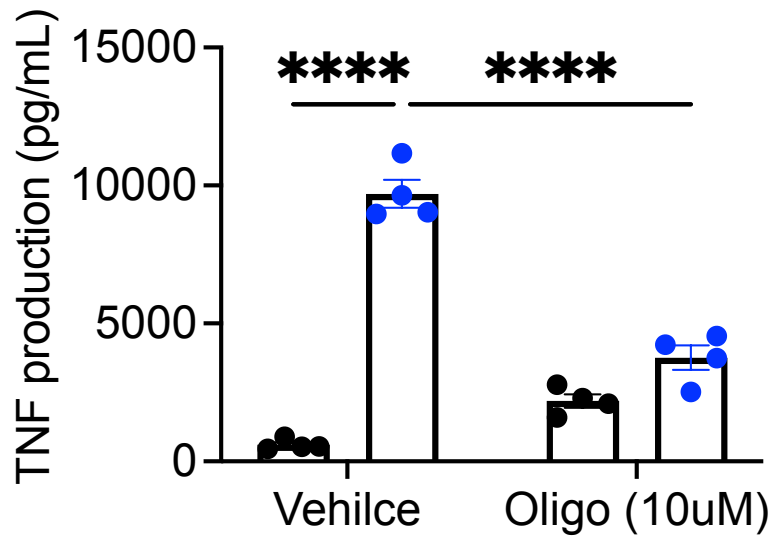
3.2.11 WGP induced trained immunity is dependent on OxPhos immunometabolic reprogramming

Having seen that WGP treated BMDMs upregulate OxPhos as well as TCA cycle genes on day 3, I decided to investigate whether inhibition of OxPhos would lead to inhibition of the trained responses we have seen. I decided to block OxPhos using two inhibitors. In order to fully inhibit OxPhos, I used an inhibitor of complex V of the electron transport chain (ETC); oligomycin (**Figure 3.36A**). Cells were treated for 24hrs with oligomycin 72hrs post WGP stimulation and allowed to rest for a further 3 days until stimulation with LPS (10ng/mL). Inhibition of complex V with oligomycin significantly reduced the training effects seen and brought the response back to the levels of the oligomycin treated (untrained) control. Interestingly, there was also a significant increase in TNF production between the untrained vehicle control and the untrained oligomycin treated group.

Next, I investigated whether inhibition of complex III of the ETC using antimycin A (AA) could significantly reduce the effects of WGP trained immunity (**Figure 3.36B**). Again, cells were treated for 24hrs with AA 72hrs post WGP stimulation and allowed to rest for 3 days until stimulation with LPS (10ng/mL). Similar to my previous result, inhibition of OxPhos and ETC with AA significantly reduced the effects of WGP induced trained immunity. Interestingly, this was again coupled with a significant increase in TNF in response to LPS between the AA treated group and the vehicle control.

Taken together this data indicates that WGP mediated trained immunity is dependent on OxPhos and the ability of trained cells to enhance TCA cycling. This indicates that metabolic intermediates may be important for epigenetic changes, at the same point I saw the highest levels of epigenetic changes linked to trained immunity. Also, interesting to point out was that inhibition of ETC lead to enhanced responses in BMDMs in response to LPS, potentially pointing towards some sort of priming mechanism occurring downstream of ETC inhibition.

A



B

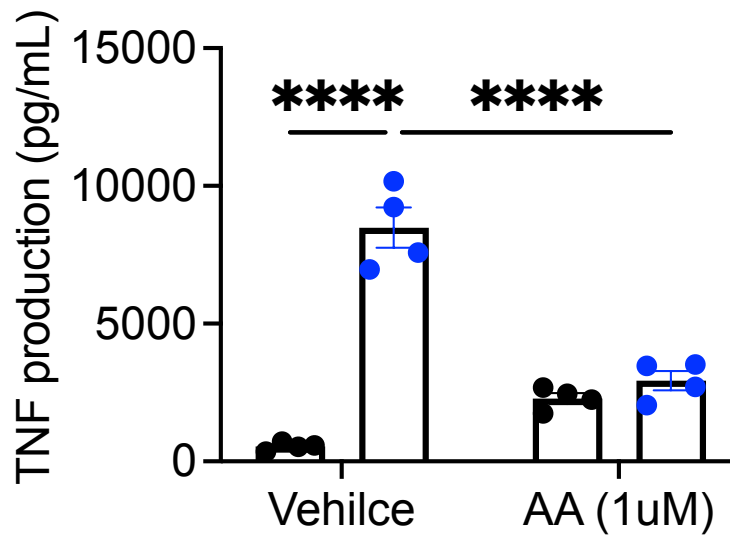


Figure 3.36 Inhibition of electron transport chain inhibits the training effect of WGP in BMDMs

BMDMs were treated for 24hr with 100 μ g/mL of WGP β -glucan (Blue) or a vehicle control (Black). WGP was washed off after 24hrs and allowed to rest for 48hrs. On day 3 BMDMs were treated with complex V inhibitor oligomycin (A) or the complex III inhibitor antimycin A (B) for 24hrs. Inhibitors were then washed off and cells were allowed to rest for 3 days before stimulation with 10ng/mL of LPS for 8hrs. Each dot is a representative of an individual experiment (Biological N = 4). ****P<0.0001; ns = not significant (Two-way ANOVA with Šídák's multiple comparisons test).

3.2.12 WGP induced trained immunity is dependent phagocytosis of β -glucan which induces metabolic reprogramming

Since I saw that WGP induced trained immunity in BMDMs was dependent on immunometabolic changes and was sensed by Dectin-1 I wanted to test whether receptor activation and phagocytosis was important for trained immunity and these metabolic changes. Therefore, I used two inhibitors for Dectin-1 binding and phagocytosis. Soluble β -glucans have been shown to competitively inhibit Dectin-1 signalling by competing for the Dectin-1 binding site, I thus used sWGP as an inhibitor of receptor binding¹⁹⁹.

To test that sWGP inhibited the ability of WGP to activate the Dectin-1 receptor I used the hDectin1b-HEK293 NF κ B-linked SEAP reporter cells (**Figure 3.37A**). As I have previously shown WGP significantly enhanced Dectin-1 activation with increased NF κ B-linked SEAP. However, pretreatment with sWGP (shown as Dectin-1 inhibitor) significantly reduced Dectin-1 activation returning the levels to unstimulated control. This confirms the ability of sWGP to inhibit Dectin-1 activation.

Next, I repeated the standard training protocol, with pretreatment of BMDMs with sWGP prior to WGP-training (**Figure 3.37B**). Untreated vehicle control cells still had significantly enhanced TNF responses upon secondary challenge with LPS, however, trained cells that had been pretreated with sWGP had significantly reduced training capability almost returning BMDM responses to untrained levels. This highlights that WGP trained immunity is dependent on Dectin-1 activation.

However, it is not only the activation of the Dectin-1 receptor that is required for innate signalling and anti-microbial responses, but also phagocytosis¹⁹⁹. Because of this work, I hypothesised that the phagocytosis of the β -glucan particle and receptor is also important for training. Therefore, I used the inhibitor of cytoskeletal actin polymerization, cytochalasin-D (CytoD), to inhibit receptor phagocytosis and trained immunity (**Figure 3.38**).

As before, I pretreated BMDMs with CytoD prior to WGP-training and then stimulated untrained and trained BMDMs with LPS for a period of 8hrs (**Figure 3.38A**). Similar to what I have shown previously there was a significant increase in IL-6 production in response to LPS. There was no significant difference between the untrained and the trained cells that had been pretreated with CytoD, showing that phagocytosis is important for inducing trained immunity.

It has been reported that blocking Dectin-1 phagocytosis can lead to increased macrophage activation due to the inhibition of the degradative phagocytic process removing Dectin-1 and its β -glucan cargo¹⁹⁹. Therefore, I returned to my RAW-Blue macrophage model and treated cells with CytoD and WGP as well as the relative vehicle controls (**Figure 3.38B**). As I have previously shown there was no significant increase in Macrophage activation upon WGP stimulation. However, upon inhibition of phagocytosis using CytoD treatment, there was a measurable and significant increase in NF κ B/SEAP reporter gene Macrophage activation after WGP stimulation. This supports the model that internalization of Dectin-1/WGP complexes by phagocytosis limits inflammatory activation.

Next, I decided to investigate the role that phagocytosis and potentially phagolysosomes have in inducing immunometabolic switching in WGP trained immunity. I first pretreated BMDMs with CytoD for 1hr prior to a 24hr WGP stimulation. Following the 24hr period I assessed the cells' ability to maintain OxPhos which I have shown at 24hrs is vital for inducing the immunometabolic switches involved in trained immunity (**Figure 3.39A**). Seahorse analysis showed that WGP treated BMDMs had maintained OxPhos after 24hrs, with maintained basal OxPhos and max respiratory rates (**Figure 3.39B-C**). However, pretreatment with CytoD lead to a significant reduction in both basal OxPhos and max respiratory rates, which may be linked with increased NF κ B activation and frustrated phagocytosis.

Finally, I hypothesized that the internalisation of WGP drives metabolic rewiring due to phagolysosome maturation. To test this hypothesis BMDMs were pretreated for 1hr with an inhibitor of phagolysosome maturation known as Bafilomycin. Cells were

then stimulated for 24hrs with WGP and their ability to maintain OxPhos was measured by Seahorse (**Figure 3.40A**). After 24hrs again WGP treated BMDMs maintained their OxPhos phenotype. However, cells pretreated with bafilomycin had a significant reduction in oxphos, as shown by the seahorse trace (**Figure 3.40A**). Basal OxPhos was not significantly reduced however, there was a significant reduction in the max respiratory rate upon pre-treatment with bafilomycin (**Figure 3.40B-C**). Taken together this data suggests that the cells ability to successfully phagocytose WGP is vital in dampening receptor linked NF κ B activation and simultaneously maintaining the OxPhos phenotype via successful phagolysosome maturation and thereby driving trained immunity.

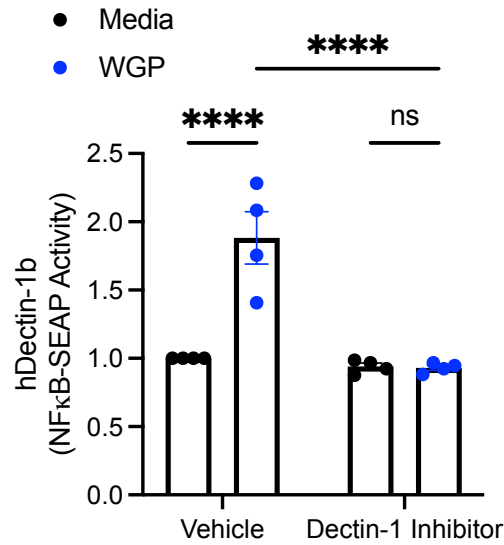
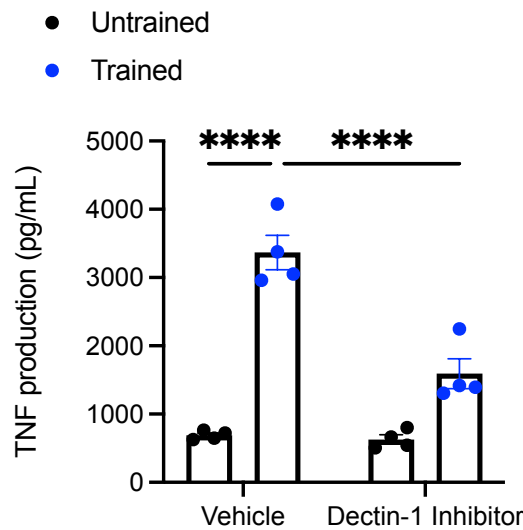
A**B**

Figure 3.37 Trained responses are dependent on Dectin-1 receptor binding and activation

Soluble WGP was used to inhibit Dectin-1 signalling and receptor binding. Cells were pretreated for 1hr with 100ug/mL of sWGP or a vehicle control followed by treatment with 100μg/mL WGP β-glucan for a period of 24hrs. (A) Supernatant was removed from hDectin-1b reporters following 24hr stim and relative NFκB-linked SEAP activity was measured at OD-655. Receptor activation is relative to NFκB-linked SEAP activity. (B) BMDMs were allowed to rest for 6 days followed by 8hr stimulation with LPS. Supernatant was taken at this time point and TNF production was measured via ELISA. Each dot is a representative of an individual experiment (Biological N = 4). ****P<0.0001; ns = not significant (Two-way ANOVA with Šídák's multiple comparisons test).

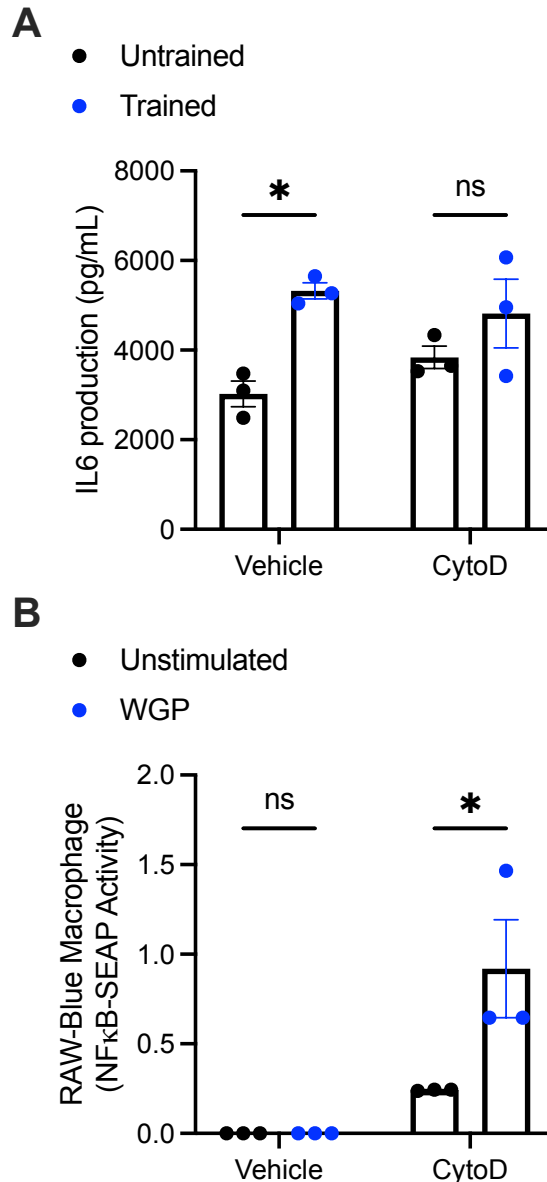


Figure 3.38 Trained responses are dependent on WGP phagocytosis and limited macrophage activation

Cytochalasin-D (CytoD) was used to inhibit phagocytosis of Dectin-1 and WGP. Cells were pretreated for 1hr with 10uM of CytoD or a vehicle control followed by treatment with 100μg/mL WGP β-glucan for a period of 24hrs. (A) BMDMs were allowed to rest for 6 days followed by 8hr stimulation with LPS. Supernatant was taken at this time point and IL-6 production was measured via ELISA. (B) Supernatant was removed from RAW-Blue Macrophages following 24hr stim and relative NFκB-linked SEAP activity was measured at OD-655. Receptor activation is relative to NFκB-linked SEAP activity. Each dot is a representative of an individual experiment (Biological N = 4). *P< 0.05; ns = not significant (Two-way ANOVA with Šídák's multiple comparisons test).

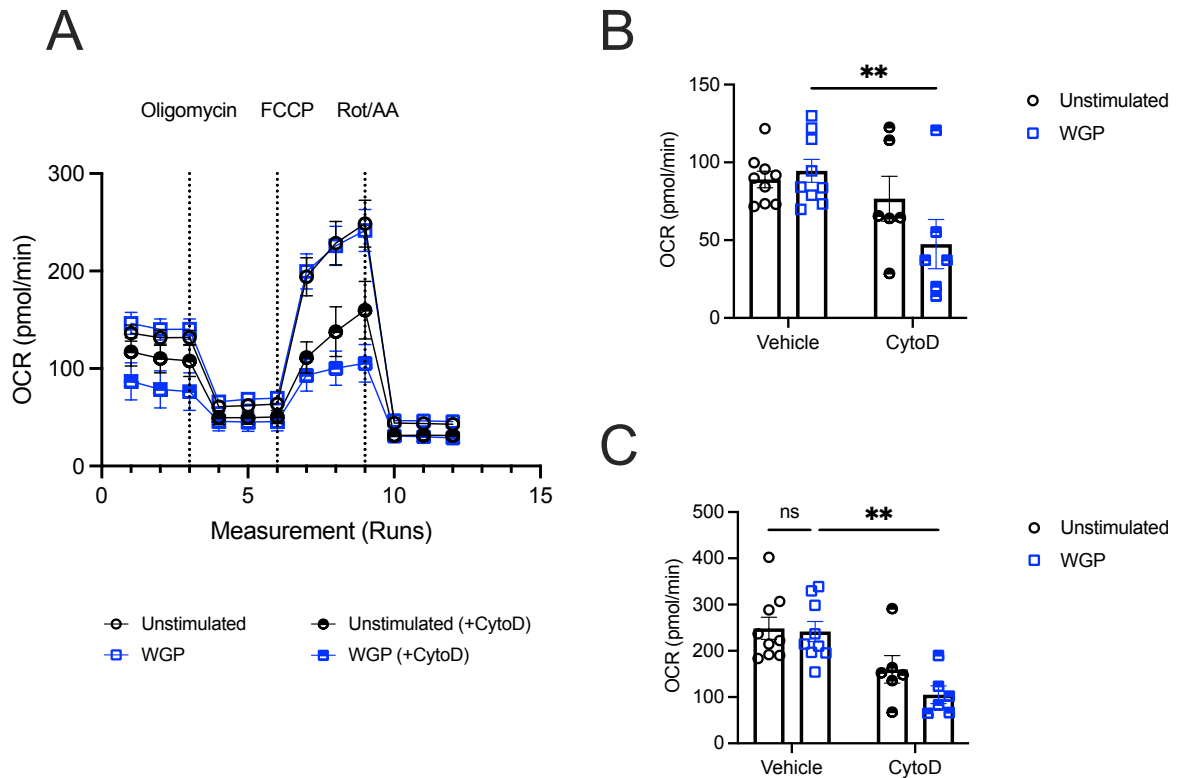


Figure 3.39 Phagocytosis is vital for maintained oxidative phosphorylation phenotype 24hrs post WGP stimulation

Cytochalasin-D (CytoD) was used to inhibit phagocytosis of Dectin-1 and WGP. Cells were pretreated for 1hr with 10uM of CytoD or a vehicle control followed by treatment with 100µg/mL WGP β-glucan for a period of 24hrs. (A) The change in oxygen consumption rate (OCR) was measured before and after various inhibitors to see changes in overall oxidative phosphorylation. (B) Basal OxPhos was measured by the difference between the last measurement before addition of oligomycin and the last measurement after Rotenone (Rot) and antimycin A (AA) when no OxPhos can occur. (C) The max respiratory rate is the last measurement after the injection of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) when mitochondrial oxphos is uncoupled. Cells were normalised by crystal violet to account for changes in cellular density during experimental procedure. Each dot is a representative of an individual experiment (Biological N = 4). **P<0.01; ns = not significant (Two-way ANOVA with Šídák's multiple comparisons test).

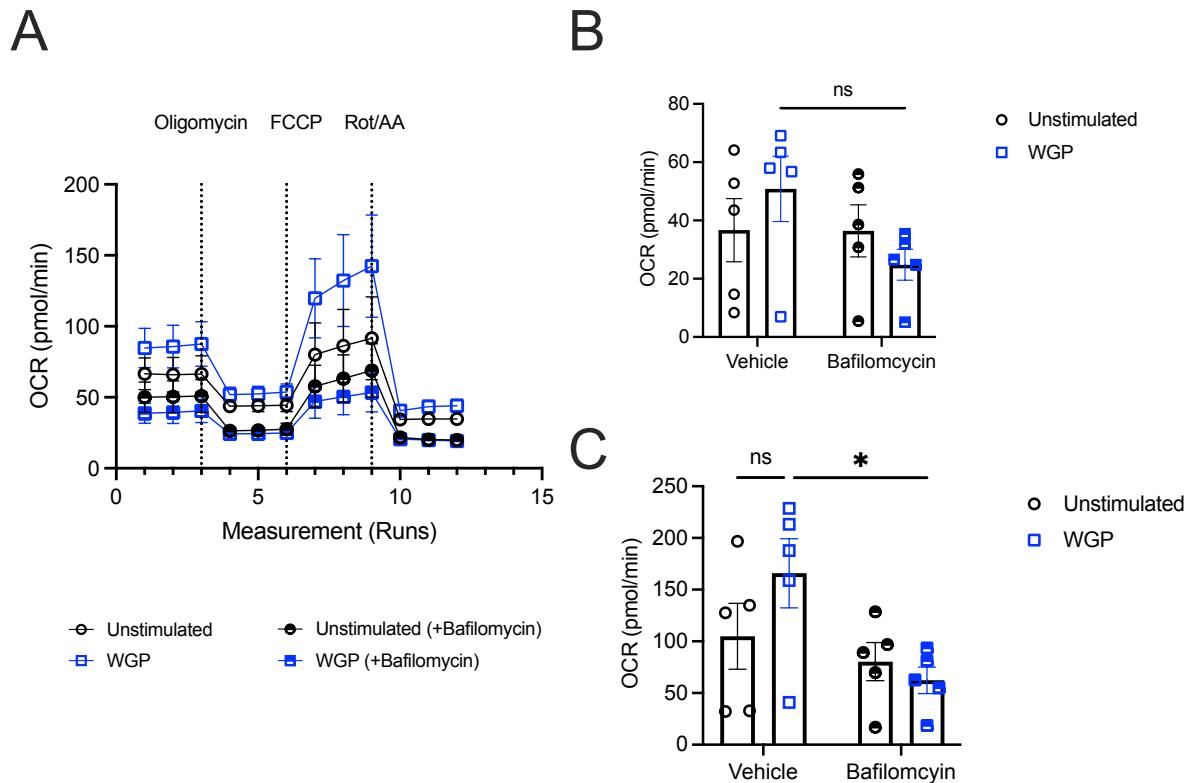


Figure 3.40 Phagolysosome maturation is vital for maintained oxidative phosphorylation phenotype 24hrs post WGP stimulation

Bafilomycin was used to inhibit phagolysosome maturation during WGP phagocytosis. Cells were pretreated for 1hr with 10uM of bafilomycin or a vehicle control followed by treatment with 100μg/mL WGP β-glucan for a period of 24hrs. (A) The change in oxygen consumption rate (OCR) was measured before and after various inhibitors to see changes in overall oxidative phosphorylation. (B) Basal OxPhos was measured by the difference between the last measurement before addition of oligomycin and the last measurement after Rotenone (Rot) and antimycin A (AA) when no OxPhos can occur. (C) The max respiratory rate is the last measurement after the injection of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) when mitochondrial OxPhos is uncoupled. Cells were normalised by crystal violet to account for changes in cellular density during experimental procedure. Each dot is a representative of an individual experiment (Biological N = 5). *P< 0.05; ns = not significant (Two-way ANOVA with Šídák's multiple comparisons test).

3.2.13 Preparation of β -glucan is more important than source of β -glucan

So far, I have shown that WGP derived from *Saccharomyces cerevisiae* can drive trained immunity via a dectin-1-phagolysosome-metabolic pathway (**Figure 3.41**). However, as I showed earlier, Zymosan which is also derived from *Saccharomyces cerevisiae*, drives a tolerogenic phenotype in macrophages. I hypothesized that this was due to activation of the NF κ B pathway due to impurities that were activating TLR-2 and TLR-4, respectively. However, I wanted to find out what happens if these impurities are removed and whether this 'depleted' form of Zymosan could drive similar training characteristics to our WGP.

To do this I used a structurally identical form of Zymosan that was produced to be a pure dectin-1 agonist, known as depleted Zymosan or dZym. I first showed that all three β -glucans could activate the β -glucan receptor Dectin-1 using the Hek-Dectin-1b reporter cell line (**Figure 3.42A**). All three could induce activation of the receptor with both WGP and dZym being able to drive significant increases in its activation. Since I knew that Zymosan contained TLR-2 and TLR-4 agonists I decided to investigate whether dZym was fully depleted of these impurities. To do this I once again employed the HEK-TLR2 and HEK-TLR4 reporter cell lines (**Figure 3.42B-C**). Only Zymosan was able to induce the activation of both receptors with no significant changes in both WGP and dZym treatment. Taken together this data indicates that removal of both the TLR2 and TLR4 agonists makes dZym a pure Dectin-1 ligand as seen with WGP.

Having seen these results I wanted to test if the different β -glucans induced different levels of innate immune memory. I have already shown that Zym induced tolerance in BMDMs with less TNF being produced by BMDMs after 4hrs stimulation with LPS (**Figure 3.43A**). However, when cells were pretreated and trained with both WGP and dZym there was a significant increase in TNF production 24hrs post LPS stimulation, indicative of trained immunity (**Figure 3.43B**). This shows that the depletion of contaminating ligands in β -glucan preparation is very important for driving a training phenotype.

Next, I investigated how the presence of certain TLR agonists could affect metabolism. I focused on OxPhos since my previous data demonstrated that maintenance of OxPhos was vital for trained immunity. Thus, I hypothesized that rescuing any perturbations to OxPhos would drive the cells from a tolerance phenotype toward a trained phenotype. To do this I stimulated BMDMs with various β -glucans or media for 24hrs and measured the cells' ability to undergo oxidative metabolism via oxygen consumption rate (OCR). As can be seen from the trace after 24hrs WGP maintained its ability to consume oxygen and thus had the same levels of OxPhos as the unstimulated control (**Figure 3.44A**). Cells treated with crude Zymosan reduced their levels of OxPhos similar to what is seen upon LPS stimulation, with a switch from OxPhos to aerobic glycolysis. However, interestingly dZym had a partial rescue of this OxPhos phenotype. This was more evident in the cells basal OxPhos (**Figure 3.44B**) and the cells max respiratory rate (**Figure 3.44C**). As shown WGP treated BMDMs maintained both basal OxPhos and max respiratory rates. In contrast crude Zymosan and LPS had a significant reduction in both respectively. dZym, however, had a significant increase in both basal OxPhos and max respiratory rates in comparison with regular zymosan showing a rescue metabolic phenotype when TLR agonists are depleted from β -glucans. Taken together this data indicates that maintenance of the OxPhos phenotype is important in driving trained immunity from β -glucans derived from *Saccharomyces cerevisiae* and that the presence of contaminating TLR ligands in β -glucan preparations can mask or inhibit potential Training effects through altering immunometabolic responses.

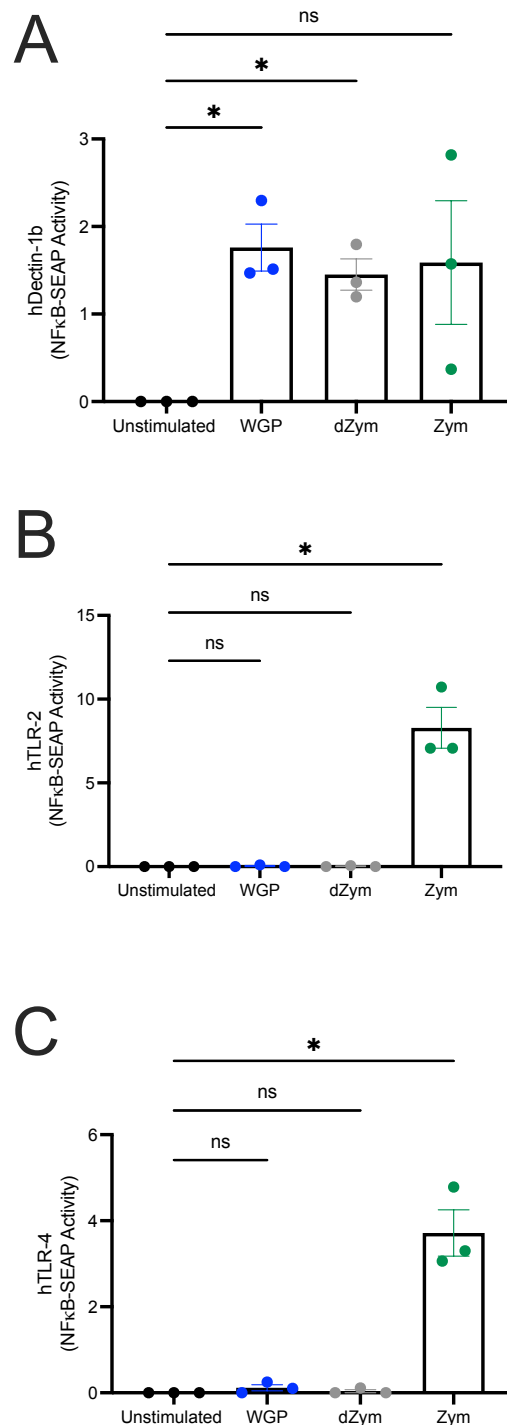
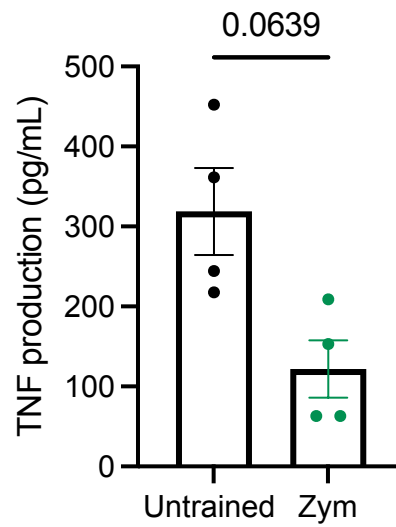


Figure 3.41 β -glucan preparations can be cleaned of TLR agonist impurities

HEK-reporter cells were stimulated with media (control), WGP, dZym or Zym for 24hrs. Media was removed and added to Quanti-blue solution to measure the levels of SEAP in the media. SEAP levels are correlated to the activity of the receptor activation. (A) Hek-Dectin1b cells were used to measure dectin-1 activation. (B) Hek-TLR2 cells were used to measure expression of TLR2 agonists in β -glucan preps. (C) Hek-TLR4 cells were used to measure expression of TLR4 agonists in β -glucan preps. Each dot is a representative of an individual experiment (Biological N = 3). *P < 0.05; ns = not significant (RM One-way ANOVA with Dunnett's multiple comparisons test).

A



B

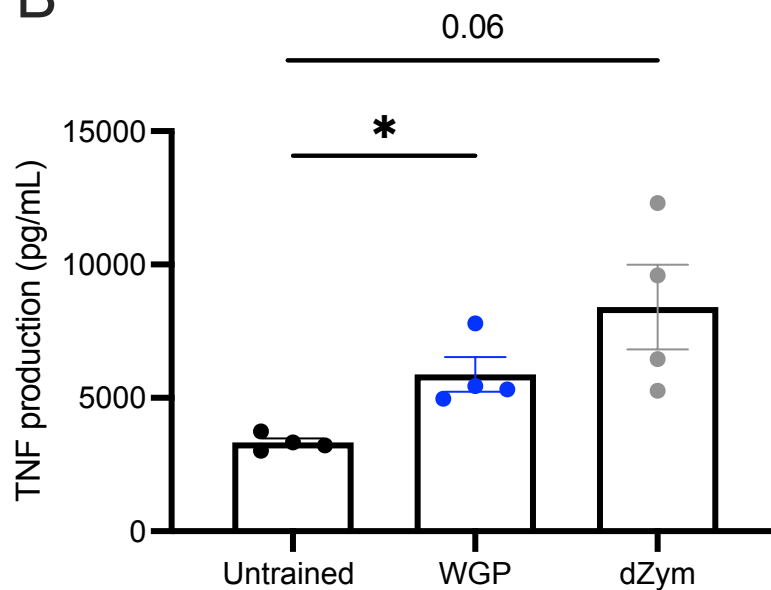


Figure 3.42 β -glucan preparation is vital for driving specific Innate Immune Memory phenotypes

BMDMs were stimulated with media (control), WGP, dZym or Zym for 24hrs. Cells were washed with PBS after 24hrs and allowed to rest for 6 days. (A) Cells that were pre-treated with media or zymosan were stimulated for 4hrs with 10ng/mL of LPS and TNF levels were measured by ELISA. (B) Cells that were trained with either media, dZym or WGP were stimulated for 24hrs with 10ng/mL LPS and TNF levels were measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). * $P < 0.05$; ns = not significant ((A) Paired T-test or (B) RM One-way ANOVA with Dunnett's multiple comparisons test).

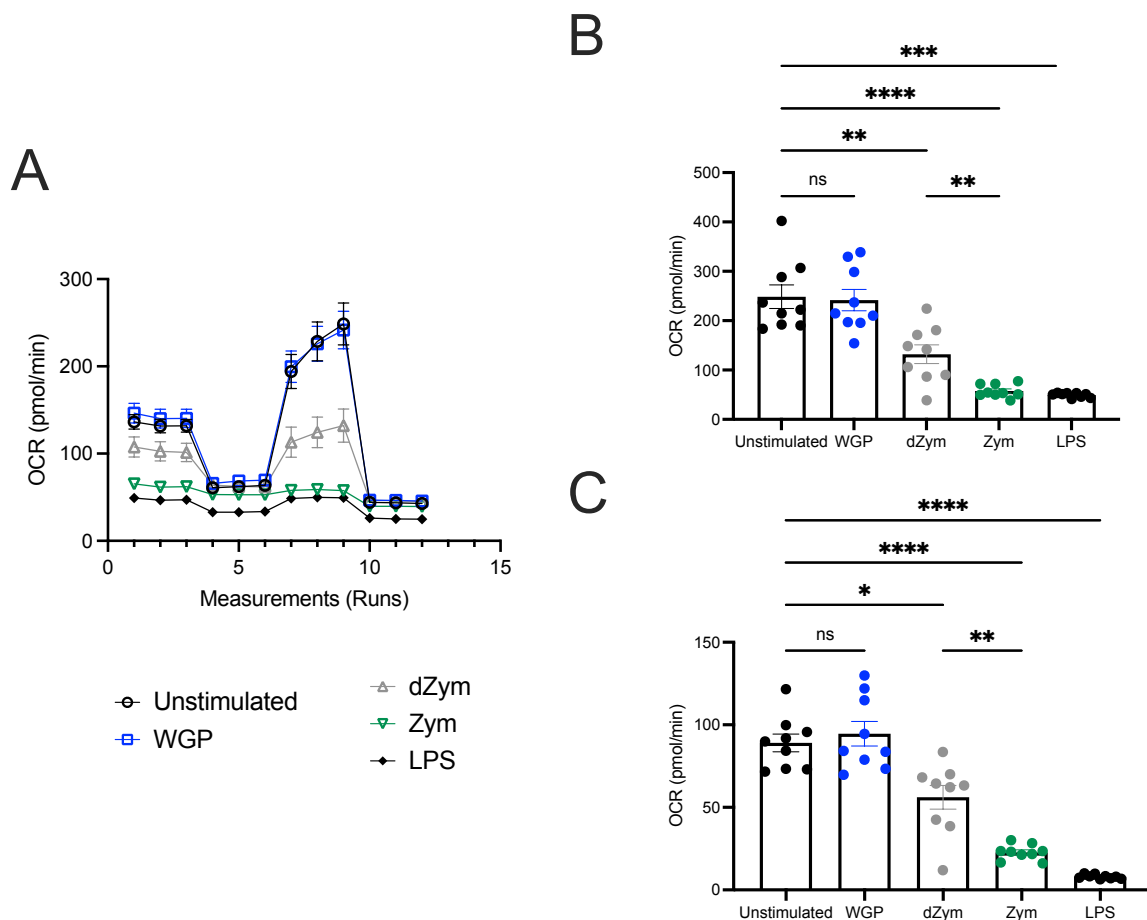


Figure 3.43 β -glucan preparation is important for driving various metabolic phenotypes

BMDMs were stimulated in a Seahorse plate with media (control), WGP, dZym, Zym or LPS for 24hrs. Cells were washed with Seahorse media prior to change of media. (A) The change in oxygen consumption rate (OCR) was measured before and after various inhibitors to see changes in overall oxidative phosphorylation. (B) Basal OxPhos was measured by the difference between the last measurement before addition of oligomycin and the last measurement after Rotenone (Rot) and antimycin A (AA) when no OxPhos can occur. (C) The max respiratory rate is the last measurement after the injection of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) when mitochondrial OxPhos is uncoupled. Cells were normalised by crystal violet to account for changes in cellular density during experimental procedure. Each dot is a representative of an individual experiment (Biological N = 9). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns = not significant (Two-way ANOVA with Šídák's multiple comparisons test).

3.3 Discussion

Work from this chapter focused on identifying the ability of WGP β -glucan to drive classical trained immunity, particularly through immunometabolic reprogramming. This work chiefly focused on identifying the optimal dosage of WGP to use which acts as the cornerstone of this thesis. Importantly, I showed that this dose was free from contaminating TLR agonists, which reduce the cell's ability to undergo trained immunity through NF κ B activation. This optimal dose drove epigenetic reprogramming through immunometabolic rewiring, particularly an enhancement of oxidative metabolism. This switch to oxidative metabolism was shown to be due to successful phagocytosis and phagolysosome maturation.

Dectin-1 has been well described in the literature to be vital in anti-fungal immunity to pathogens such as *Candida albicans*²⁰². Most work surrounding trained immunity in the literature focuses on β -glucans derived from these pathogenic strains^{83, 196}. However, our work in The Sheedy Lab focused on sources of β -glucans from less pathogenic strains, such as *Saccharomyces cerevisiae* or *Agaricus bisporus* mushroom^{99, 197}. This differs from the literature and importantly I was able to show in an in-vitro setting that *Saccharomyces cerevisiae* β -glucans, such as WGP, are capable of driving a trained phenotype in mature cells like BMDMs. In my screens of Dectin-1 activation I was able to show that these non-pathogenic strains are capable of successfully engaging the receptor driving activation. However, I showed that careful preparation of β -glucans is vital for a successful trained response, since impurities in a structurally identical β -glucan, Zymosan, failed to induce trained immunity and was shown to contain TLR-2 and TLR-4 ligands. This is a vital addition to the literature in identifying β -glucans that have potential for driving trained immunity as well as overcoming any potential errors in interpreting data from metabolic assays.

As mentioned Dectin-1 is the primary receptor involved in the recognition of multiple forms of β -glucans stemming from various organism, such as fungal, bacterial or algae^{83, 203, 204}. The ability of Dectin-1 to recognise the β -1,3/ β -1,6 linkages of β -glucans allows the immune response to distinguish non-pathogenic strains of β -

glucan, such as oats and barley, from potential pathogenic strains such as *Candida albicans* and *Saccharomyces cerevisiae*^{98, 195}. The activation of this receptor leads to NFκB activation downstream of SYK-CARD9-BCL-10 which enables inflammatory responses to fungal infections, while preventing the same activation to inert β-glucans¹⁹⁵. However, I have shown that this same receptor is vital for driving the trained response since inhibition of this receptor prevents trained immunity. WGP represents an artificial β-glucan that has been stripped of any contaminating ligands, such as mannans or endotoxins leaving a pure β-glucan rich shell. Therefore, unlike Zymosan this β-glucan chiefly activates the Dectin-1 receptor alone thus preventing excessive NFκB activation, inflammation and importantly aerobic glycolysis. Potentially, the findings that pure β-glucans drive training while contamination with ligands such as mannans or endotoxins drive tolerance highlights an evolutionary conserved mechanism by which bacteria, fungi and yeast hide their β-glucan rich cell walls to evade innate immune cell memory and establish infection. It has been shown that mannans on *Candida albicans* act as a shield preventing myeloid cells from recognizing their β-glucans, thus putting this theory into practise²⁰⁵.

It has been well documented at this point that metabolic reprogramming and epigenetic changes are vital in driving a trained innate immune response^{120, 121, 196}. However, there has been inconsistencies in the literature arguing between the importance of aerobic glycolysis vs oxidative metabolism in trained immunity^{120, 196}. The results in this chapter highlight that in terms of WGP induced trained immunity, OxPhos is important, whereas glycolysis seems to be a by-product of an activated cell. One area which I failed to investigate fully was the role glutaminolysis may play in WGP induced trained immunity. In fact, not appreciated in the field of trained immunity is the ability of cells that ramp up glutaminolysis to convert glutamine to lactate via TCA cycle intermediates and exit of malate into the cytosol, whereby it can be converted to pyruvate and lactate via the cytosolic malic enzyme^{206, 207}. This process has been shown to occur in transformed cells and malate may exit the TCA cycle via the dicarboxylate carrier²⁰⁷. This pathway was originally worked out to explain TCA cycle balance during insulin secretion since pancreatic islets cannot undergo gluconeogenesis as their source of Pyruvate²⁰⁸. This may help explain why

inhibition of glycolysis via 2DG had no effect on WGP-mediated trained immunity, even though Seahorse analysis showed increased extracellular acidification rates and colorimetric assays showed increased lactate production.

The importance of understanding these changes in metabolism, as well as appreciating that heightened glutaminolysis can induce errors in interpreting glycolytic results with lactate as a readout, is vital for our understanding of epigenetic modifications. It's been well acknowledged in the training field that TCA cycle intermediates are vital for driving the epigenetic machinery necessary for trained immunity^{121, 128, 209, 210}. Therefore, it makes sense that maintenance of OxPhos and TCA cycle is vital for trained immunity and a switch towards aerobic glycolysis would negatively impact this, since TCA cycle intermediates would not be able to be as efficiently accumulated. It makes sense therefore that our epigenetic results line up with max oxphos turnover and increased TCA cycle genes 3 days post WGP stimulation. This also explains why MTA inhibition works at this timepoint but not during the initial stimulation with WGP. In contrast CPH inhibition either has an off-target effect on cellular metabolism or has a longer transient effect on epigenetics than MTA, which explains how it can inhibit trained immunity during the first 24hr stimulation with WGP.

One limitation of our work to date has been our lack of identifying what specific genes H3K4me3 and H3K27ac are targeting. Potentially, these genes could be associated with various inflammatory genes that may be novel to WGP stimulation or may be linked to metabolism which could explain the maintained metabolic phenotype. Another limitation is that we only focused on H3K4me3 and H3K27ac and no other markers such as negative regulators of inflammation or H3K4me1. We know that WGP enhances the expression of SET7 which has been shown to epigenetically target succinate dehydrogenase (SDH) and malate dehydrogenase 2 (MDH2) via H3K4me1. Therefore, it would be useful to explore more in depth how metabolism and epigenetics are linked in WGP-mediated trained immunity.

The work in this chapter found that yeast-derived β -glucan particles play an immunomodulatory role by promoting metabolic reprogramming in BMDMs, which is necessary for trained immunity. This could have an impact on methods that use

WGP or related β -glucans to induce innate immune training and could help to increase innate immune resistance to infection. Interestingly, even differences in the physical structure of WGP had an impact on ability to drive Dectin-1 activation, metabolic changes and training effects. The soluble form of WGP had a poor ability to drive Dectin-1 activation, did not impact metabolic changes and also could not drive trained immunity. These findings also suggest that the pathway underlying trained immunity may have developed as a bioenergetic response to perceived threats by primitive pattern recognition receptors. This implies that only when legitimate infectious threats are specifically identified, are metabolic and energetic resources dedicated to long-term innate memory-like responses activated. Further examination of these traits across a variety of molecules now known to drive trained immunity in other contexts may reveal better ways to modify this response for greater therapeutic benefit, even though pathogens may have evolved to counteract this in their ligand presentation strategies.

Chapter 4. WGP β -glucan drives non-canonical anti-viral trained immunity via type I interferon signalling

4. 1 Introduction

The power of trained immunity stems from its ability to drive non-specific affects. Most typically, trained immunity has been studied in the context of anti-bacterial and anti-tumour immunity^{34, 100}. Since the COVID-19 pandemic, a greater need for the development of novel strategies to tackle emerging viral pathogens has evolved. One area of growing interest in terms of anti-viral immunity is the induction of trained immunity. There has been some work between the two fields in both human and mouse models, however, not much is known about how or why vaccine or fungal related ligands can protect against viral infections^{86, 211}.

One emerging field in trained immunity is the ability of type I interferons to drive trained responses and memory phenotypes in both mature cells, like macrophages, but also in stem cells, such as hematopoietic stem and progenitors (HSPCs)^{119, 211}. Some work has been published on the ability of dendritic cells to induce type I interferons in response to crude β -glucan preps, such as Zymosan and curdlan¹⁶⁸. However, not much is known about the ability of macrophages, the primary cell type used in training, to induce type I interferons in response to fungal ligands. Secondly, it is not known whether purified preparations of β -glucan (like WGP) can induce the similar responses seen in Zymosan.

The ability of β -glucans to drive type I interferon in myeloid cells is a fascinating prospect for boosting anti-viral responses. Our lab is interested in how macrophages can be primed to induce non-specific protection against multiple forms of pathogens and this bridge between anti-fungal and anti-viral immunity is an interesting area to explore. Therefore, I hypothesize that WGP can induce an anti-viral state in macrophages dependent on dectin-1 recognition. In addition, I also hypothesize that this enhanced anti-viral state will be triggered by type I interferon signalling either in an autocrine or paracrine manner.

4.2 Results

4.2.1 WGP β -glucan training enhances pro-inflammatory responses to viral ligands

In the previous chapter I showed that yeast derived β -glucan particles enhance inflammatory responses to fungal and bacterial ligands through immunometabolic rewiring. However, little is known about the benefits of trained immunity in the context of viral infection and the pathways that orchestrate this. Therefore, I decided to investigate whether WGP training led to a greater response to viral ligands 24hrs after stimulation. I used four different TLR agonists to explore whether trained immunity against viral ligands had any specificity. The first agonist I used was CpG oligodeoxynucleotide (ODN) which mimics foreign DNA and is recognised by TLR-9 (**Figure 4.01A**). In response to CpG BMDMs increased their production of TNF. However, WGP-trained macrophages produced significantly more TNF than the untrained control. This confirms that β -glucan trained macrophages enhance their inflammatory potential in response to TLR sensing of DNA agonists. I next investigated their ability to respond more robustly to RNA agonists. I first observed trained responses to the TLR3 agonist (naked) pIC (**Figure 4.01B**). As seen with CpG, cells trained with WGP had heightened TNF responses to pIC, showing that WGP trained immunity had no specificity to either DNA or RNA responses for enhanced TNF production. In order to test whether intracellular RIG-I Like receptors (RLRs) were also sensitive to WGP trained immunity, I transfected pIC (TpIC) in a liposome and stimulated BMDMs. Transfection of pIC enables the activation of RLRs such as MDA5 and RIG-I. Trained BMDMs also had heightened TNF responses to TpIC (**Figure 4.01C**). Finally, I also stimulated BMDMs with the TLR-7/8, RNA sensor, agonist R848. Again, I saw that trained macrophages had heightened TNF responses as compared to the untrained control (**Figure 4.01D**). This confirmed that WGP training enhanced TNF responses for both anti-viral and anti-bacterial immunity.

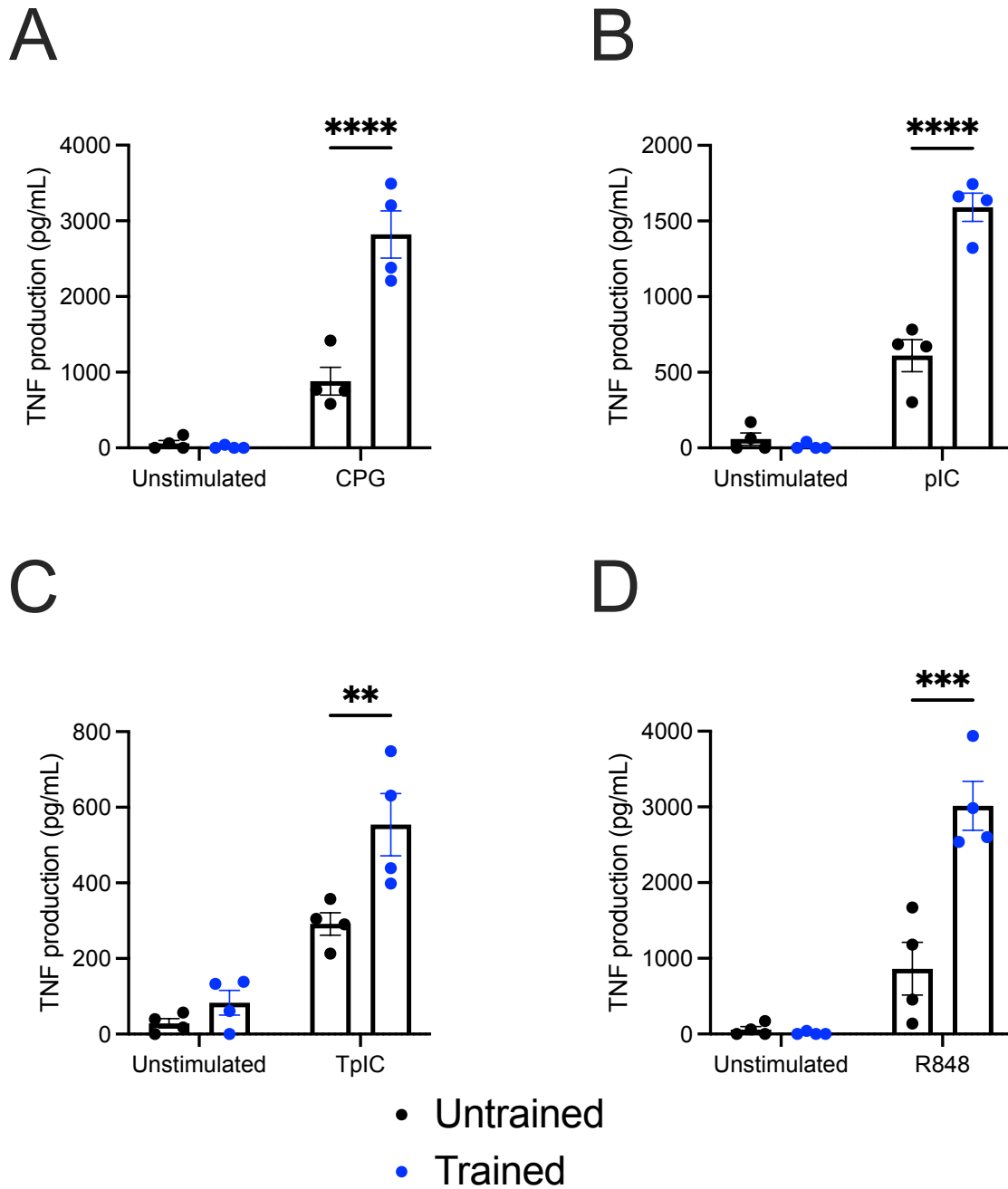


Figure 4.1 WGP enhances BMDM TNF production in response to different RNA and DNA agonists

BMDMs were treated 100ug/mL WGP or media control for a period of 24hrs. Cells were washed and allowed to rest for 6 days. BMDMs were then stimulated with various ligands, 1μM CpG (A), 10μg/mL pIC (B), 1μg/mL TpIC (C) and 5ng/mL R848 (D) for 24hrs followed by measurement of TNF via ELISA. Each dot is a representative of an individual experiment (Biological N = 4). **P<0.0078; ***P=0.0001; ****P<0.0001; (Paired Two-way ANOVA with Šídák's multiple comparison test).

4.2.2 WGP β -glucan training enhances anti-viral cytokine responses to viral ligands

Since I had seen that WGP training enhances TNF responses to viral ligands I next wanted to see if type I interferon (IFN1) responses were also sensitive to WGP training. In order to test this in WGP-trained BMDM I used various RNA and DNA ligands which are well known to drive a IFN1 response and measured IFN β by ELISA as a readout 24hrs after stimulation. Four IFN1 inducing ligands were selected, including pIC and TpIC, as before when measuring TNF responses. I also used a cyclic GMP-AMP synthase (cGAS) G3-ended Y-form short DNA (G3-YSD) and an agonist of stimulator of interferon genes (STING) known as 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) – strong specific inducers of type I interferons. I firstly showed that upon pIC stimulation, WGP trained BMDMs significantly increased their production of IFN β as measured by ELISA (**Figure 4.02A**). WGP trained BMDMs also had enhanced IFN β production 24hrs after stimulation with TpIC (**Figure 4.02B**). This data indicates that in response to RNA agonists WGP trained macrophages can not only enhance their ability to produce pro-inflammatory cytokines such as TNF but also they have an enhanced capacity to upregulate their production of IFN1. Next, I wanted to investigate whether WGP trained macrophages could enhance their IFN1 response to DNA agonists. To do this I used the two agonists previously mentioned, G3-YSD and DMXAA) to investigate the cGAS-STING response pathway to DNA antagonism. Surprisingly, when I stimulated macrophages with G3-YSD cells trained with WGP did not significantly enhance their IFN1 response (**Figure 4.02C**). This suggests that the cGAS pathway may not be sensitive to WGP training. However, when macrophages were stimulated with DMXAA they significantly increased their IFN1 response (**Figure 4.02D**). This specificity in responses to DNA agonists, especially in one pathway, suggests that WGP training may act downstream of cGAS and enhance STING responses independently of cGAS.

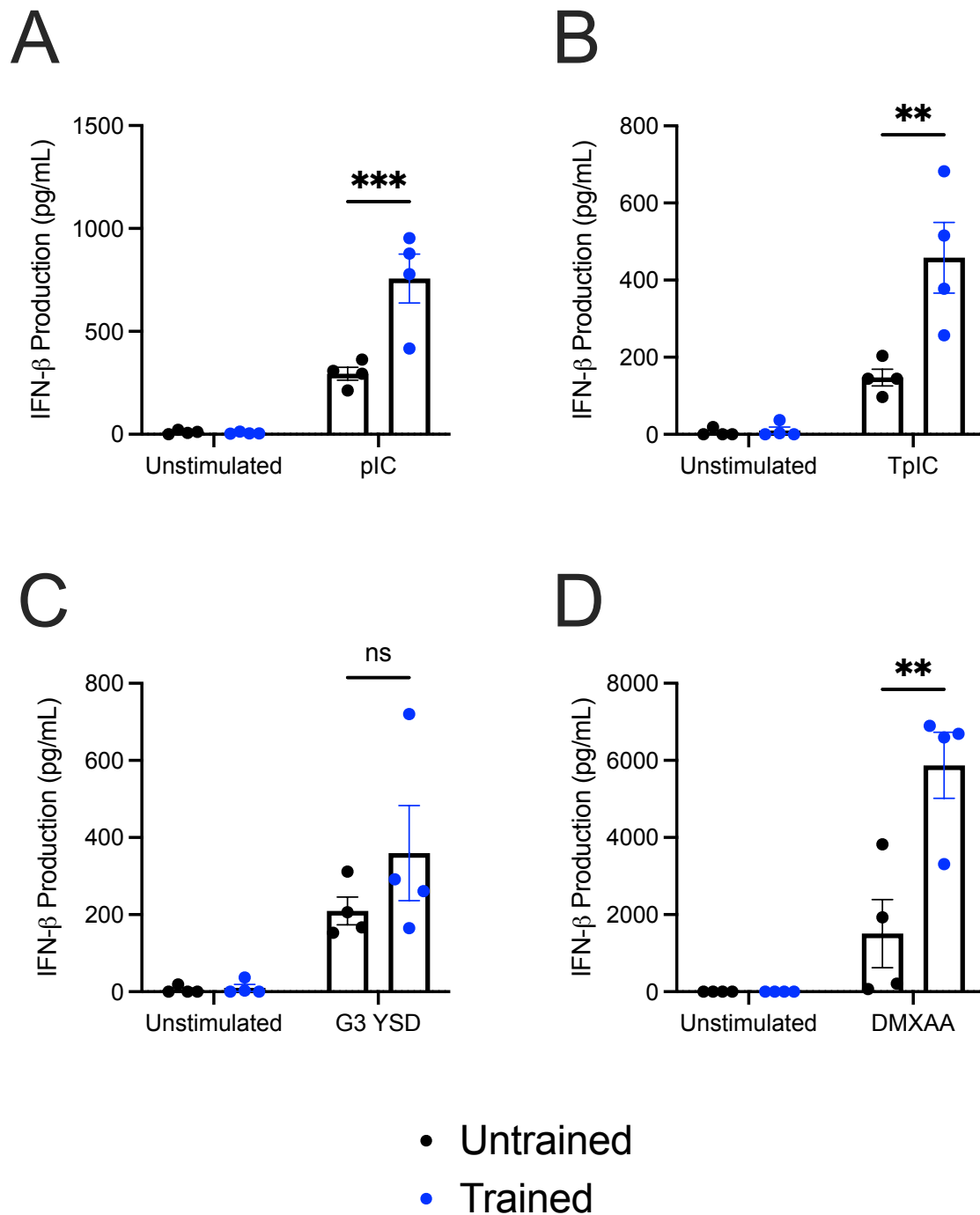


Figure 4.2 WGP enhances BMDM IFN- β production in response to different RNA and DNA agonists

BMDMs were treated 100ug/mL WGP or media control for a period of 24hrs. Cells were washed and allowed to rest for 6 days. BMDMs were then stimulated with various ligands, 10 μ g/mL pIC (A), 1 μ g/mL TplC (B), G3-YSD 1 μ g/mL (C) and DMXAA 100 μ g/mL (D) for 24hrs followed by measurement of IFN- β via ELISA. Each dot is a representative of an individual experiment (Biological N = 4). Ns= Not significant, **P<0.0078; ***P=0.0001; (Paired Two-way ANOVA with Šídák's multiple comparison test).

4.2.3 WGP training enhances overall responses to TLR-3 agonist poly I:C

Since I saw the most consistent results with pIC, I decided to use this ligand for further experiments to better characterize the molecular basis for this in more detail. I first wanted to understand the immune response of trained BMDMs to this ligand. An effective immune response to viruses requires a potent, yet balanced response. Therefore, I decided to measure the production of pro-inflammatory and anti-inflammatory cytokines as well anti-viral cytokine responses to pIC. As I have previously shown trained BMDMs enhance their TNF responses to pIC, as shown by significantly enhanced TNF responses (**Figure 4.03A**). The dichotomous cytokine IL-6, which plays a dual role in viral infections, had a trend towards significant enhancement in trained BMDMs (**Figure 4.03B, P-value = 0.069**). Therefore, in response to pIC trained BMDMs show an overall enhanced pro-inflammatory response.

Next, I wanted to see if trained BMDMs enhanced anti-inflammatory responses to viral ligands as well, since this balance between inflammation and repair is vital in macrophages during viral infection. First, I measured the anti-inflammatory cytokine IL-10. Trained BMDMs did not enhance their overall production of IL-10, however, importantly they also did not decrease their production and therefore maintained some sort of anti-inflammatory control (**Figure 4.03C**). I next measured the anti-inflammatory cytokine IL-1Ra. Two observations were made from this. Firstly, unstimulated trained BMDMs had enhanced IL-1Ra production versus their untrained unstimulated counterparts as seen previously (Fig 3.10D). Secondly, in response to pIC these trained cells still had enhanced IL-1Ra responses (**Figure 4.03D**). This data implies that trained cells are in a basal anti-inflammatory state prior to infection and that they can maintain or enhance anti-inflammatory responses during reactions to viral agonists.

Next, I wanted to see if trained BMDMs enhanced their broader anti-viral responses. As I have previously shown trained BMDMs enhance their production of IFN- β in response to pIC stimulation (**Figure 4.03E**). However, it is not just IFN- β that these cells can enhance in terms of an anti-viral response. I decided to measure IP-10,

also known as CXCL10, in trained cells. This chemokine is upregulated in response to IFN signalling and thus acts as an interferon stimulated gene (ISG). Trained BMDMs enhanced their production of this chemokine, showing that overall, these cells are anti-viral in nature (**Figure 4.03F**).

Finally, since cell number may affect cytokine output, I also measured the mRNA expression of *IFN- β* via qPCR (**Figure 4.04A**). *IFN- β* gene expression trended towards a significant increase in pIC stimulated trained BMDMs as compared with *IFN- β* gene expression in untrained BMDMs (p-value = 0.0616), highlighting that cytokine increases are not dependent on cell number changes and may arise as a result of increased transcriptional activity of ISGs.

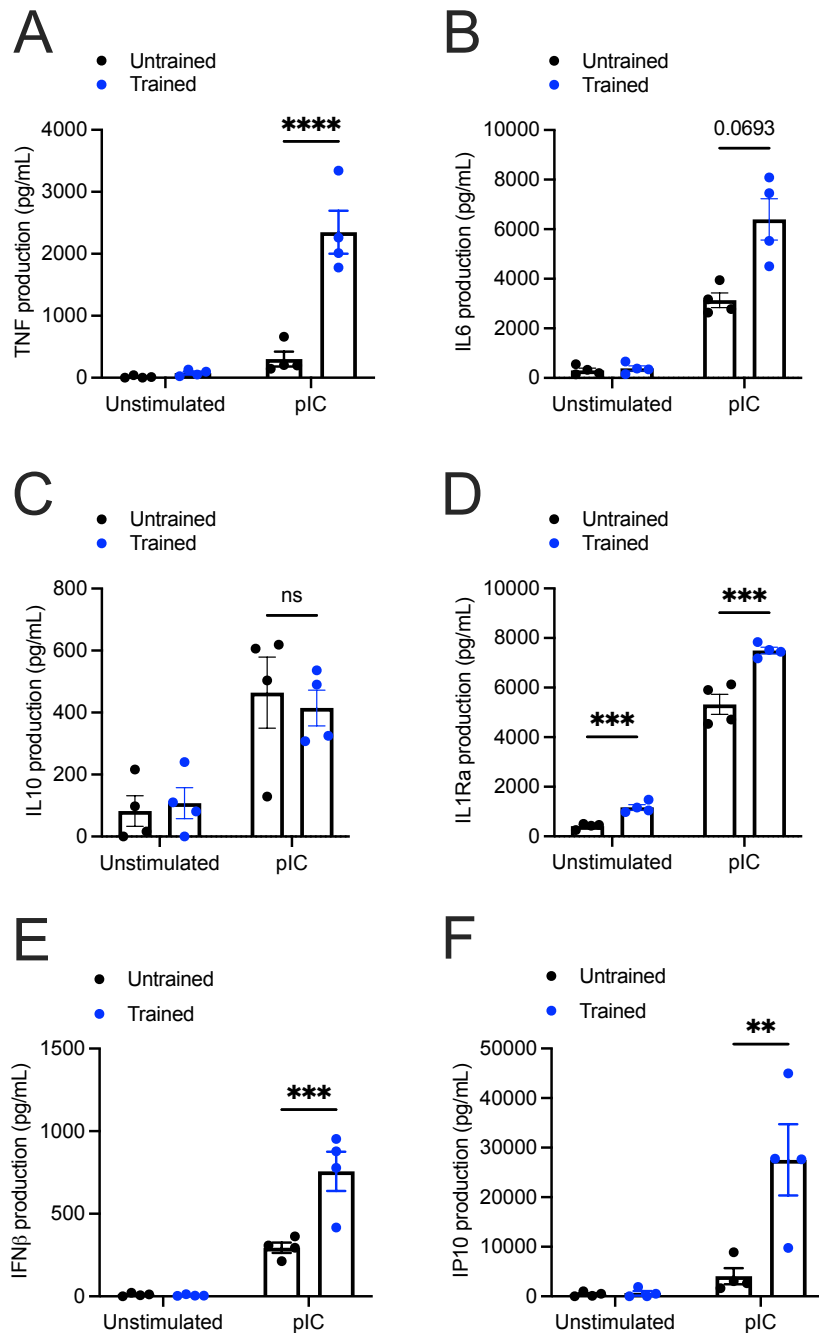


Figure 4.3 WGP training enhances production of overall immune responses to viral ligands in BMDMs

BMDMs were treated 100ug/mL WGP or media control for a period of 24hrs. Cells were washed and allowed to rest for 6 days. BMDMs were then stimulated with pIC for 24hrs followed by measurement of TNF (A), IL-6 (B), IL-10 (C), IL1-Ra (D), IFN- β (E) and IP-10 (F) via ELISA. Each dot is a representative of an individual experiment (Biological N = 4). Ns= Not significant, **P<0.0078; ***P=0.0001; (Paired Two-way ANOVA with Šídák's multiple comparison test).

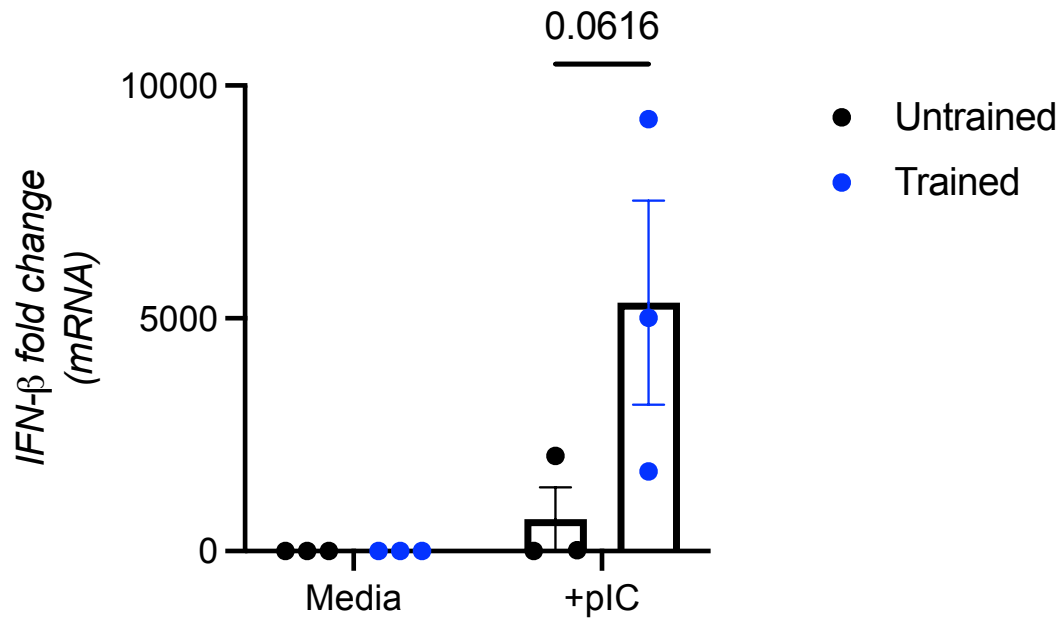


Figure 4.4 mRNA Trained response to pIC secondary challenge showing enhanced IFN- β mRNA expression 4hrs post stimulation

mRNA expression was measured from untrained vs trained BMDMs in response to 10 μ g/mL pIC. BMDMs were treated with 100 μ g/mL WGP β -glucan for a period of 24hrs and left to rest for 6 days before a secondary challenge with pIC. RNA was extracted from BMDMs 4hrs post pIC stimulation and IFN- β gene expression was measured via qPCR. Each dot is a representative of an individual experiment (Biological N = 3). Exact P-value shown; (Paired Two-way ANOVA with Šídák's multiple comparisons test).

4.2.4 Trained BMDMs are anti-viral and are less susceptible to viral infections

Having seen that BMDMs trained with WGP enhance their responses to viral ligands I hypothesized that these cells will be more anti-viral and be better protected from viral infection. In order to test this hypothesis two viral models of infection were used, including the double-stranded (ds) DNA virus *Herpes simplex virus 1* (HSV1) and the negative sense single stranded (-SS) RNA virus *Vesicular stomatitis virus* (VSV). BMDMs were trained as normal and were lifted and counted on the day of viral infection to ensure an accurate multiplicity of infection (MOI). I first measured the ability of infected cells to mount an IFN- β response to both viruses. HSV1 infection induced type I interferon responses in both untrained and trained BMDMs, however, trained cells had significantly increased levels of IFN- β production in comparison to untrained BMDMs (**Figure 4.05A**). Similarly, VSV infection induced the production of IFN- β in BMDMs in response to the virus in both untrained and trained cells. However, trained BMDMs again had a significantly heightened IFN response in comparison with untrained BMDMs (**Figure 4.05B**). This data confirms that trained macrophages enhance their type I IFN responses to both viral ligands, as well as live viral infection.

However, enhanced IFN production is not a suitable readout for viral containment, viruses have evolved mechanisms of subverting this response. Therefore, I decided to measure the expression of viral mRNA that were found within infected cells relative to uninfected cells. In this way I was able to quantify whether WGP trained immunity could protect macrophages from viral infection. BMDMs were readily infected by HSV1 in untrained conditions, however, there was a significant reduction in the viral genome in the trained cells (**Figure 4.05C**). This shows that trained BMDMs have heightened anti-viral responses to DNA viruses. I also saw a significant reduction in the viral genome number in trained cells infected with VSV (**Figure 4.05D**). Taken together this data indicates that WGP trained immunity protects macrophages from infection from both DNA and RNA viruses.

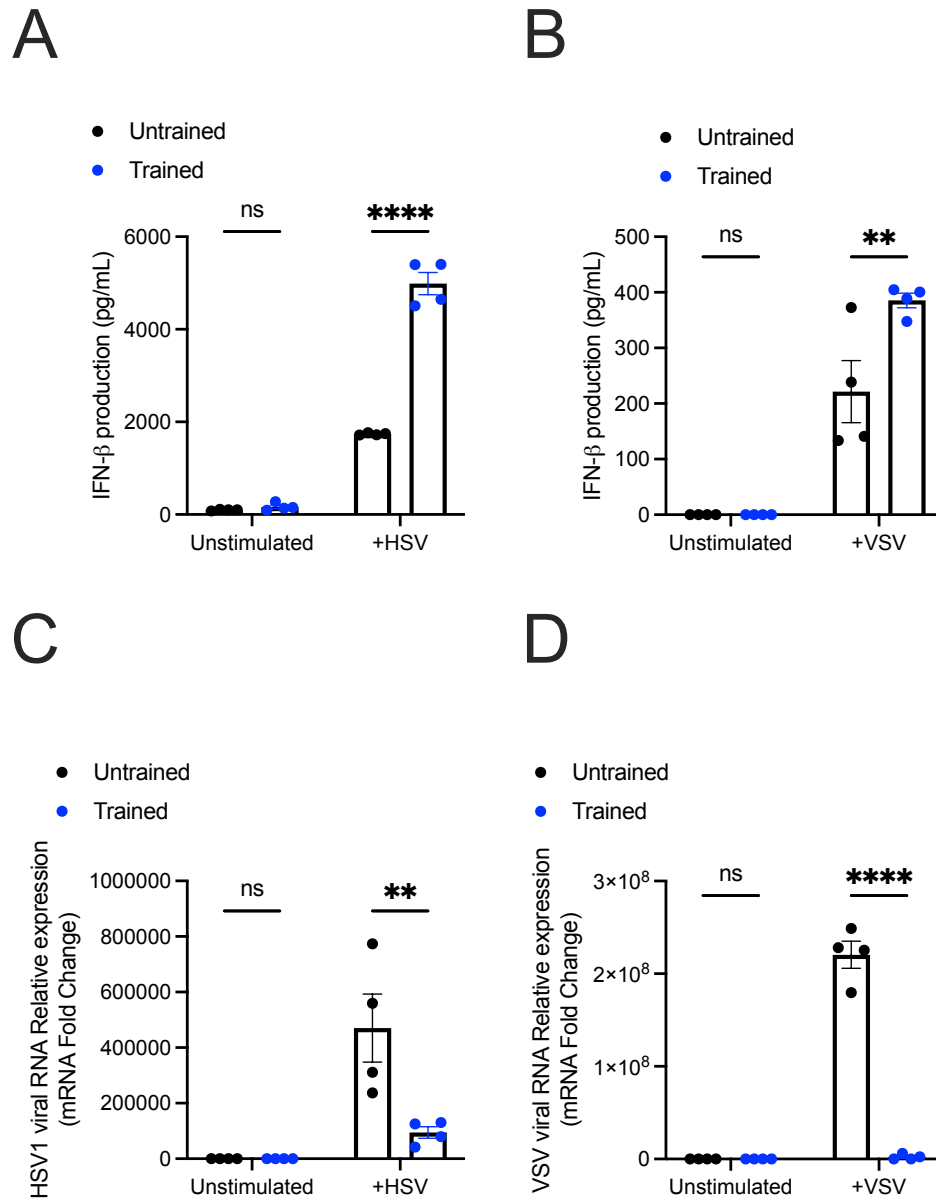


Figure 4.5 WGP training enhances type I IFN production during viral infection and protects against both DNA and RNA viral infections in-vitro

BMDMs were treated 100ug/mL WGP or media control for a period of 24hrs. Cells were washed and allowed to rest for 6 days. On day 6 BMDMs were replated into 24 well plates at a concentration of 500,000 cells per well. Cells were infected for 24hrs with an MOI of 10 (HSV) and an MOI of 1 (VSV) respectively. After 24hrs supernatant was removed for cytokine analysis and RNA was frozen for qPCR. IFN- β production was measured (A-B) and expression of viral RNA was measured by qPCR (C-D). Each dot is a representative of an individual experiment (Biological N = 4). Ns= Not significant, **P= <0.0064 ; ****P=0.0001; (Paired Two-way ANOVA with Šídák's multiple comparison test).

4.2.5 WGP β -glucan training is associated with wide transcriptional changes in BMDMs

Having identified that WGP could induce both classical and non-classical antiviral immunity I wanted to identify a transcriptional phenotype that could explain these enhanced responses. To do this I utilised a workflow as shown (**Figure 4.06**) where I utilised next-generation bulk RNA-sequencing. RNA was extracted from BMDMs trained +/- WGP as previously described 144hr post WGP stimulation as well as 4hrs post pIC stimulation. Samples were sequenced by Novogene and analysed. Analysis of pIC treated groups were performed in-house. Here I wanted to explore the transcriptional changes that occur at the end of the training process. Papers focusing on the transcriptional changes induced by trained immunity either focus on initial changes 24hrs post treatment or during the secondary stimulation, often with LPS²¹², or making Two-Way comparisons with unstimulated and stimulated groups on day 6 masking any effects that β -glucan alone might have on the RNA signature¹⁹³. Therefore, I wanted to investigate the impact WGP has on the transcriptional profile of macrophages, without any other comparisons as well as investigating the genes that are enhanced rapidly in WGP trained macrophages upon stimulation with pIC (4hrs). Firstly, I looked at the variation between treated and untreated groups as well as variation among biological replicates (N=4). To do this, I utilized principal component analysis (PCA) (**Figure 4.07**). In short, this analysis allows me to analyse a high dimensional data set and capture the most important data from this.

As shown from the graph, the transcriptomes of WGP treated macrophages were significantly different to that of untreated controls (**Figure 4.07**). This was identified based off PC1 with 93% variation between groups. In contrast, there was very little differences between PC2, with only a 3% difference, showing that within groups there was little changes amongst biological replicates.

Having identified that WGP treatment causes a change in the transcriptional landscape of BMDMs, I next analysed the changes based on differentially expressed genes using DeSeq2 analysis (**Figure 4.08**). Here, I set the pAdjusted

cut off to ≤ 0.05 and the Log_2 Fold change cut off to ≥ 1 . This data was expressed as a volcano plot. Overall, this analysis revealed 643 significantly upregulated genes and 798 downregulated genes in the WGP trained BMDMs with 20,274 genes not being significantly altered.

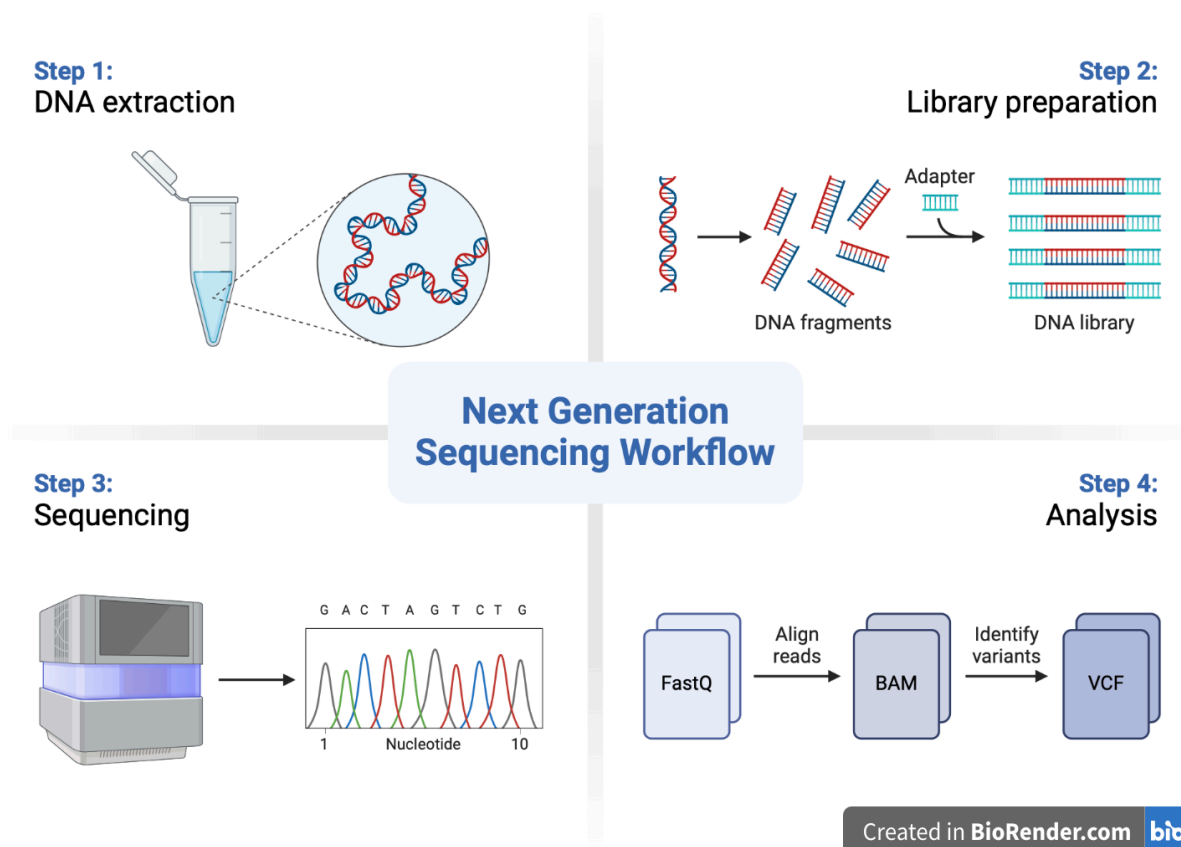


Figure 4.6 Graphical representation of RNA-seq workflow performed by Novogene

Samples were prepared and shipped in house as previously described. Upon delivery RNA was tested via quality control steps as described by Novogene and converted into complimentary DNA (cDNA). Libraries were prepared via mRNA enrichment, poly (A) enrichment and RNA fragmentation and reverse transcription to cDNA. Samples were then sequenced using Illumina NovaSeq platforms. Downstream analysis was then performed including Desq2 analysis, principal component analysis (PCA), GO enrichment, KEGG pathway analysis and Reactome analysis. This was performed either by Novogene or in-house. In-house analysis was performed using R-Studio and codes are listed in material and methods section.

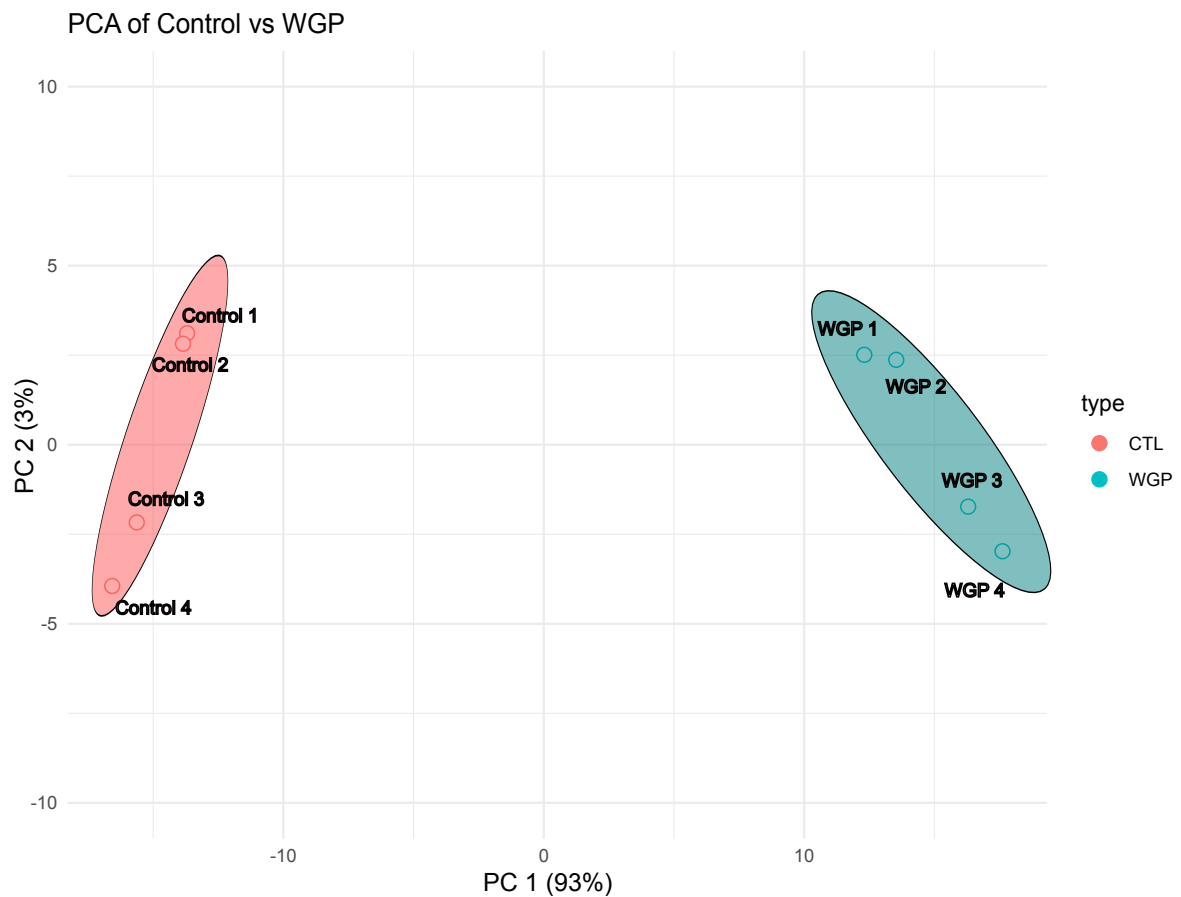


Figure 4.7 Principal component analysis of BMDMs 6 days post WGP mediated training

Principal component analysis (PCA) reveals major differences in RNA transcriptomes between media treated or WGP trained BMDMs (N = 4). PCA reveals no major outliers within biological replicates of both untrained and trained groups.

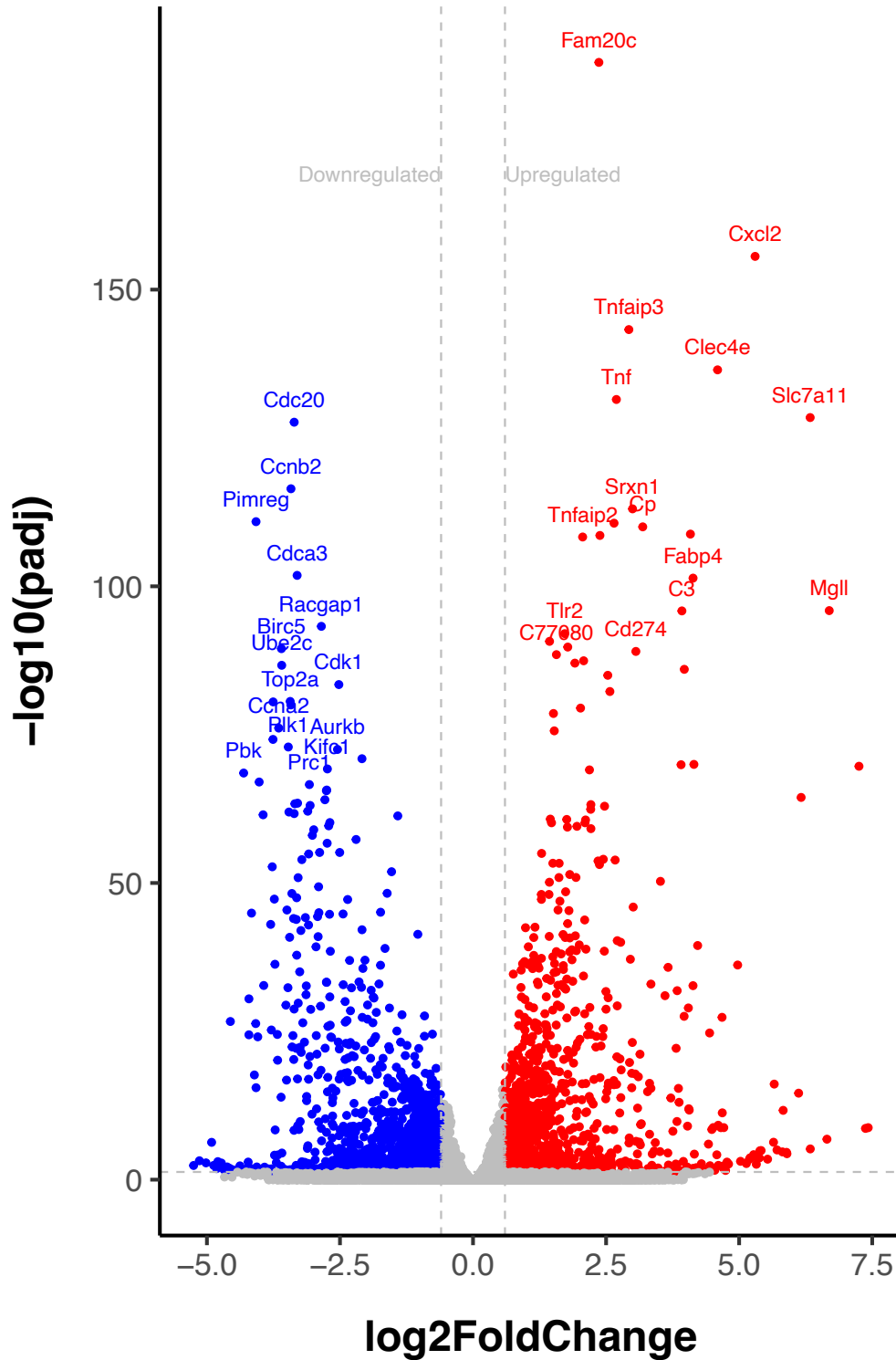


Figure 4.8 Volcano plot showing the differentially expressed genes (DEGs) between WGP vs Media treated BMDMs

Volcano plot highlights DEGs that are upregulated (Red) in WGP trained macrophages at baseline as well as DEGs that are downregulated (Blue) during WGP training. Genes in grey do not reach statistical significance. Upregulated genes (643), Downregulated genes (798) and no significant differences (20,274). Log2Fold change cut off ≥ 1 and pAdjusted cut off ≤ 0.05 . Biological replicates N = 4

4.2.6 Gene Ontology enrichment analysis reveals processes that are significantly altered due to WGP mediated trained immunity

Having seen that WGP training induces changes in gene expression I wanted to further analyze this data using Gene Ontology (GO) enrichment analysis. In short, GO allows us to identify the biological processes into which the significantly altered genes fall into. GO analysis revealed significant upregulated and downregulated processes into which the differentially expressed genes (DEGs) fell into (**Figure 4.09**). The top enriched processes included responses to bacteria, adaptive immune responses, T cell activation and positive regulation of cytokine production. There were also downregulated processes. The top downregulated processes included chromosome segregation, organelle fission, nuclear division and extracellular matrix.

Next having seen that there were significant up and downregulated processes I also performed gene set enrichment analysis (GSEA) on the genes from gene ontology. This revealed several processes that were significantly upregulated in the WGP treated group. For simplicity, I selected 4 gene sets from this. Processes enriched in WGP treated BMDMs included acute inflammatory responses (**A**), cytokine production involved in immune responses (**B**), response to external biotic stimulus (**C**) and I Kappa Kinase NF kappa signalling (**D**) (**Figure 4.10 A-D**).

Since these enriched processes contain at least 100 genes I decided to show the top upregulated genes from each data set as a heat map with any downregulated genes from this data set shown as a dot chart. The first biological process I investigated was the acute inflammatory response (**Figure 4.11**). Inflammatory genes of interest here include cytokine genes such as *Tnf*, *Il1a* and *Il1b* and chemokines such as *Cxcl1* and *Ccl5*. Also of interest were the significant increase in the metabolic gene *Ass1* as well as the tissue factor gene *F3*. As well as these upregulated genes, there were also 4 significantly downregulated genes in the “acute inflammatory response” process. These included *Alox5*, *Sigirr*, *Ccr5* and *Cd163* (**Figure 4.12A-D**).

The next process identified was cytokine production in immune responses (**Figure 4.13**). There were similar overlaps from the previous gene set, especially pro-inflammatory cytokines such as *Tnf* and *Il1b*. There was also some increased expression of receptor genes associated with inflammation including *Cd55*, *Gprc5b* and *Tnfrsf1b*. Also seen were increases in genes associated with haem metabolism such as *Hmox1* and *Acp5*. Nucleotide-binding oligomerisation domain-like receptor signalling were also increased with higher gene expression of the intracellular bacterial and viral receptors *Nod1* and *Nod2* as well as downstream effector signalling genes such as *Ripk2*. Similarly, there was an increase in genes that encode nucleic acid sensing receptors, such as *Ddx58*, and genes that encode anti-viral proteins, such as *Ddx21*. However, there were also 9 downregulated genes from this data set as well. These included genes that encode for chemokine receptors such as *Ccr2*, genes that encode for anti-inflammatory cytokines such as *Il10* and *Il4* and genes involved in iron storage such as *Hfe* (**Figure 4.14A-D**).

Surprisingly, I also saw that WGP stimulation led to a significant enrichment of genes associated with NF- κ B signalling by day 6 (**Figure 4.15**). This is interesting since I have shown that WGP does not induce NF- κ B signalling during the initial 24hr stimulation with β -glucan and the accepted model of Training is that cells return to baseline inflammatory activity following exposure to primary stimuli. These results indicate a more long-term transcriptional imprinting can occur in response to training stimuli. Important genes of interest here include genes that encode regulators of NF- κ B signalling such as *Trim13*, *Nfkbia*, *Nfkbib* and *Tnfrsf1b*. Genes that were involved in the NF- κ B signalling cascade were also enriched including *Relb*, *Malt1* and *Irk2*. There were also a couple of genes involved in anti-viral responses including *Ikbke*, *Irf1* and *Optn*. There was also significant reduction in genes associated with negative regulation of the inflammatory cascade, such as *Trim59*, as well as genes associated with cell cycle and death, *S100a4*, *Pidd1* and *Ripk3* (**Figure 4.16A-D**).

Taken together this data confirms previous results in chapter 3, with increased expression of inflammatory pathways. This may point towards a reason for the increased responses discussed in chapter 3.

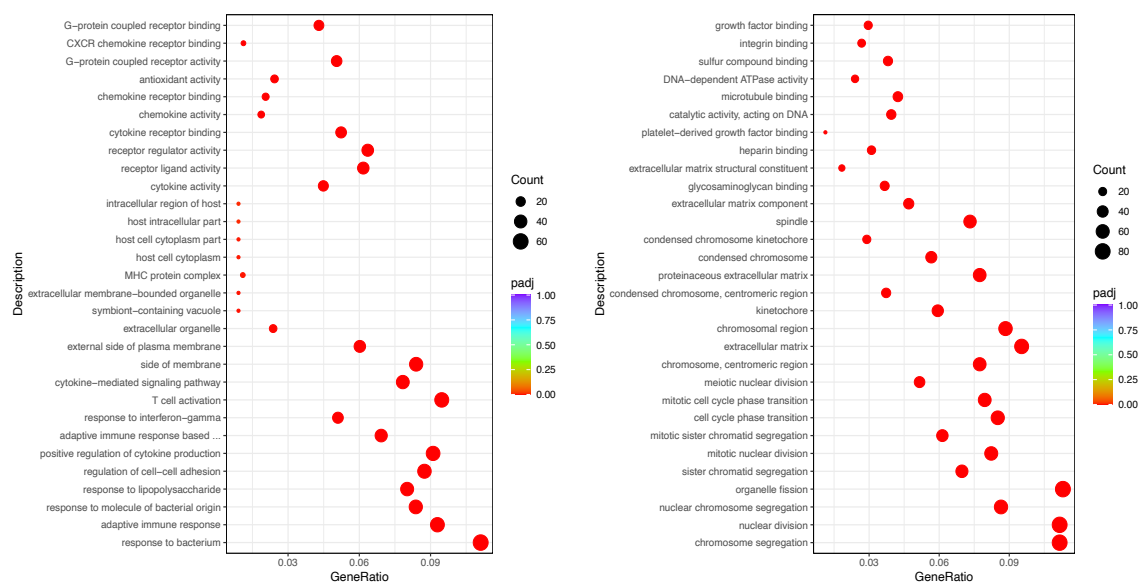
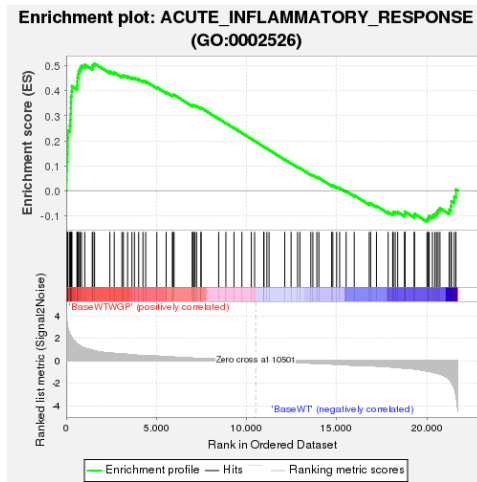


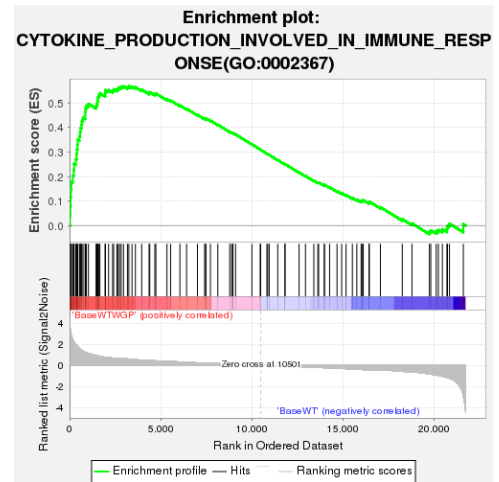
Figure 4.9 Gene ontology analysis showing the top 30 upregulated and downregulated processes involved in WGP mediated trained immunity

Dot plot highlights DEGs in processes that are upregulated (Left) in WGP trained macrophages at baseline as well as DEGs in processes that are downregulated (Right) during WGP mediated training. N = 4.

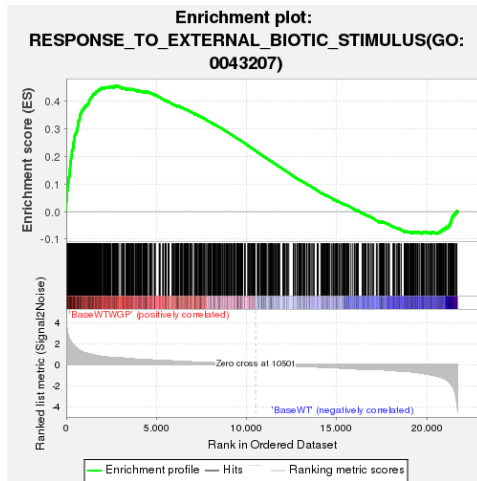
A



B



C



D

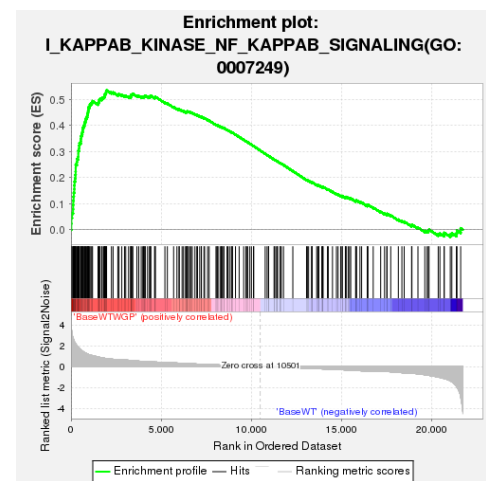


Figure 4.10 Gene ontology enrichment plots showing increased genes in different signalling pathways in trained BMDMs

GO analysis reveals various immunological processes that are upregulated in WGP mediated trained immunity. (A) Enrichment plot showing heightened gene expression associated with acute inflammatory responses in BMDMs. (B) Enrichment plot showing increased gene expression associated with cytokine production involved in immune responses. (C) Enrichment plot showing increased gene expression associated with responses to external biotic stimulus. (D) Enrichment plot showing increased gene expression associated with I Kappa B kinase (NF- κ B) signalling. N = 4.

WGP Media

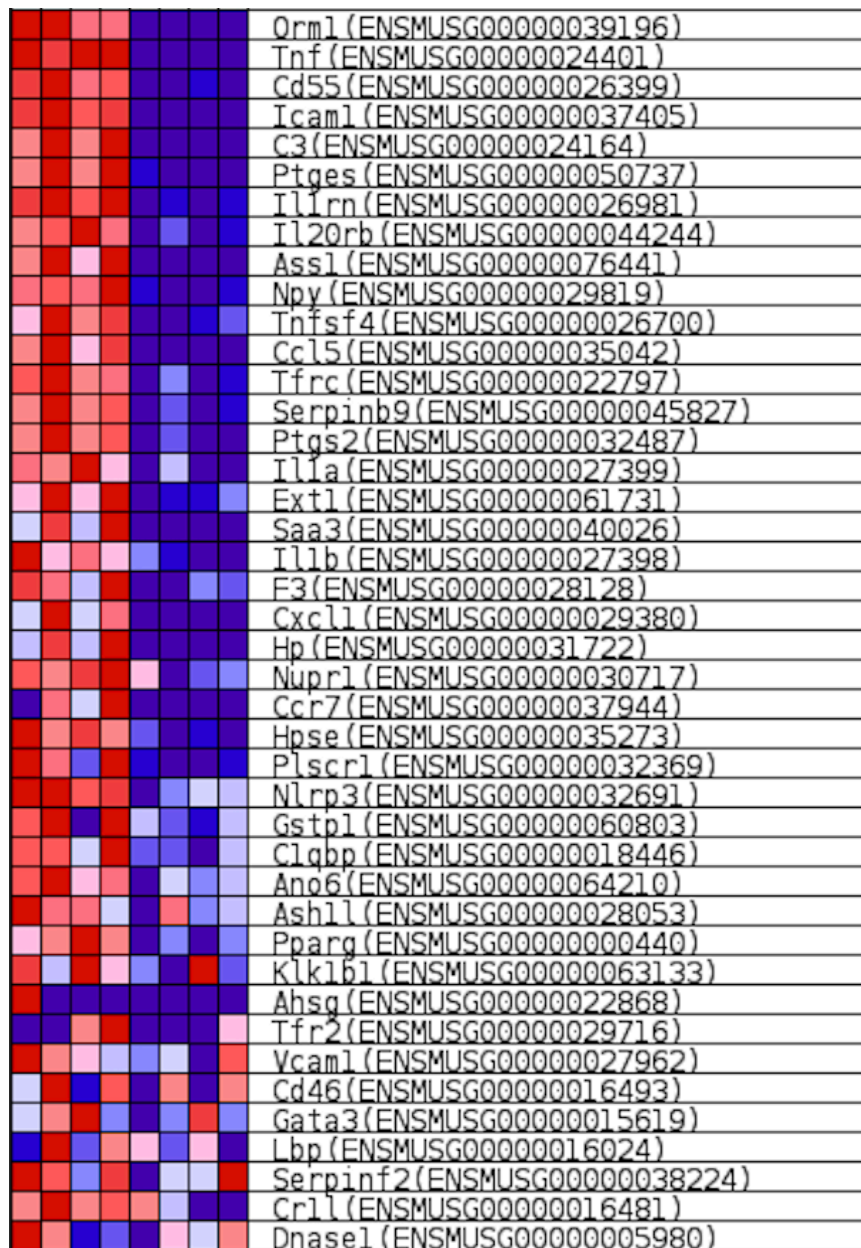


Figure 4.11 Heatmap showing genes expressed in WGP trained BMDMs under the immune process Acute inflammatory response

GO analysis reveals gene expression in WGP mediated trained immunity. Heatmap shows WGP trained vs untrained BMDMs and upregulated genes shown are part of the acute inflammatory response. N = 4.

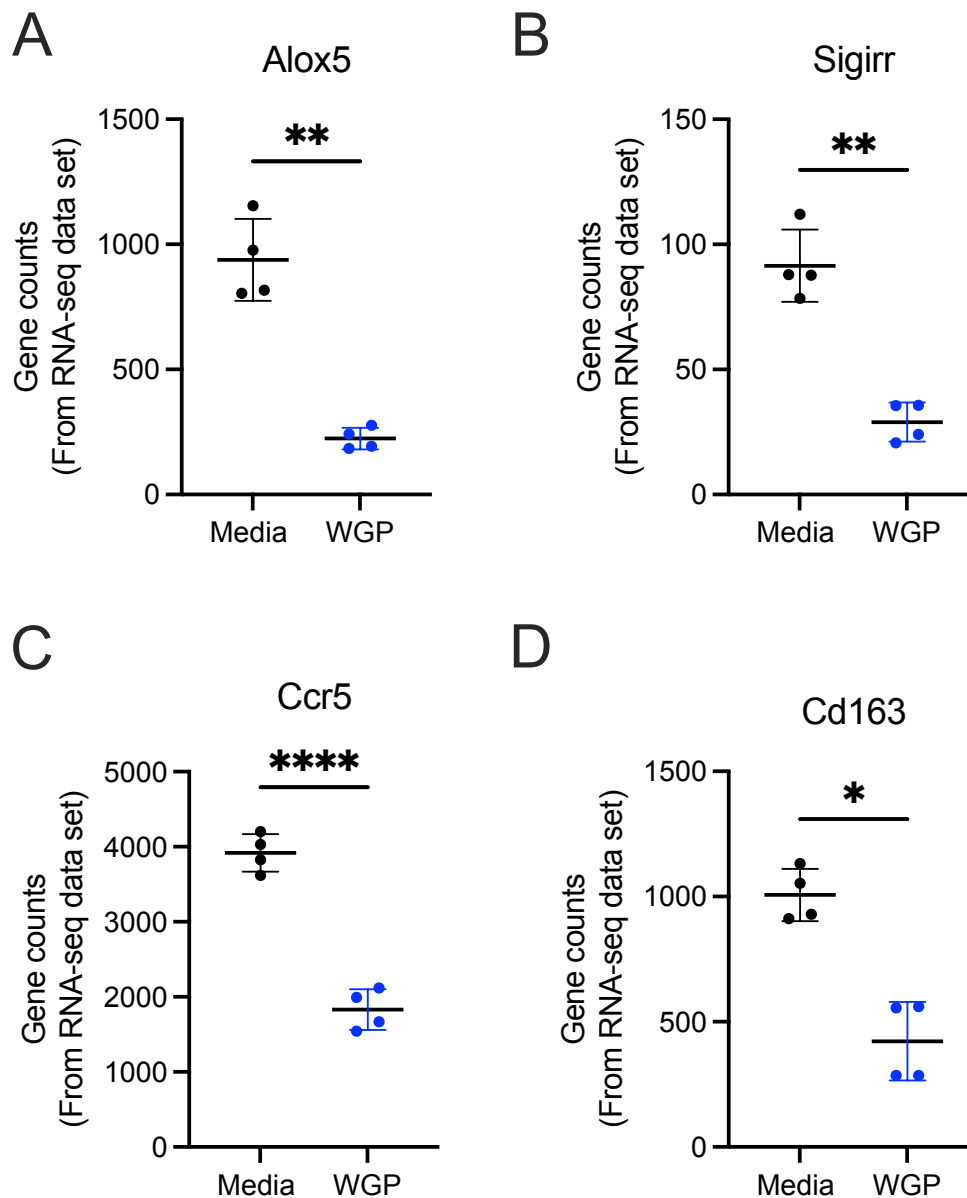


Figure 4.12 Downregulated genes from GO analysis involved in the acute inflammatory response processes

GO analysis reveals gene expression in WGP mediated trained immunity. (A) Raw gene count taken from RNA-seq data analysis showing expression of *Alox5*. (B) Raw gene count taken from RNA-seq data analysis showing expression of *Sigirr*. (C) Raw gene count taken from RNA-seq data analysis showing expression of *Ccr5*. (D) Raw gene count taken from RNA-seq data analysis showing expression of *Cd163*. Paired student T-test. N = 4. *P-value <0.0332, **P-value <0.0021 & ***P-value <0.0001.

WGP Media

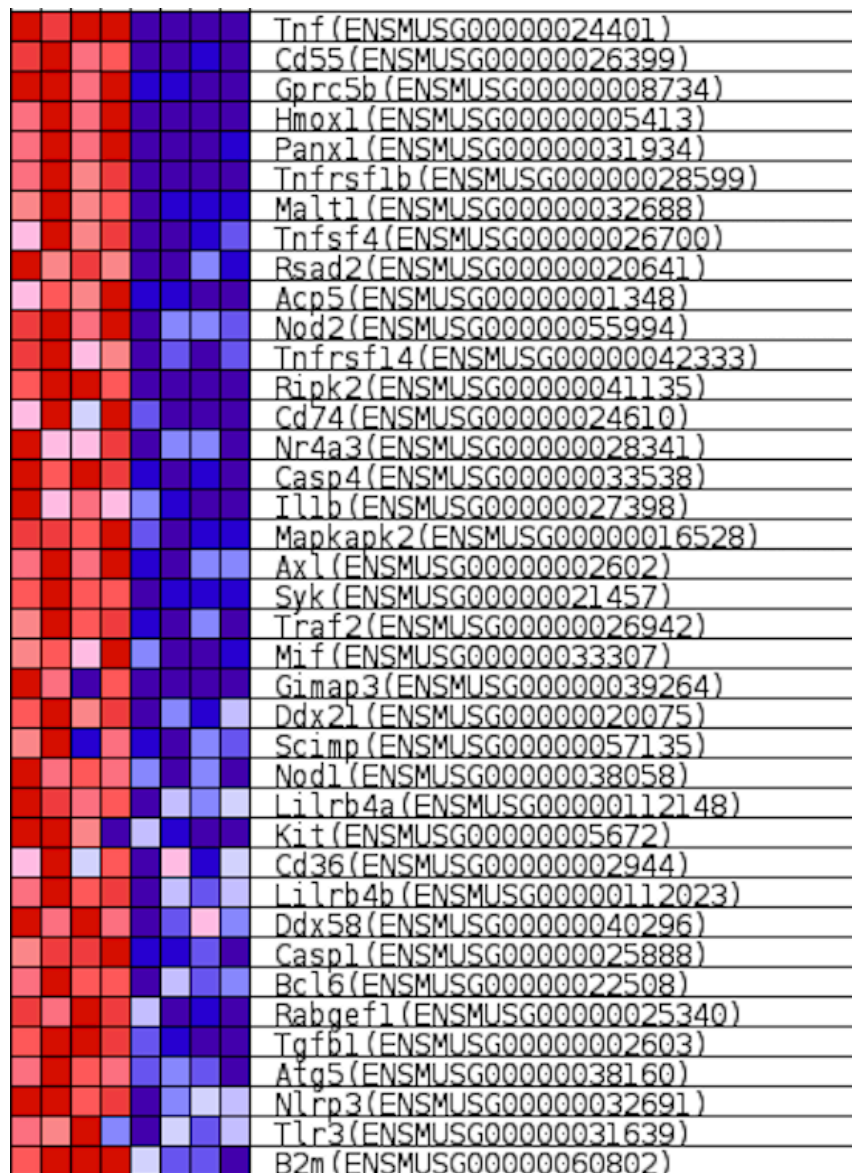


Figure 4.13 Heatmap showing genes expressed in WGP trained BMDMs under the immune process Cytokine production involved in immune response

GO analysis reveals gene expression in WGP mediated trained immunity. Heatmap shows WGP trained vs untrained BMDMs and upregulated genes shown are part of the cytokine production in immune response processes. N = 4.

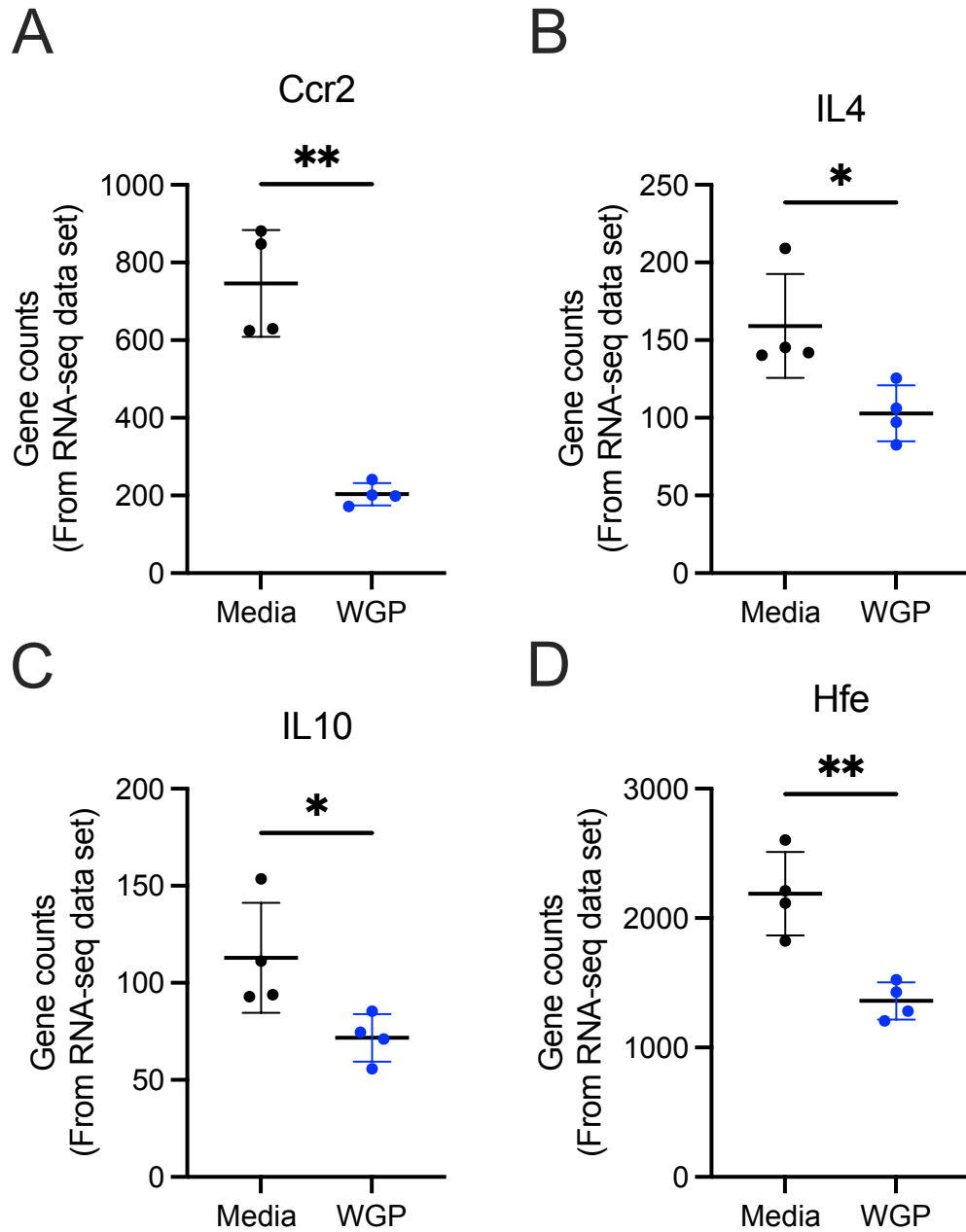


Figure 4.14 Downregulated genes from GO analysis involved in the acute inflammatory response processes

GO analysis reveals gene expression in WGP mediated trained immunity. (A) Raw gene count taken from RNA-seq data analysis showing expression of *Ccr2*. (B) Raw gene count taken from RNA-seq data analysis showing expression of *Il4*. (C) Raw gene count taken from RNA-seq data analysis showing expression of *Il10*. (D) Raw gene count taken from RNA-seq data analysis showing expression of *Hfe*. Paired student T-test. N = 4. *P-value <0.0332 & **P-value <0.002.

WGP Media

						Tnfrsf3(ENSMUSG000000019850)
						Tnfrsf3(ENSMUSG000000024401)
						Gprc5b(ENSMUSG000000008734)
						Trim13(ENSMUSG000000035235)
						Nfkb1a(ENSMUSG000000021025)
						Tlr2(ENSMUSG000000027995)
						Tnfrsf3(ENSMUSG000000044162)
						Relb(ENSMUSG000000002983)
						Tnfrsf1(ENSMUSG000000020400)
						Irak2(ENSMUSG000000060477)
						Ikbke(ENSMUSG000000042349)
						Malt1(ENSMUSG000000032688)
						Cd4(ENSMUSG000000023274)
						Traf3(ENSMUSG000000021277)
						Tfrc(ENSMUSG000000022797)
						Traf1(ENSMUSG000000026875)
						Irf1(ENSMUSG000000018899)
						Sqstm1(ENSMUSG000000015837)
						Lpar1(ENSMUSG000000038668)
						Nod2(ENSMUSG000000055994)
						Clec4n(ENSMUSG000000023349)
						Ripk2(ENSMUSG000000041135)
						Clec4d(ENSMUSG000000030144)
						Bcl3(ENSMUSG000000053175)
						Cd74(ENSMUSG000000024610)
						Ppm1n(ENSMUSG000000030402)
						Optn(ENSMUSG000000026672)
						Illa(ENSMUSG000000027399)
						Saa3(ENSMUSG000000040026)
						Illb(ENSMUSG000000027398)
						Nfkb1b(ENSMUSG000000030595)
						Tank(ENSMUSG000000064289)
						Zc3h12a(ENSMUSG000000042677)
						Cflar(ENSMUSG000000026031)
						Cttnb1(ENSMUSG00000006932)
						Htr2b(ENSMUSG000000026228)
						Traf2(ENSMUSG000000026942)
						Ripk3(ENSMUSG000000024404)
						Cd40(ENSMUSG000000017652)
						Prkcb(ENSMUSG000000052889)
						Mtdh(ENSMUSG000000022255)
						Nkiras1(ENSMUSG000000021772)
						Nfkb1d(ENSMUSG000000036931)
						Ddx21(ENSMUSG000000020075)
						Tnfrsf10(ENSMUSG000000039304)
						Gapdh(ENSMUSG000000057666)
						Edn1(ENSMUSG000000021367)
						Ltf(ENSMUSG000000032496)
						Nod1(ENSMUSG000000038058)
						Lilrb4a(ENSMUSG000000112148)
						Cd36(ENSMUSG000000002944)
						Ikbkg(ENSMUSG000000004221)
						Lilrb4b(ENSMUSG000000112023)
						Tle1(ENSMUSG000000008305)
						Sirpa(ENSMUSG000000037902)
						Nup62(ENSMUSG000000109511)
						Tlr6(ENSMUSG000000051498)
						Perl(ENSMUSG000000020893)
						Rela(ENSMUSG000000024927)
						Stat1(ENSMUSG000000026104)
						Trim30d(ENSMUSG000000057596)
						Casp1(ENSMUSG000000025888)
						Usp25(ENSMUSG000000022867)
						Peli1(ENSMUSG000000020134)
						Usp10(ENSMUSG000000031826)
						Tlr3(ENSMUSG000000031639)

Figure 4.15 Heatmap showing genes expressed in WGP trained BMDMs under the immune process I Kappa B Kinase NF- κ B signalling

GO analysis reveals gene expression in WGP mediated trained immunity. Heatmap shows WGP trained vs untrained BMDMs and upregulated genes shown are part of the I Kappa B Kinase NF κ B signalling processes. N = 4.

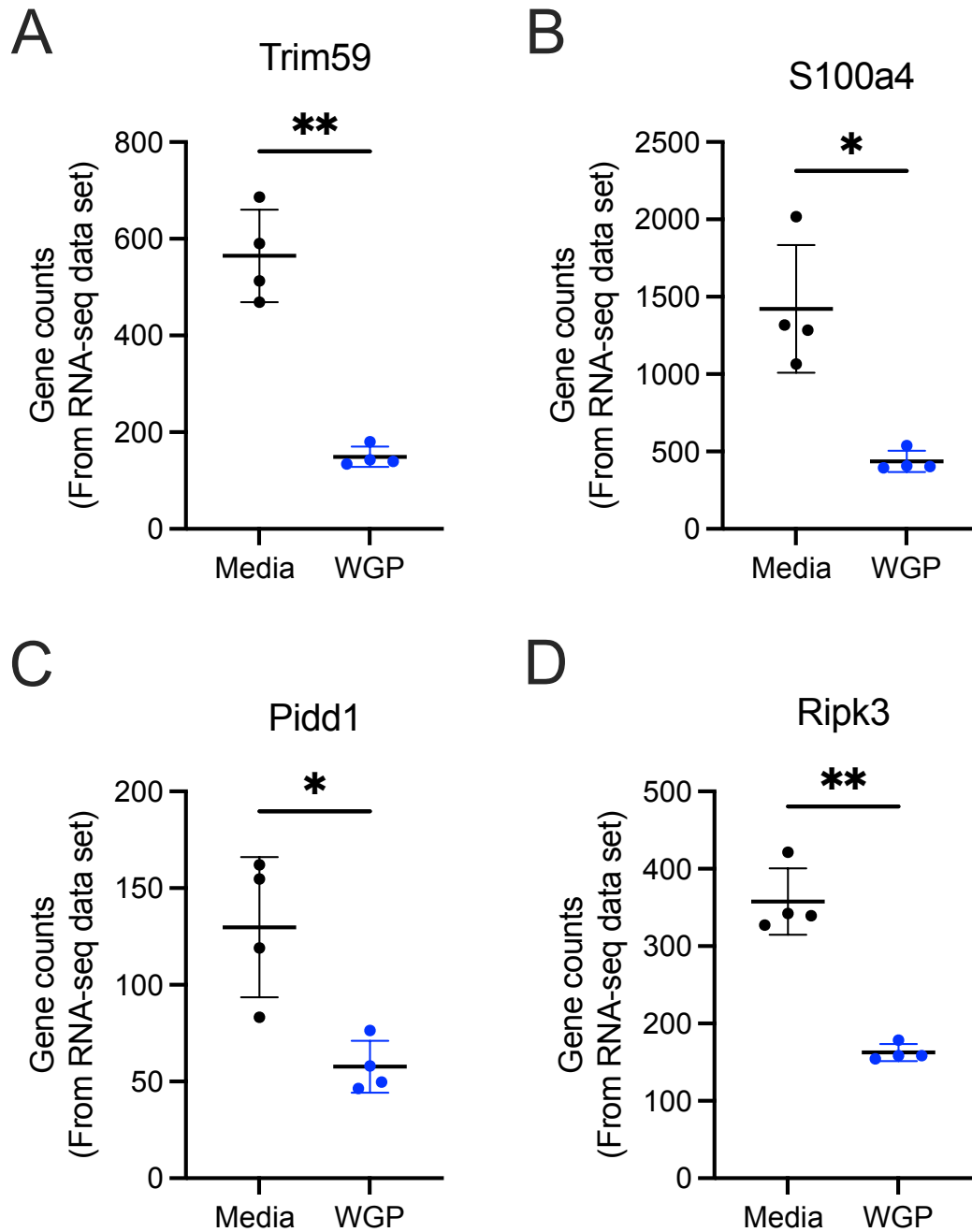


Figure 4.16 Downregulated genes from GO analysis involved in I Kappa B Kinase NF- κ B signalling process

GO analysis reveals gene expression in WGP mediated trained immunity. (A) Raw gene count taken from RNA-seq data analysis showing expression of *Trim59*. (B) Raw gene count taken from RNA-seq data analysis showing expression of *S100a4*. (C) Raw gene count taken from RNA-seq data analysis showing expression of *Pidd1*. (D) Raw gene count taken from RNA-seq data analysis showing expression of *Ripk3*. Paired student T-test. N = 4. *P-value <0.0332 & **P-value <0.0021.

4.2.7 Kyoto Encyclopaedia of Genes and Genome analysis reveals pathways that are significantly altered due to WGP mediated trained immunity

To get a better understanding of the precise biological pathways involved in WGP mediated trained immunity I used Kyoto Encyclopaedia of Genes and Genome analysis. This form of analysis allowed me to identify the top upregulated and downregulated biological processes that occur 6 days post WGP stimulation and highlight the top genes that are altered prior to restimulation. This approach allows for a better understanding of why trained BMDMs have a heightened response.

KEGG analysis revealed several interesting pathways that were altered 6 days post WGP stimulation. Similar to what was revealed by GO analysis pathways such as NF κ B signalling, cytokine-cytokine interactions and TNF signalling were all enhanced (**Figure 4.17A**). However, there were also a number of biological pathways upregulated that have already been associated with trained immunity including lipid and atherosclerosis, C-type lectin receptor signalling pathway and tuberculosis (**Figure 4.17A**). Also upregulated were pathways that have not yet been associated with trained immunity; however, these pathways are linked with infiltrating inflammatory innate immune cells such as monocytes and neutrophils and thus maybe linked with innate immune training. These include Rheumatoid arthritis, allograft rejection and type 1 diabetes mellitus (**Figure 4.17A**). Finally, there are also non-classical pathways that were found to be enriched which were associated with viral infection and type 1 interferon responses. These included Epstein-Barr virus infection, Staphylococcus aureus infection and viral protein interaction with cytokine and cytokine receptor. KEGG analysis also revealed a number of downregulated pathways involved in WGP mediated trained immunity including cell cycle, PI3K-AKT signalling pathway and focal adhesion, however, for the purpose of my thesis I decided to concentrate on the upregulated pathways (**Figure 4.17B**).

I also identified HIF-1 signalling pathway as upregulated in my data set and since this has been associated with trained immunity, I decided to investigate the genes involved in this pathway as well. Enrichment plots were generated in R studio using

GSEA revealing significant positive correlation between C-type lectin receptor signalling pathway, HIF-1 signalling pathway, lipid and atherosclerosis, tuberculosis and trained BMDMs (**Figure 4.18A-D**). To better understand the genes involved in these pathways I generated KEGG analysis plots to present visually significantly altered genes of interest in each data set. Since there were genes that might overlap between pathways, I selected 4 genes randomly that had no overlap between data sets and presented them as gene counts showing significant changes between untrained (Media treated) and trained (WGP treated) groups.

The first pathway of interest was C-type lectin receptor signalling pathway. KEGG analysis plots revealed several genes that were significantly enriched (**Figure 4.19**). Many of these genes were either associated with different C-type lectin receptors (CLR), such as Dectin-1 and Dectin-2 or with inflammatory processes such as TNF or IL1 β . The DNA-binding transcription factor *Erg2/3* was significantly altered. There was also an increase in the expression of the mitochondrially encoded cytochrome C oxidase II (*Mt-co2*) also referred to as *Cox2*. There was also alterations in the gene *Ikba* which is involved in the negative regulation of NF κ B signalling.

GSEA revealed that both the genes that encode for Dectin-1 (*Clec7a*) and IL1 β were significantly enriched (**Figure 4.20 A-B**). Also, upregulated were genes involved in downstream receptor signalling such as the tyrosine kinase *Src* as well as genes involved in interferon regulation such as *Irf1* (**Figure 4.20 C-D**). Again, this data highlights that WGP mediated trained immunity drives changes at the transcriptional level to poise the cell for enhanced responses to fungal and bacterial challenges through upregulation of CLR signalling pathways.

Next, I investigated genes that were common in both WGP mediated trained immunity and lipid and atherosclerosis (**Figure 4.21**). As expected, there were several genes that were commonly altered across the two pathways such as inflammatory genes like TNF and IL1 as well as downstream signalling genes like *Src*. Genes that encode adhesion molecules were also increased such as *Icam1*. WGP trained BMDMs also showed enhancement in the gene that encodes the

oxidised low density lipoprotein receptor 1 (*Olr1*) also known as *Lox-1*. Also, changed was the gene associated with downstream TNF signalling *Traf3*.

GSEA revealed a significant increase in genes involved in superoxide processing as well. One such gene was *Sod1* which was significantly upregulated in WGP trained BMDMs (**Figure 4.22A**). Similarly, there was a significant upregulation of genes involved in downstream, signalling pathways associated with extracellular receptors. One such upregulated gene was *Pi3kcb* (**Figure 4.22B**). There was also an increase in genes that encoded for chemokines including *Cxcl3* and *Cxcl2* (**Figure 4.22C-D**).

There were also a number of genes that were common across pathways involved in tuberculosis infection and WGP mediated trained immunity (**Figure 4.23**). Again, I saw that CLR receptor genes were enhanced such as *Clec7a* and that pro-inflammatory genes such as TNF and IL1 were also altered. There were also changes in Toll-like receptor (TLR) genes as well with alterations to TLR-2. Downstream tyrosine kinase genes such as *Src* were also altered as has been common across all pathways. Interestingly, there were also changes in antigen presentation with enrichment of MHC class II. Genes involved in phagosome maturation were also altered including *CamkII* and *V-Atpase*.

GSEA revealed several other genes that were also significantly enriched that were common to tuberculosis infection (**Figure 4.24**). There was a significant change in the gene counts 6 days post WGP treatment for the gene that encodes Mincle known as *Clec4e* (**Figure 4.24A**). Genes that were common to NF κ B signalling and that are involved in downstream IL1 signalling were also significantly increased including *Irak2* (**Figure 4.24B**). Genes involved in the recognition of bacterial (LPS/MDP) and viral (ssRNA) ligands were also increased, such as *Nod2* (**Figure 4.24C**). Finally, there was also an increase in the gene count for *Nos2* which enhances the production of nitric oxide important in both the anti-bacterial and anti-tumour response (**Figure 4.24D**).

KEGG pathway analysis revealed a number of genes that were common in HIF-1 signalling pathways and WGP mediated trained immunity (**Figure 4.25**). Some of these genes were common with other pathways I have previously shown including iNOS and Nox. Calcium/Calmodulin Dependent Protein Kinase (*CamK*) was also significantly altered suggesting a role for calcium signalling in WGP trained immunity. Iron and heme metabolic genes were also significantly increased with changes in *Tfrc* and *Hmox1* respectively. There was also an overall change in *Phd* which is made up of three PHD genes including *Phd1*, *Phd2* and *Phd3*. However, GSEA reveals that only *Phd2* and *Phd3* were significantly changed.

GSEA revealed that many metabolic genes that were associated with HIF-1 signalling were increased in WGP trained BMDMs (**Figure 4.26**). There was a significant increase in the gene count in WGP trained cells for the gene *Slc2a1*, which encodes for the glucose transporter Glut1 (**Figure 4.26A**). The gene count for HIF-1 α itself was also significantly enhanced post WGP stimulation (**Figure 4.26B**). There was also a significant increase in the gene count for *Ldhb* a subunit of the gene that encodes for lactate dehydrogenase 3 (LDH3) (**Figure 4.26C**). Finally, I also saw a significant change in the gene count for the gene *Cybb*, which plays an important role in anti-bacterial killing through superoxide (**Figure 4.26D**).

Taken together these results highlight pathways that are commonly associated with a classical trained phenotype, which I have previously explored in chapter 3. They also point towards the role of maladaptive trained immunity in various diseases, with lipids and atherosclerosis pathways being upregulated.

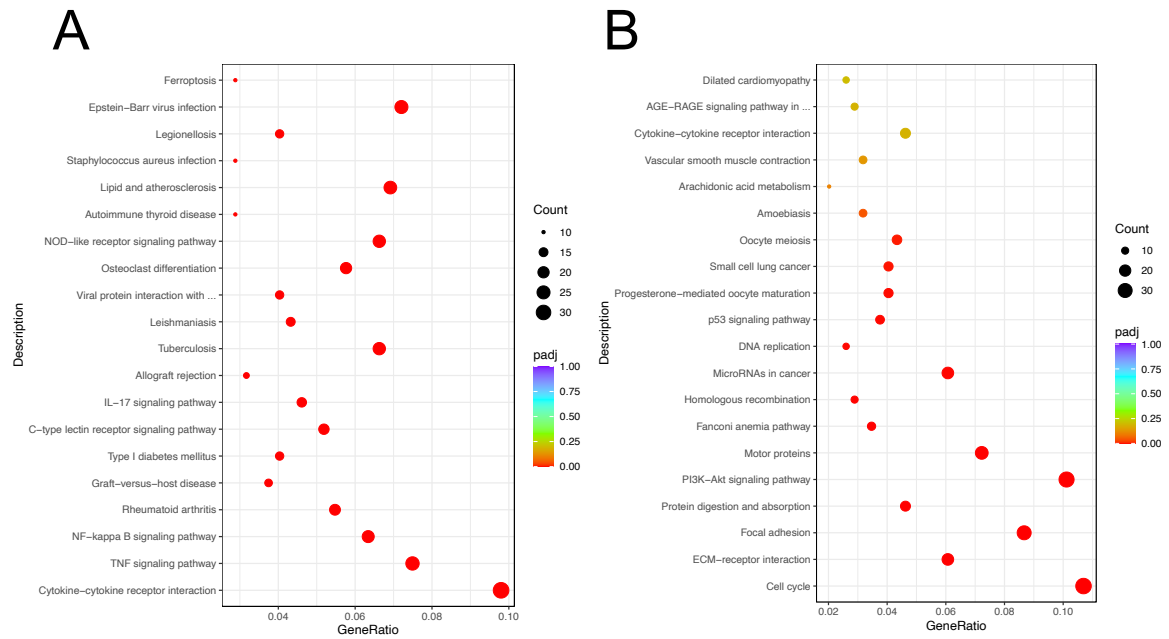


Figure 4.17 Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis showing the top 20 upregulated and downregulated pathways involved in WGP mediated trained immunity

Dot plot highlights pathways that are upregulated (A) in WGP trained macrophages at baseline as well as pathways that are downregulated (B) during WGP mediated training. N = 4.

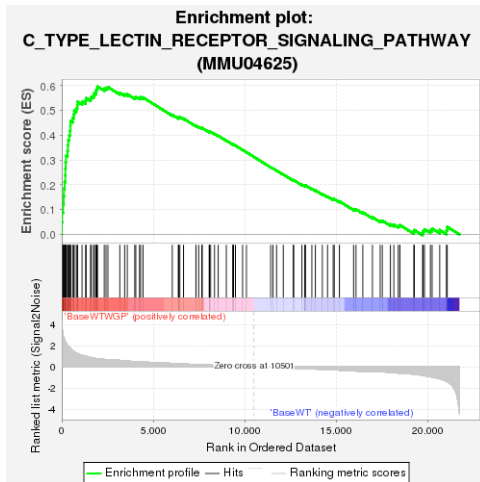
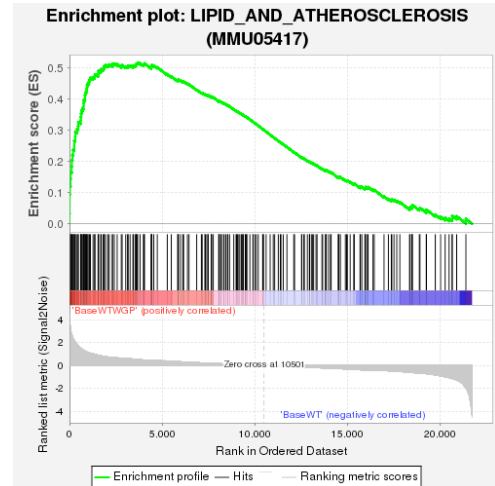
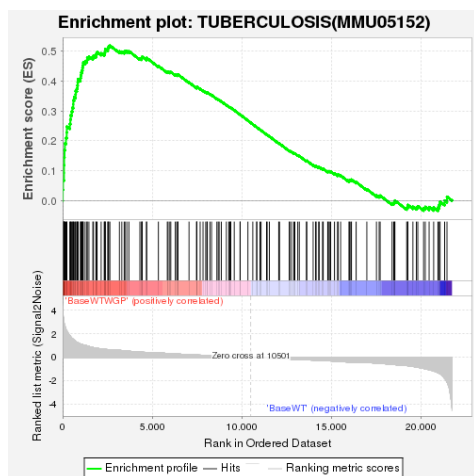
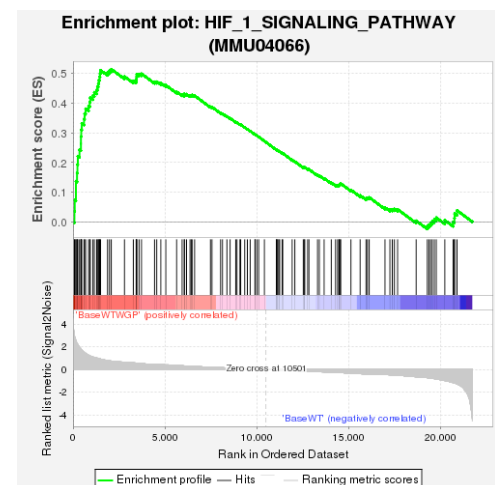
A**B****C****D**

Figure 4.18 KEGG enrichment plots showing increased genes in different signalling pathways classically associated with trained macrophages

KEGG analysis reveals various immunological processes that are upregulated in WGP mediated trained immunity that are also commonly associated with a trained phenotype. (A) Enrichment plot showing heightened gene expression associated with C-type lectin signalling pathways. (B) Enrichment plot showing increased gene expression associated with hypoxia inducible factor 1 alpha (HIF-1 α) signalling pathway. (C) Enrichment plot showing increased gene expression associated with lipid and atherosclerosis. (D) Enrichment plot showing increased gene expression associated with tuberculosis. N = 4.

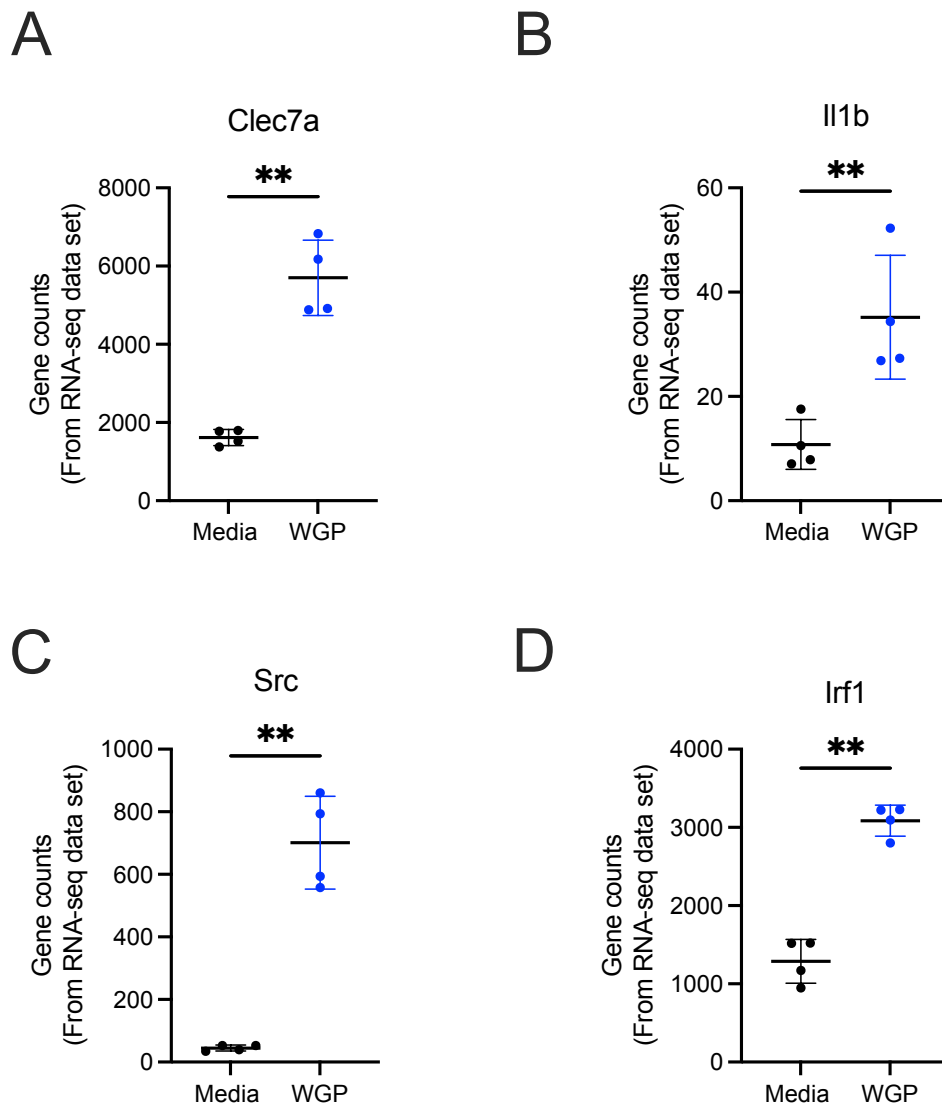


Figure 4.20 Significantly increased genes involved in the C-type lectin signalling pathways

GSEA analysis reveals genes upregulated in the C-type lectin signalling pathway induced by WGP mediated trained immunity. (A) Raw gene count taken from RNA-seq data analysis showing expression of *Clec7a*. (B) Raw gene count taken from RNA-seq data analysis showing expression of *Il1b*. (C) Raw gene count taken from RNA-seq data analysis showing expression of *Src*. (D) Raw gene count taken from RNA-seq data analysis showing expression of *Irf1*. Paired student T-test. N = 4. *P-value <0.0332 & **P-value <0.0021.

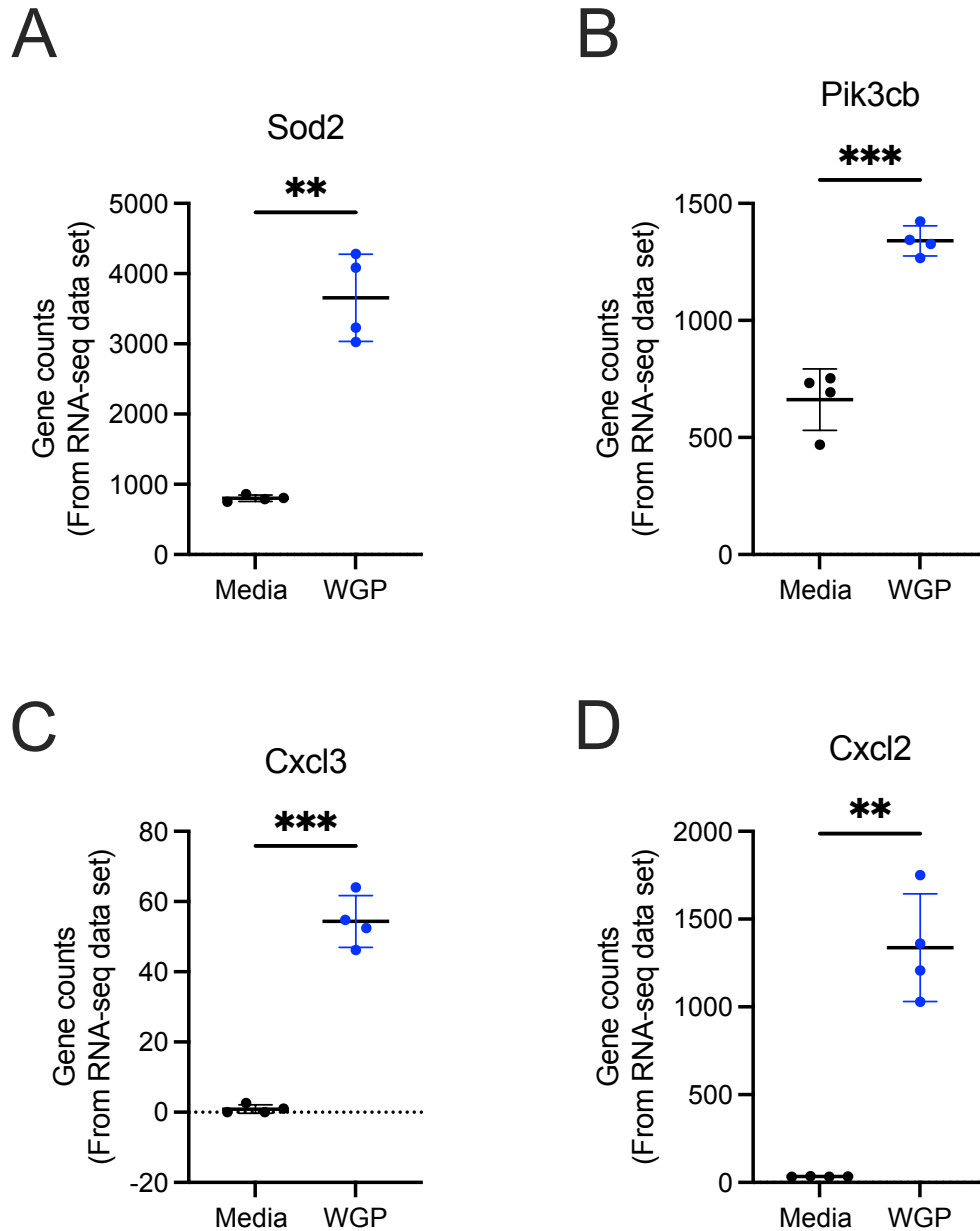


Figure 4.22 Significantly increased genes involved in lipid and atherosclerosis

GSEA analysis reveals genes upregulated in the lipid and atherosclerosis pathway induced by WGP mediated trained immunity. (A) Raw gene count taken from RNA-seq data analysis showing expression of *Sod2*. (B) Raw gene count taken from RNA-seq data analysis showing expression of *Pik3cb*. (C) Raw gene count taken from RNA-seq data analysis showing expression of *Cxcl3*. (D) Raw gene count taken from RNA-seq data analysis showing expression of *Cxcl2*. Paired student T-test. N = 4. *P-value <0.0332, **P-value <0.0021 & ***P-value <0.0002.

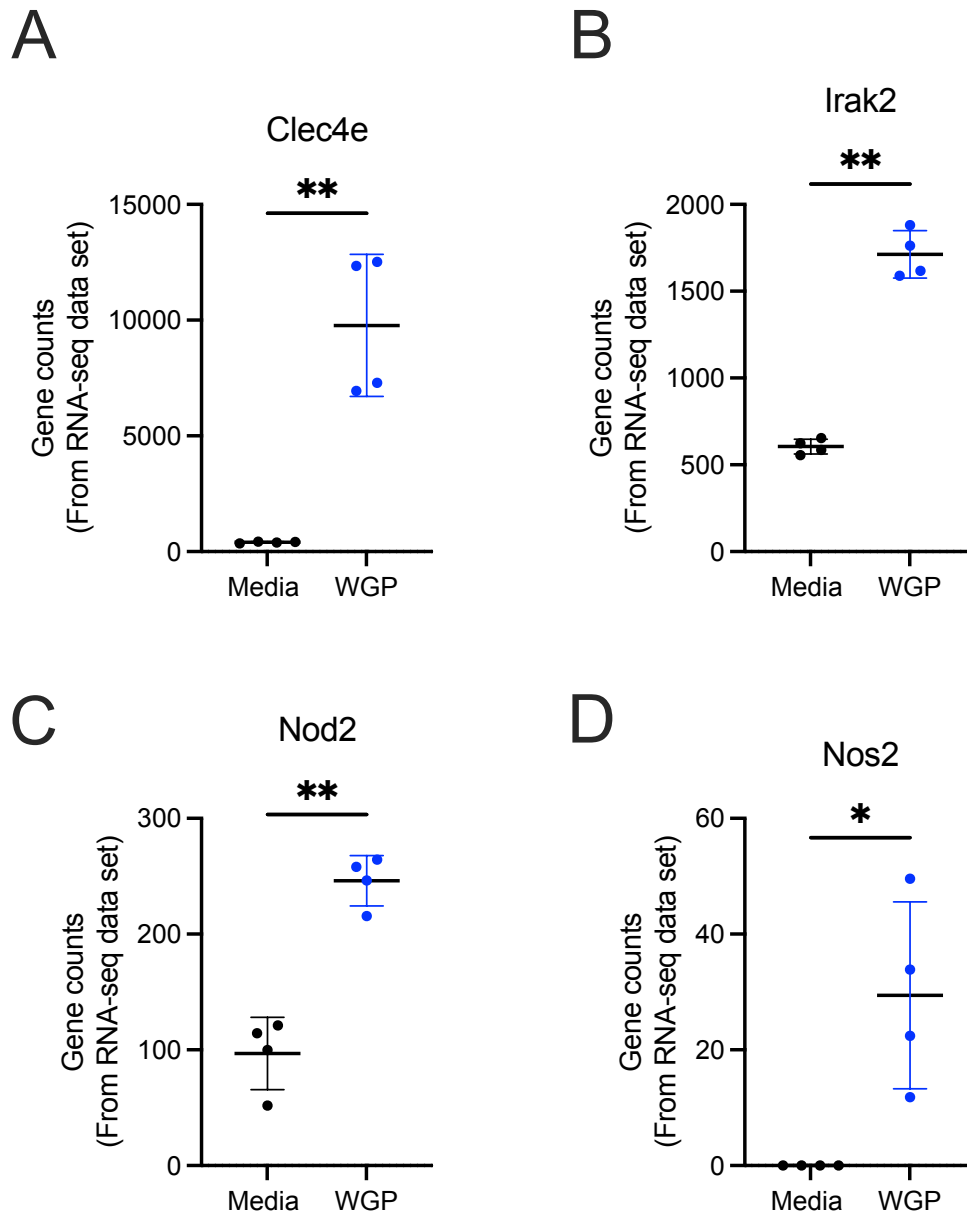


Figure 4.24 Significantly increased genes involved in tuberculosis

GSEA analysis reveals genes upregulated in tuberculosis induced by WGP mediated trained immunity. (A) Raw gene count taken from RNA-seq data analysis showing expression of *Clec4e*. (B) Raw gene count taken from RNA-seq data analysis showing expression of *Irak2*. (C) Raw gene count taken from RNA-seq data analysis showing expression of *Nod2*. (D) Raw gene count taken from RNA-seq data analysis showing expression of *Nos2*. Paired student T-test. N = 4. *P-value <0.0332 & **P-value <0.0021.

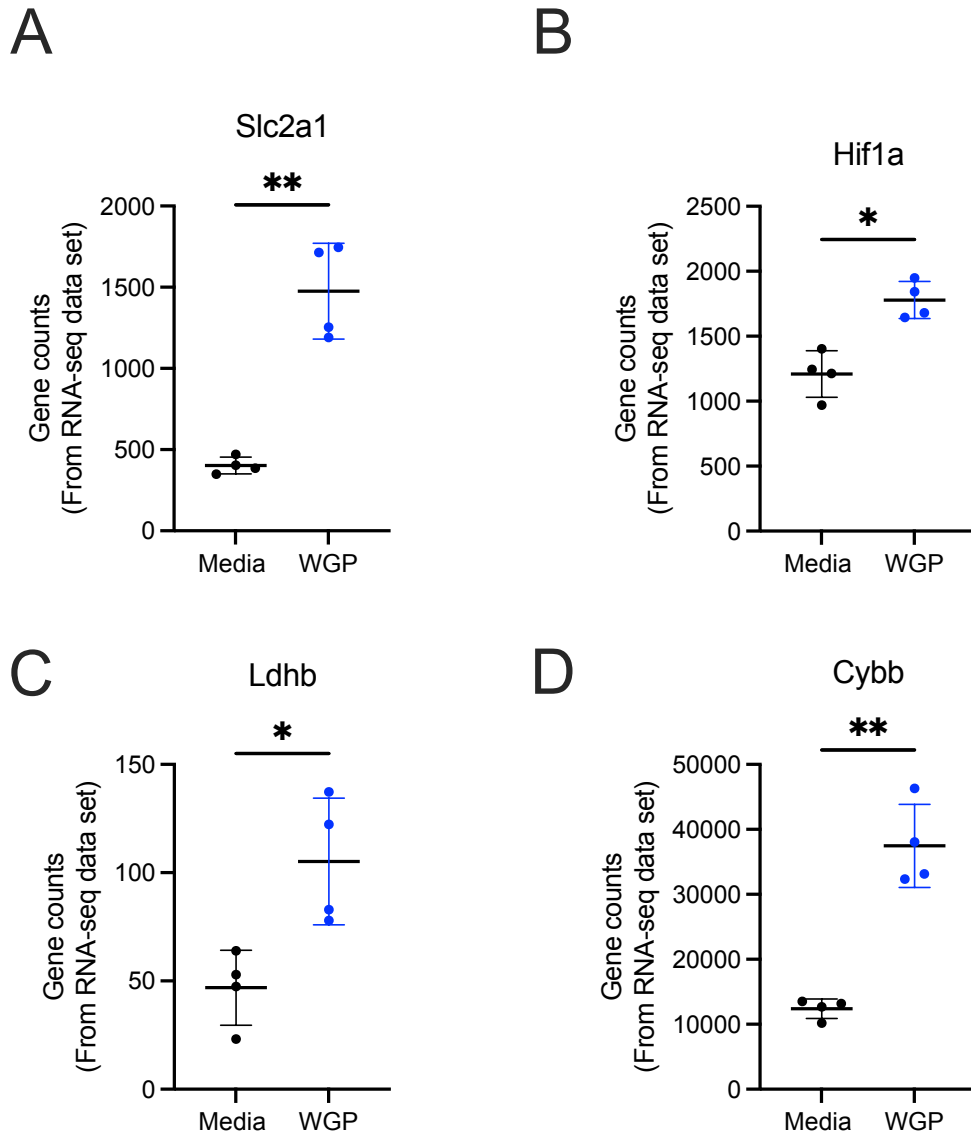


Figure 4.26 Significantly increased genes involved in the Hif-1 α signalling pathways

GSEA analysis reveals genes upregulated in the Hif-1 α signalling pathway induced by WGP mediated trained immunity. (A) Raw gene count taken from RNA-seq data analysis showing expression of *Slc2a1*. (B) Raw gene count taken from RNA-seq data analysis showing expression of *Hif1a*. (C) Raw gene count taken from RNA-seq data analysis showing expression of *Ldha*. (D) Raw gene count taken from RNA-seq data analysis showing expression of *Cybb*. Paired student T-test. N = 4. *P-value <0.0332 & **P-value <0.0021.

4.2.8 Kyoto Encyclopaedia of Genes and Genome analysis reveals non-classical trained immunity pathways induced via WGP stimulation

As well as classical pathways associated with Trained immunity, KEGG pathway analysis revealed several non-classical anti-viral pathways upregulated 6 days post WGP stimulation. Out of these I identified four major pathways of interest due to either their ability to enhance interferon signalling or due to their association in the literature with phenotypes similar to or associated directly with Trained immunity^{93, 213}. These included Cytosolic DNA sensing pathway, Epstein-Barr virus infection, Hepatitis C and Influenza A (**Figure 4.27A-D**). Each of these pathways showed a positive association of genes upregulated in the aforementioned pathways as well as in day 6 WGP stimulated BMDMs.

KEGG pathway analysis of genes associated with cytosolic DNA sensing pathway revealed a number of upregulated and downregulated genes in this pathway. The top genes that were altered included genes that I have previously described, genes that were involved in the regulation of the NF- κ B pathway including *I κ B* and *IKK ϵ /I* (**Figure 4.28**). There was also an increase in *IL1 β* which I have previously described but also an increase in chemokines such as *CCL5* and *CXCL10*, both associated with interferon signalling²¹⁴. Finally, there was also an overall increase in Pyrin which is commonly found in many cytosolic nucleic acid sensors but also plays an important role in anti-bacterial responses.

In contrast there were also significantly downregulated genes identified. The main gene here was found to be *RIPK3*. This gene is associated with cell death particularly necroptosis due to viral infections.

When I ran GSEA on this pathway I found that there were also a number of genes that were significantly increased using this form of analysis. There was a significant increase in the the gene *IKBK ϵ* in BMDMs that had been treated with WGP and allowed to rest for 6 days (**Figure 4.29A**). I also saw the increase in the enrichment of the gene that encodes the intracellular RNA sensor RIG-I, *Ddx58* (**Figure 4.29B**).

The DNA sensor that acts as an RNA polymerase to inhibit viral replication was also significantly altered known as *Polr3d* (**Figure 4.29C**). Finally, I also identified the enrichment of *Zbp1* which interacts with viral Z-shaped nucleic acids to drive an interferon response (**Figure 4.29D**). In this way, both forms of analysis identified enrichment of genes involved in cytosolic DNA sensing pathways potentially showing that WGP training enhances responses to not just bacteria but also viral infections.

Next, I decided to look at another pathway upregulated in WGP treated cells. This pathway was associated with Epstein-Barr viral infection and was analysed and identified via KEGG pathway analysis (**Figure 4.30**). Viral infection with Epstein-Barr virus is common in humans with an estimated 95% of the population being seropositive for the virus by adulthood²¹⁵. Therefore, genes that are upregulated by this pathway that are associated with trained immunity are an attractive area to study in the context of autoimmune or autoinflammatory diseases. As was expected a number of these upregulated genes are common for the NFκB pathway or inflammatory genes including *Tlr2*, *Tnf*, *Cxcl10*, *Nfkb*, *A20* and *Ikb*. Interestingly, there was also an increase in genes associated with antigen processing such as *Mhcl* and *MhcII*. There were also increases in expression of genes associated with chemotaxis, responses to intracellular pathogens and adhesion such as *Lfa1II*, *Gadd45* and *Icam1*.

GSEA analysis on this pathway identified a host of significantly upregulated genes associated with WGP training (**Figure 4.31A-D**). First, I identified that *Nfkbie* was significantly enriched (**Figure 4.31A**). This gene is involved in regulation of the NFκB pathway and thus may play a role in regulating inflammatory responses in trained cells. *Cxcl10* was also seen to be upregulated (**Figure 4.31B**). This gene is important in the recruitment of immune cells but is also activated by type I and type II interferons and thus is an ISG. Also upregulated was the expression of the gene *Tnfaip3* which is also known as A20 (**Figure 4.31C**). This gene is another important regulator of inflammation again highlighting a balance in inflammatory and anti-inflammatory genes in trained cells. Similar to *Cxcl10*, there was also an increase

in the expression of *Traf3* (**Figure 4.31D**). This is a key anti-viral signalling molecule and thus highlights why innate memory can be highly non-specific.

There was also an increase in genes associated with Hepatitis C, again highlighting that viral infections may induce similar pathways and genes as trained immunity (**Figure 4.32**). Like previous pathways I have explored previously most of these upregulated genes were linked with the pro-inflammatory response. However, there were a few novel genes that were enriched. One example is *Isg56*, also known as *Isgp56* or *Ifit1*. The translated protein of this gene is vital in preventing viral transcription through binding of viral single stranded-(ss)-RNA. There was also enrichment of the anti-viral response protein Viperin, which is encoded by the gene *Rsad2*. This again highlights the potential WGP has in driving macrophages into an anti-viral state. Interestingly, there was also an increase in $\text{LXR}\alpha$ which is important in macrophage lipid metabolism and cholesterol biosynthesis which has been associated with trained immunity.

As revealed by KEGG pathway analysis, GSEA analysis of this pathway highlighted genes that were significantly upregulated in BMDMs 6 days post WGP stimulation. There was an increase in the gene *Nr1h3* which encodes the protein $\text{LXR}\alpha$ (**Figure 2.33A**). Although commonly linked with cholesterol metabolism this gene has also been associated with anti-viral protection²¹⁶. Also, as I have previously shown there were increases in the genes *Rsad2* and *Ifit1* (**Figure 2.33B-C**). Finally, there was an increase in the anti-viral gene involved in RNA cap binding known as *Ifit1b1* (**Figure 2.33D**).

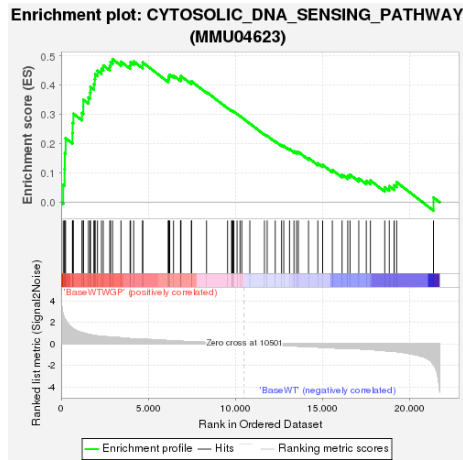
Finally, there was also a significant enrichment of genes in the pathway associated with Influenza A virus (**Figure 2.34**). This is of significant interest since it has been recently shown that infection with influenza can leave innate immune training signatures in hosts, be it in a health or disease setting^{92, 93}. For this reason, this pathway was of particular interest to me to not just understand how trained immunity can be harnessed to protect against viral infections but also to understand how viruses and other pathogens can be associated with and lead to autoimmune/autoinflammatory diseases. KEGG pathway analysis showed similar

results to what we have seen previously with enhanced genes associated with inflammation (*Il1*, *Tnf*, *Rantes*, *Icam1* and *Mhcll*). There were also increases in NF κ B signalling genes such as *Ikk ϵ* and *Ikk β* . There were also increased expression of anti-viral pathways with changes in *Trail*, *Viperin* and *Traf3*. Finally, there was a reduction in the expression of a family of genes known as Importin. These genes encode for nucleocytoplasmic transporters which viruses can hijack for their trafficking and replication²¹⁷.

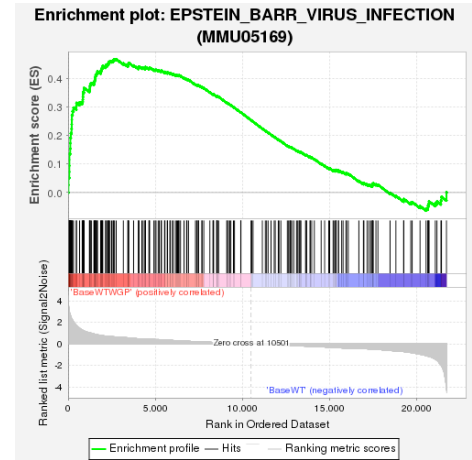
GSEA analysis revealed a few other genes that were significantly enriched (**Figure 2.35A-D**). The first gene I identified was the ISG *Mx1* which is a GTPase that has been shown to inhibit *Influenza* replication (**Figure 2.35A**). The enhanced enrichment of this gene again adds to the growing body of evidence that WGP can exert a potent anti-viral response. The next three genes I identified, *Ciita*, *Jak2* and *Ifngr2*, were all part of the type II interferon response. *Ciita* acts downstream of IFN γ and JAK/STAT signalling to upregulate the expression of MHCII, important in antigen processing and presentation (**Figure 2.35B**). *Jak2* is one of the key signalling kinases that is activated downstream of IFN γ signalling and thus plays a vital role in intracellular pathogen immunity (**Figure 2.35C**). Finally, I also saw an increase in *Ifngr2* which encodes the protein for one half of the IFN γ receptor IFNGR, split into two subunits IFNGR1 and IFNGR2 (**Figure 2.35D**). Taken together this transcriptomic data highlights the non-specific nature of WGP trained immunity, with a broad spectrum increase in genes associated with both anti-bacterial and fungal immune responses as well as anti-viral responses.

Of particular interest for the rest of this chapter and thesis in general are the changes in anti-viral signalling pathways. Of particular interest was the changes in cytosolic DNA sensing pathways which will be explored further in chapter 5.

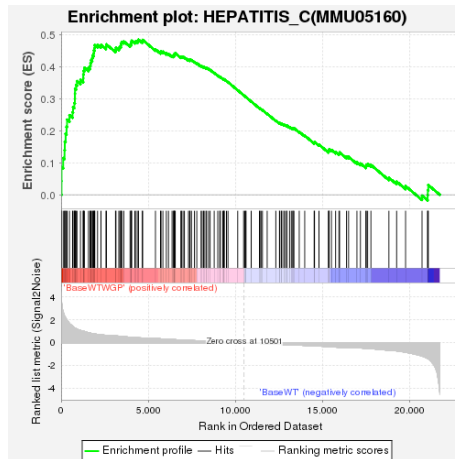
A



B



C



D

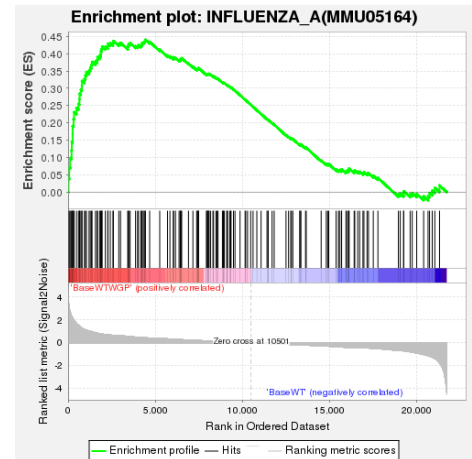


Figure 4.27 KEGG enrichment plots showing increased novel genes not classically associated with trained immunity

KEGG analysis reveals various immunological processes that are upregulated in WGP mediated trained immunity that are not commonly associated with a trained phenotype. (A) Enrichment plot showing heightened gene expression associated with cytosolic DNA sensing pathways. (B) Enrichment plot showing increased gene expression associated with Epstein-Barr virus infection. (C) Enrichment plot showing increased gene expression associated Hepatitis C. (D) Enrichment plot showing increased gene expression associated with Influenza A. N = 4.

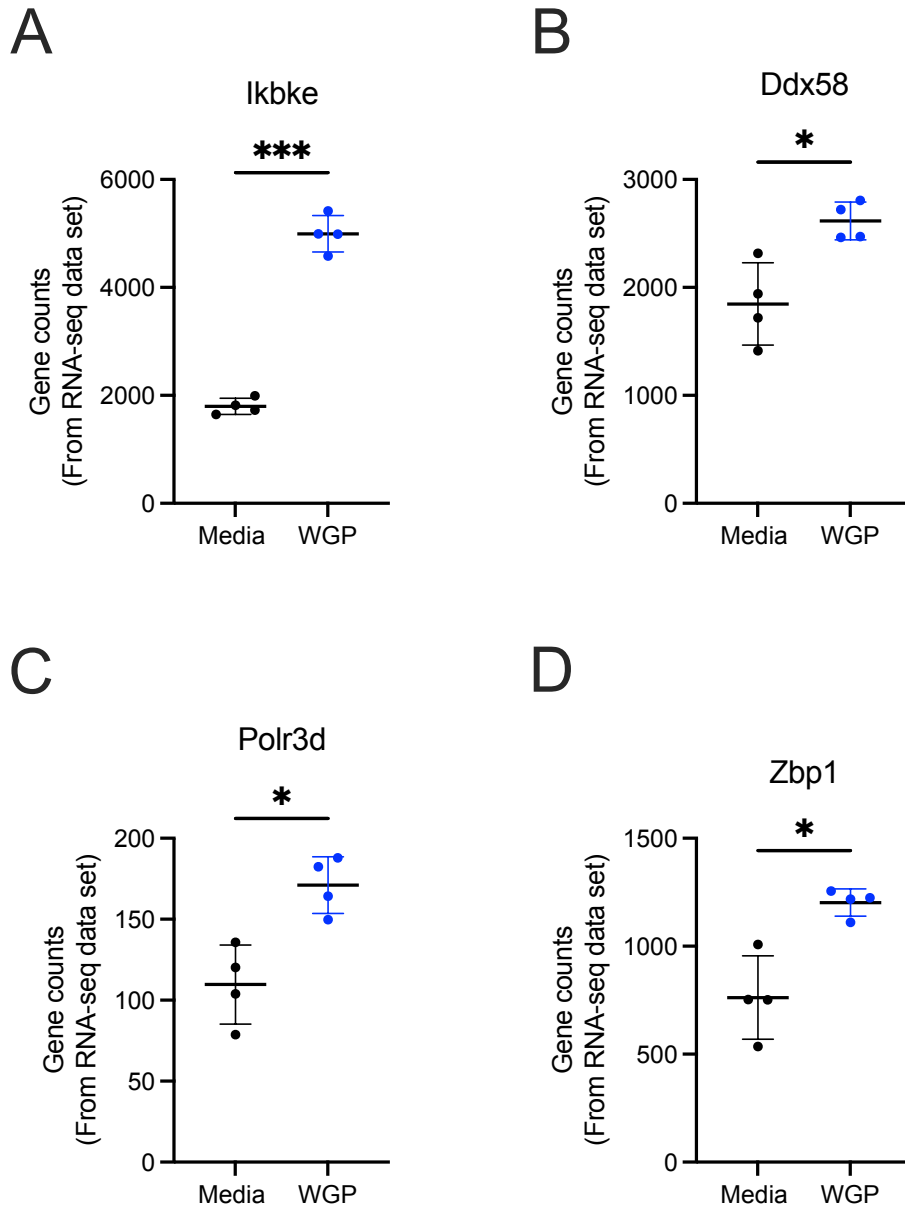


Figure 4.29 Significantly increased genes involved in cytosolic DNA sensing pathway

KEGG analysis reveals genes upregulated in the cytosolic DNA sensing pathway induced by WGP mediated trained immunity. (A) Raw gene count taken from RNA-seq data analysis showing expression of *Ikbke*. (B) Raw gene count taken from RNA-seq data analysis showing expression of *Ddx58*. (C) Raw gene count taken from RNA-seq data analysis showing expression of *Polr3d*. (D) Raw gene count taken from RNA-seq data analysis showing expression of *Zbp1*. Paired student T-test. N = 4. *P-value <0.0332 & ***P-value <0.0002.

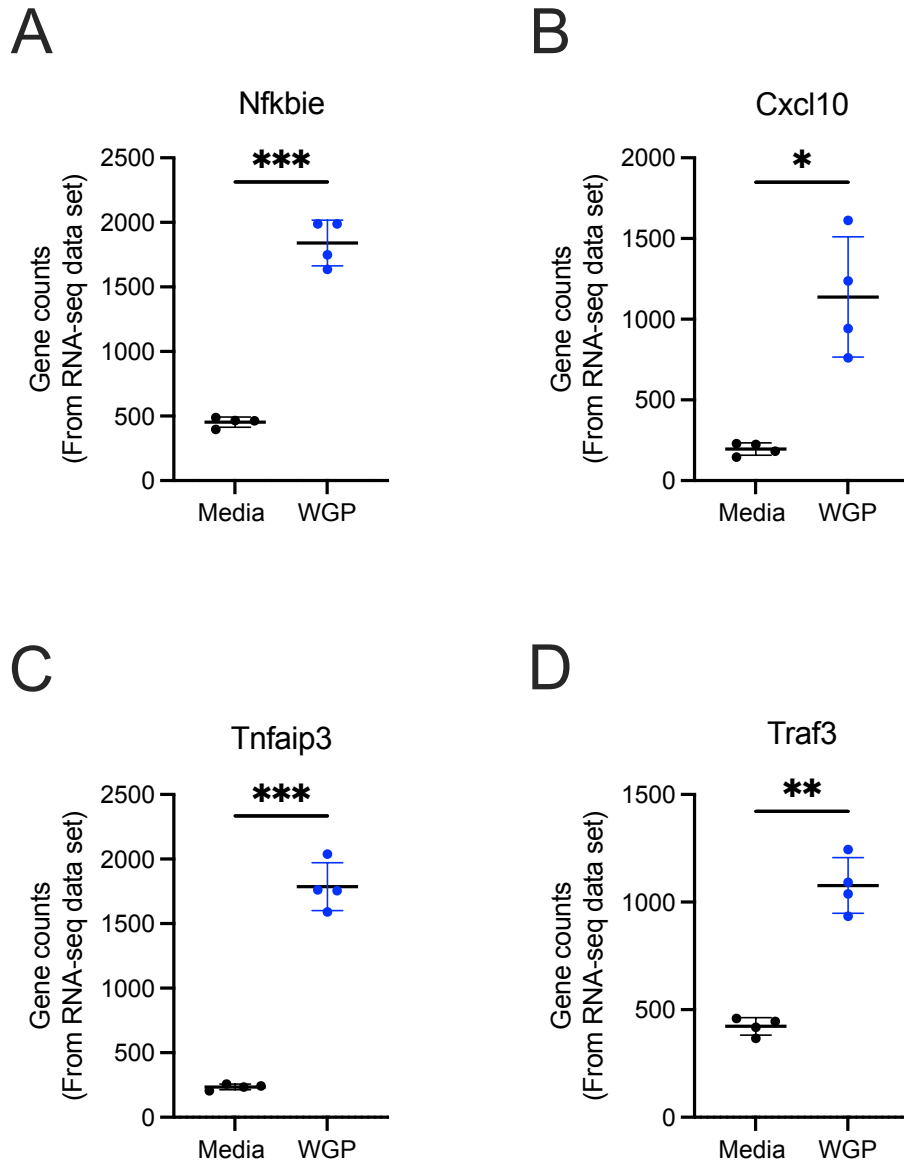


Figure 4.31 Significantly increased genes involved in Epstein-Barr virus infection

KEGG analysis reveals genes upregulated in Epstein-Barr virus infection induced by WGP mediated trained immunity. GSEA analysis on this pathway highlighted a number of significantly enriched genes. (A) Raw gene count taken from RNA-seq data analysis showing expression of *Nfkbie*. (B) Raw gene count taken from RNA-seq data analysis showing expression of *Cxcl10*. (C) Raw gene count taken from RNA-seq data analysis showing expression of *Tnfaip3*. (D) Raw gene count taken from RNA-seq data analysis showing expression of *Traf3*. Paired student T-test. N = 4. *P-value <0.0332, **P-value <0.0021 & ***P-value <0.0002.

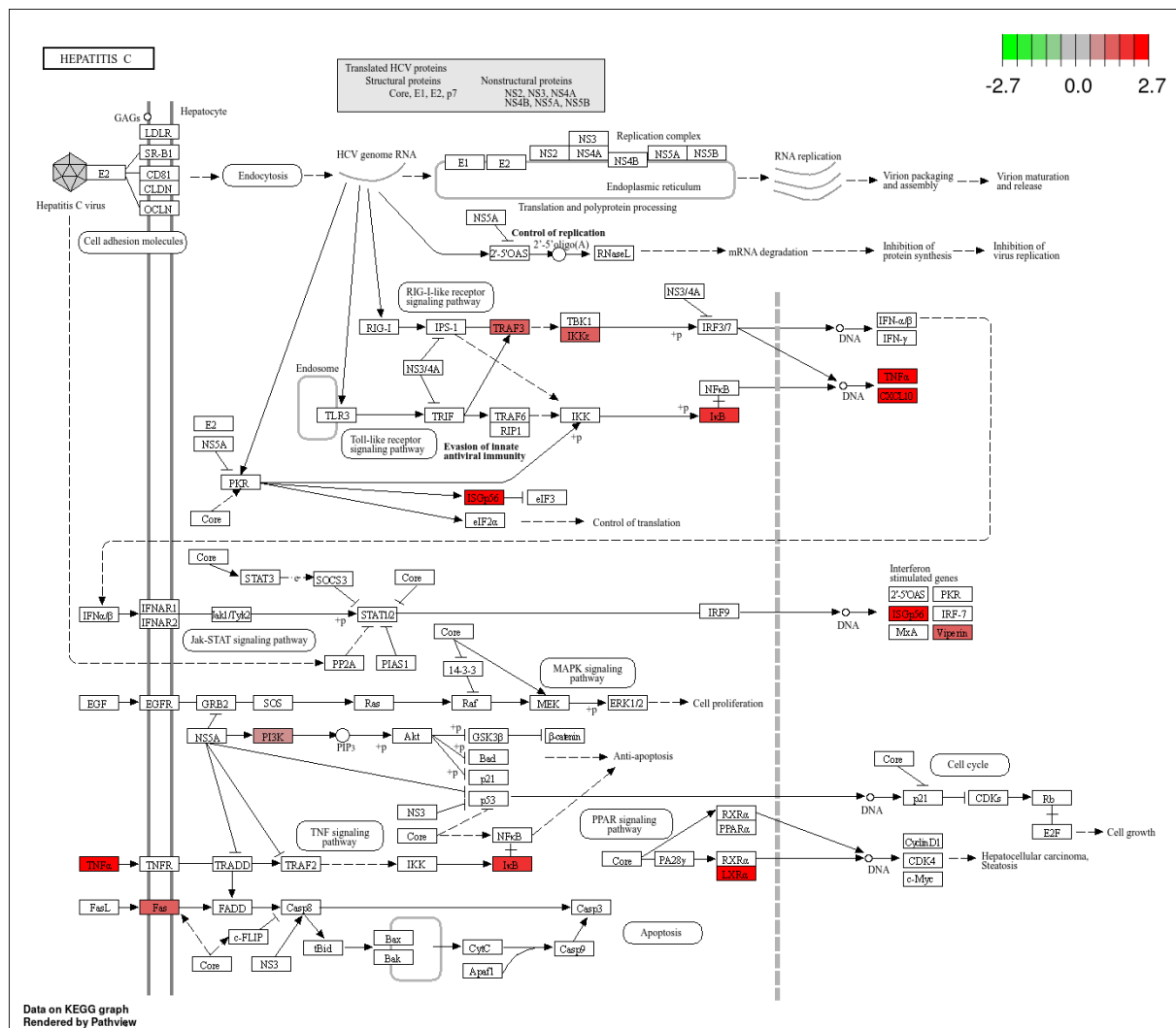


Figure 4.32 KEGG analysis reveal novel training pathway associated with Hepatitis C

KEGG analysis reveals changes in gene expression associated with Hepatitis C not commonly associated with trained immunity. N = 4.

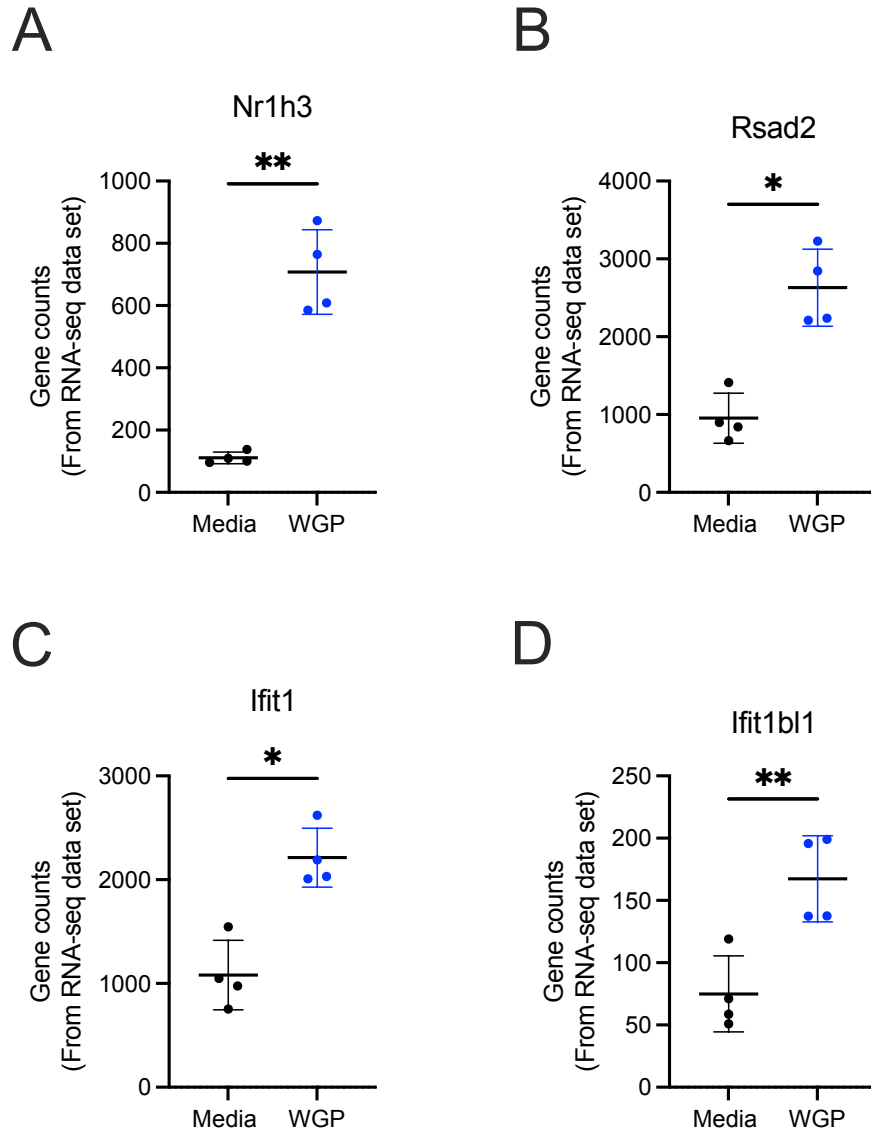


Figure 4.33 Significantly increased genes involved in Hepatitis C

KEGG analysis reveals genes upregulated in Hepatitis C induced by WGP mediated trained immunity. (A) Raw gene count taken from RNA-seq data analysis showing expression of *Nr1h3*. (B) Raw gene count taken from RNA-seq data analysis showing expression of *Rsad2*. (C) Raw gene count taken from RNA-seq data analysis showing expression of *Ifit1*. (D) Raw gene count taken from RNA-seq data analysis showing expression of *Ifit1b1*. Paired student T-test. N = 4. *P-value <0.0332 & **P-value <0.0021.

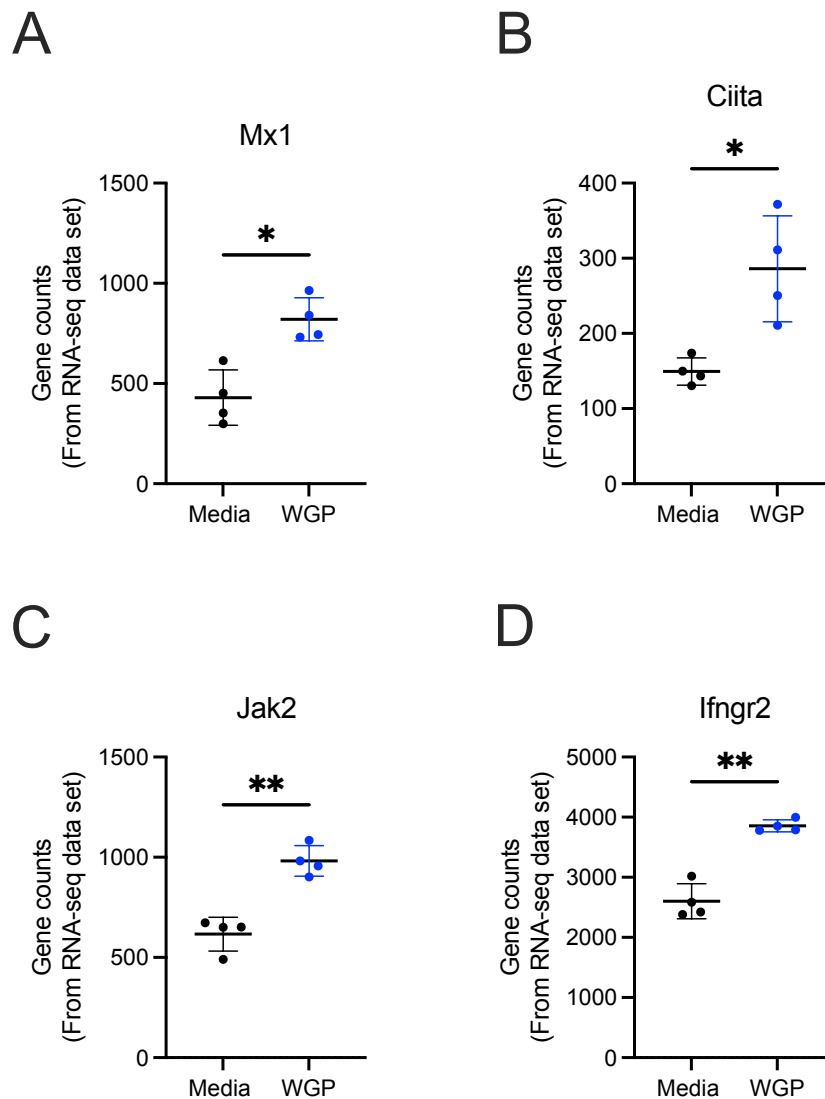


Figure 4.35 Significantly increased genes involved in Influenza A

KEGG analysis reveals genes upregulated in Influenza A induced by WGP mediated trained immunity. (A) Raw gene count taken from RNA-seq data analysis showing expression of *Mx1*. (B) Raw gene count taken from RNA-seq data analysis showing expression of *Ciita*. (C) Raw gene count taken from RNA-seq data analysis showing expression of *Jak2*. (D) Raw gene count taken from RNA-seq data analysis showing expression of *Ifngr2*. Paired student T-test. N = 4. *P-value <0.0332 & **P-value <0.0021.

4.2.9 RNA sequencing reveals substantial transcriptional differences in trained vs untrained BMDMs stimulated with pIC

Having seen that WGP training enhances the expression of both classical training pathways and novel anti-viral pathways in resting macrophages, I wanted to investigate whether trained BMDMs stimulated with a viral ligand alter their transcriptome, quantitatively. One area of trained immunity poorly understood is what occurs during the restimulation responses; therefore, I hypothesized that trained BMDMs stimulated with pIC would look transcriptionally different to untrained BMDMs stimulated with pIC. Again, cells were trained for 6 days as per our training protocol and for this experiment cells were stimulated for 4hrs with pIC. Bulk RNA-seq was performed on RNA from these cells and untrained BMDMs were compared to trained BMDMs. PCA analysis revealed that there were no significant outliers within the two groups with less than 6.5% variation between each biological replicate (**Figure 4.36**). There was however a significant difference between BMDMs that were trained with WGP and stimulated with pIC and BMDMs that were untrained and stimulated with pIC with greater than 80% variation between the groups.

Deseq2 analysis revealed a number of transcriptional differences between untrained and trained BMDMs, with 701 genes being upregulated in trained BMDMs and 927 genes being downregulated. As can be seen on the volcano plot the top few upregulated genes were associated with the proinflammatory response, such as TNF, C-type lectin signalling, such as Clec5a, as well as reactive oxygen synthesis, such as NOS2 (**Figure 4.37**). However, interestingly there were also upregulated genes associated with antigen processing, such as H2-M2, metabolic processes, such as Ass1 and genes involved in protection from influenza such as Saa3.

However, there were also a number of downregulated genes. These included agonists of receptors such as IL4RA and IL17RA, genes involved in the antioxidant response such as Selenop and genes that encode for gap junction proteins such as Gja1. Therefore, trained BMDMs stimulated with pIC maintain an altered

transcriptional profile with both increased and decreased gene expression in comparison with untrained controls.

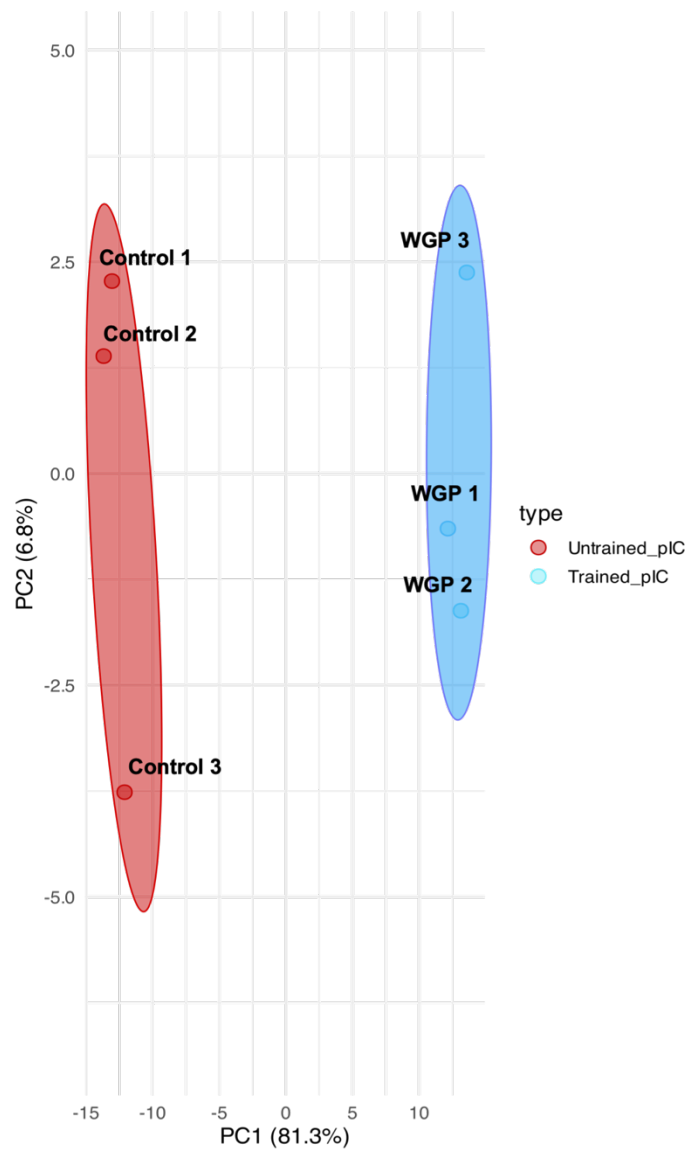


Figure 4.36 Principal component analysis of BMDMs 6 days post WGP mediated training stimulated with pIC

Principal component analysis (PCA) reveals major differences in RNA transcriptomes between media treated or WGP trained BMDMs stimulated with pIC (N = 3). PCA reveals no major outliers within the two main groups.

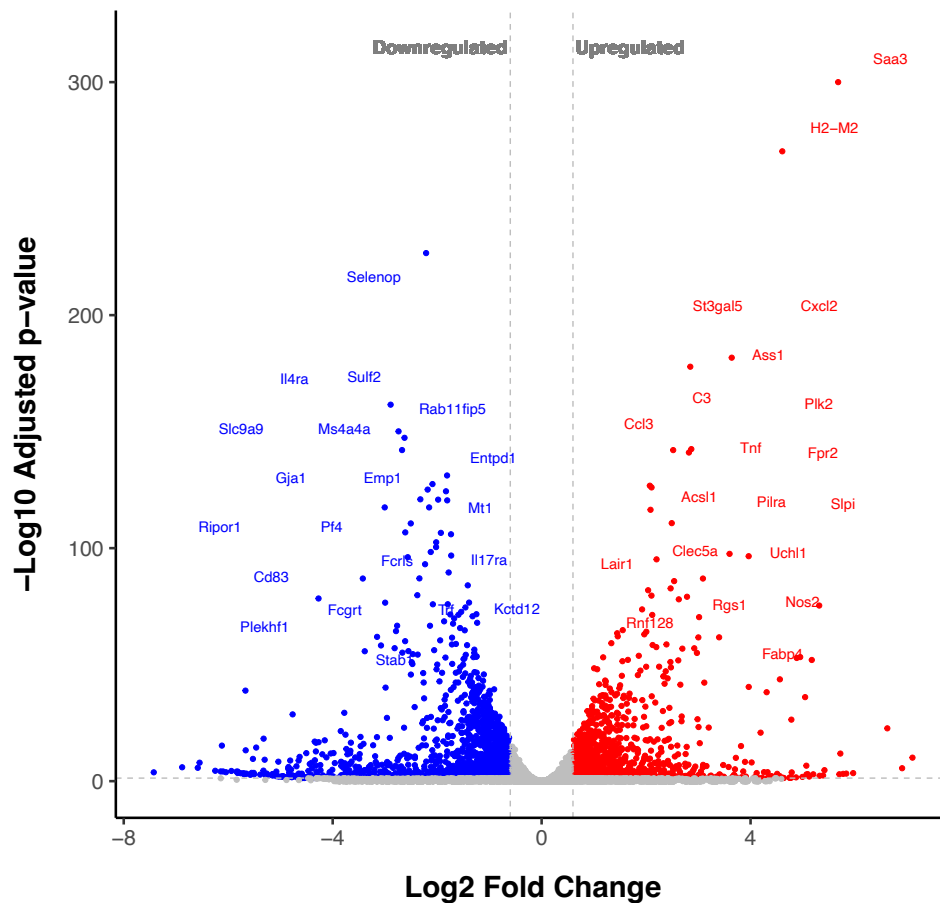


Figure 4.37 Volcano plot of significantly altered genes between trained and untrained BMDMs stimulated with pIC

Volcano plot highlights DEGs that are upregulated (Red) in WGP trained macrophages 4hrs post pIC stimulation as well as DEGs that are downregulated (Blue). Genes in grey do not reach statistical significance. Upregulated genes (701), Downregulated genes (927) and no significant differences (18,899) (N = 3). Log2Fold change cut off ≥ 1 and pAdjusted cut off ≤ 0.05 .

4.2.10 RNA sequencing reveals that metabolic processes are some of the major differences in trained vs untrained BMDMs stimulated with pIC (4hrs)

Since I had seen that WGP trained macrophages maintained their transcriptional differences after a rechallenge with the viral ligand pIC, I decided to investigate what pathways these changed genes fell into. To do this I again used GO and KEGG to investigate various pathways and to confirm that what I saw in one method of analysis was consistent across another. When I looked at the list of processes that were upregulated in trained macrophages stimulated with pIC I saw that a number of metabolic processes were upregulated. These included lipid biosynthetic processes, monocarboxylic acid metabolic processes and fatty acid metabolic processes (**Figure 4.38A**). This was further confirmed by KEGG pathway analysis since I also saw that PPAR signalling pathway, fatty acid biosynthesis and pyruvate metabolism were all upregulated in the trained macrophages stimulated for 4hrs with pIC (**Figure 4.38B**). Taken together this suggests that trained macrophages alter their metabolic transcriptome during viral ligand stimulation, potentially pointing towards a mechanism for anti-viral trained immunity.

In order to investigate these metabolic processes in more detail I performed GSEA on these data sets and found two pathways of interest while looking at both the GO and KEGG pathways. Firstly, In the GO analysis GSEA revealed that carboxylic acid transmembrane transport was significantly enriched (**Figure 4.39A**). WGP training and pIC stimulation seemed to significantly enrich this pathway mainly with an upregulation of genes associated with fatty acid metabolism (*Acs11* and *Cln8*), amino acid transporters (*Slc6a9*, *Slc38a* and *Slc7a5*), ATP dependent transporters of cAMP and cGMP (*Abcc5*) as well as key transporters involved in transport of metabolites from the cytosol to the mitochondria or vice versa (*Slc25a11*, *Slc1a1*, *Slc25a1*, and *Slc25a12*) (**Figure 4.39B**).

Secondly, I used GSEA of KEGG pathways to investigate what genes were enhanced in the metabolic pathways I had seen. One pathway of interest I saw was oxidative phosphorylation, which highlights the results I have previously shown in chapter 3. Most of the genes clustered towards being significantly enhanced as can

be seen by the enrichment plot (**Figure 4.40A**). Many of the genes involved in this pathway were associated with different complexes of the electron transport chain (ETC) including *Ndufa6*, *Sdhb*, *Uqcrb*, *Cox7a2* and *Atp5b*, as shown by the heatmap (**Figure 4.40B**). Taken together this data highlights the importance metabolism plays in enhancing the response to viral ligands in WGP trained cells. These changes in metabolic pathways in trained macrophages in response to an anti-viral ligand are particularly interesting and the role of metabolism to drive a successful anti-viral responses will be explored further in chapter 5.

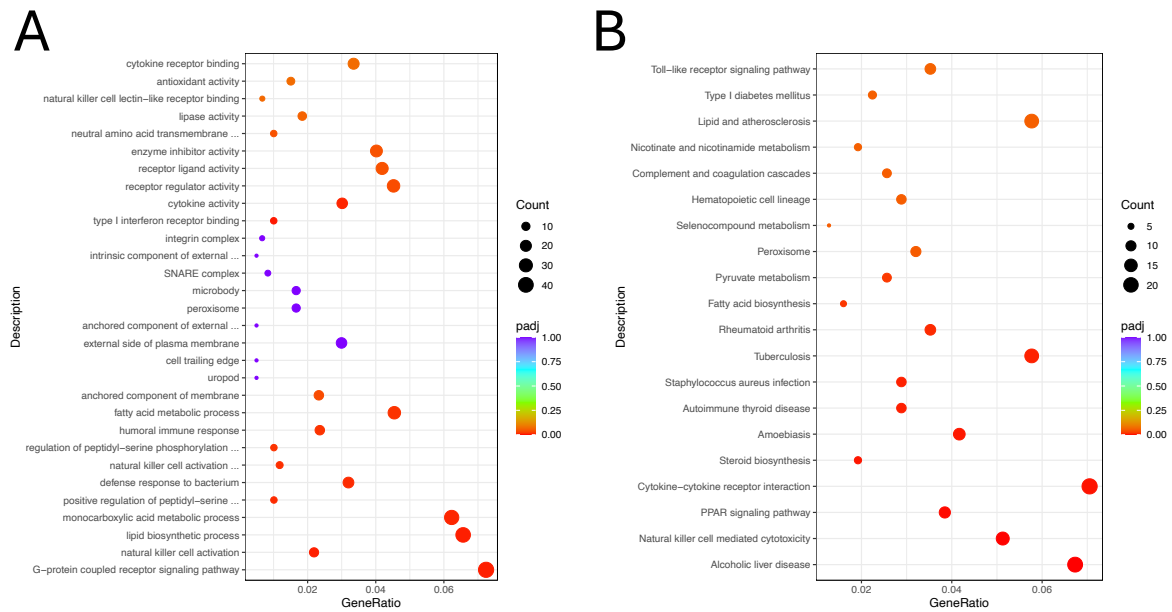
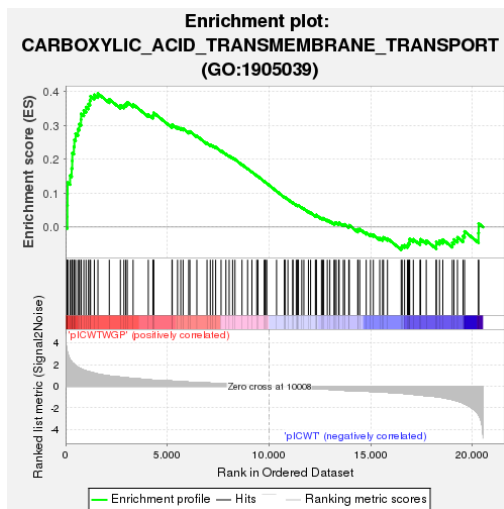


Figure 4.38 Pathway analysis showing the top 20 upregulated pathways from GO and KEGG pathway analysis

Dot plot highlights GO pathways that are upregulated in WGP trained macrophages 4hrs post pIC stimulation (A) as well as KEGG pathways that are upregulated during WGP mediated training and 4hr stimulation with pIC (B). N = 3.

A



B

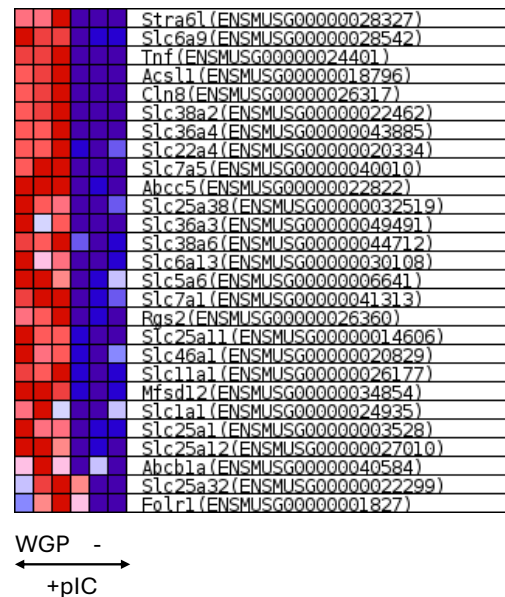
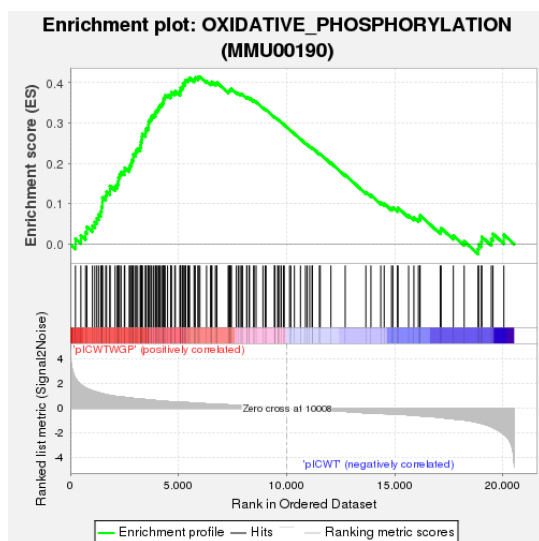


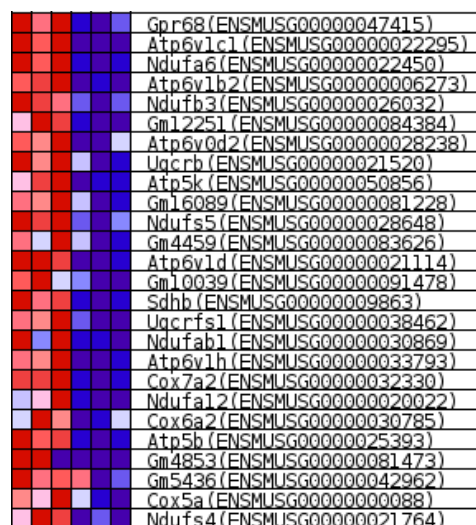
Figure 4.39 GSEA enrichment of GO pathways reveal carboxylic acid transmembrane transport as being significantly altered in Trained BMDMs stimulated with pIC (4hrs)

GSEA reveals a number of genes significantly altered during WGP training and restimulation with pIC for 4hrs. Genes are shown as upregulated or downregulated via enrichment plot (A) and the top genes are highlighted by the heatmap (B). N = 3.

A



B



WGP -
 ↔
 +pIC

Figure 4.40 GSEA enrichment of KEGG pathways reveal oxidative phosphorylation as being significantly altered in Trained BMDMs stimulated with pIC (4hrs)

GSEA reveals a number of genes significantly altered during WGP training and restimulation with pIC for 4hrs. Genes are shown as upregulated or downregulated via enrichment plot (A) and the top genes are highlighted by the heatmap (B). N = 3.

4.2.11 Trained interferon alpha/beta receptor (IFNAR) knockouts are transcriptionally different to wild-type trained BMDMs

Having seen that WGP training could enhance anti-viral gene transcription both in resting cells, as well as post viral ligand stimulation, I next wanted to investigate whether these long-term changes were dependent on type I interferon signalling. To do this, interferon alpha/beta receptor knock-out (IFNAR KO) macrophages were differentiated from bone marrow and trained as previously described. A limitation of this study is that I compared trained wild-type (WT) to trained IFNAR KO cells, meaning that changes observed maybe associated with genotype instead of phenotype. RNA sequencing was then performed on trained BMDMs from WT mice as well as trained BMDMs from IFNAR KO mice (6-days post WGP-training). Principal component analysis (PCA) was first performed which identified that the two groups were transcriptionally different from each other, with 81.3% variance between the trained BMDMs from WT and trained BMDMs from IFNAR KO (**Figure 4.41**). I also showed that there were no significant differences within both sets, with only a max variation of 6.8% as shown by PC2.

After running DeSeq2 analysis to normalize raw gene counts and to identify significantly differentiated genes I plotted these genes in a volcano plot (**Figure 4.42**). As was to be expected there were a number of genes that were significantly different in the WT vs the KO trained BMDMs. There were 213 genes that were positively differentially expressed in the WT trained BMDMs. The majority of these genes were associated with the anti-viral immune responses. There were also 159 genes that were negatively differentially expressed in WT trained BMDMs. These genes were interestingly associated with cellular metabolic responses.

Taken together this data suggests that type I interferon signalling is vital for the anti-viral transcriptional changes associated with WGP trained immunity. This data also suggests that interferon signalling induced by WGP trained immunity is linked to immunometabolic changes.

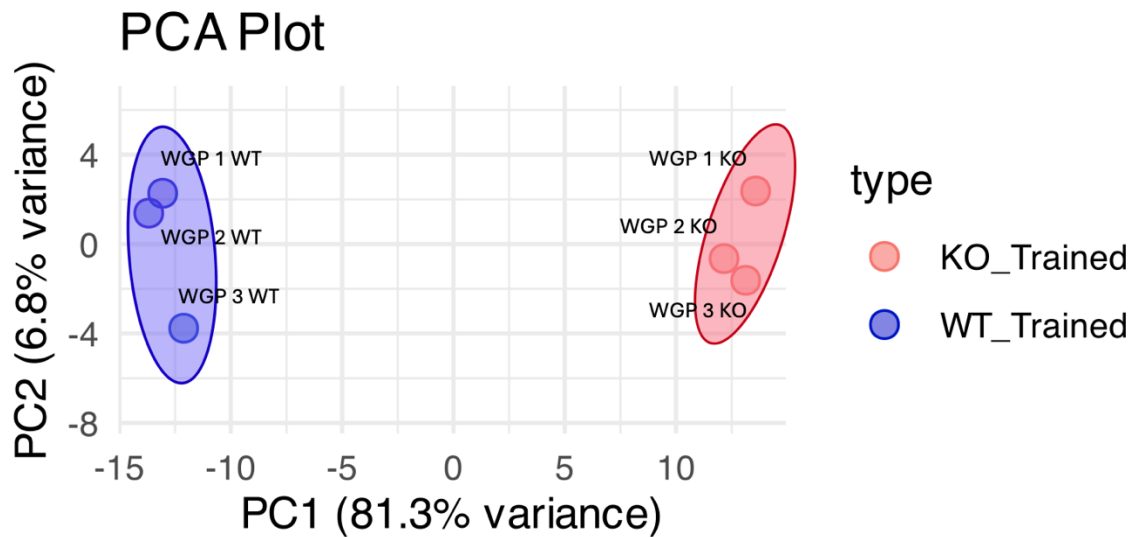


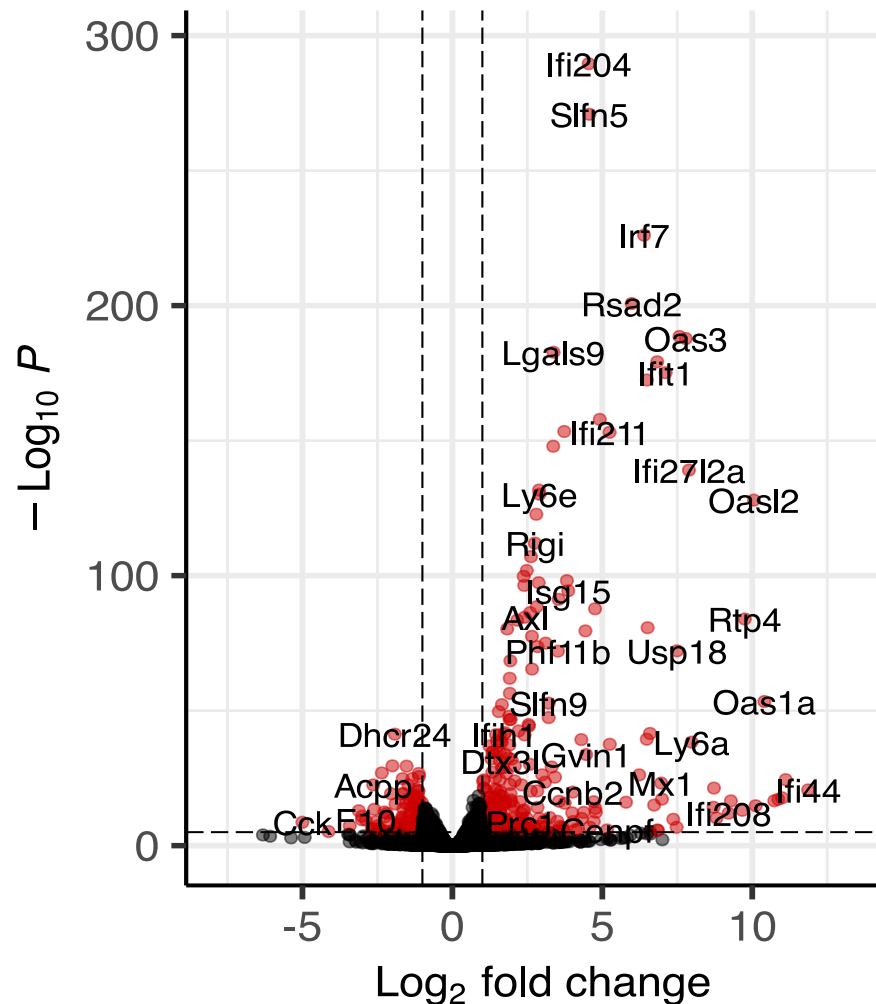
Figure 4.41 Principal component analysis of BMDMs 6 days post WGP mediated training in wild-type (WT) vs IFNAR Knock-outs (KO)

Principal component analysis (PCA) reveals major differences in RNA transcriptomes between WGP treated WT BMDMs or WGP trained IFNAR KO BMDMs (N = 3). PCA reveals no major outliers within the two main groups.

WT WGP vs KO WGP

EnhancedVolcano

● NS ● Log₂ FC ● p-value ● p – value and



total = 15454 variables

Figure 4.42 Volcano plot of significantly altered genes between trained BMDMs from wild-type vs IFNAR Knock-out mice

Volcano plot highlights DEGs that are upregulated (Right) in Wild-type WGP trained macrophages as well as DEGs that are downregulated (Left). Genes in black do not reach statistical significance. Upregulated genes (213), Downregulated genes (159) and no significant differences (29,101) (N = 3). Log2Fold change cut off ≥ 1 and pAdjusted cut off ≤ 0.05 .

4.2.12 Interferon signalling via IFNAR is vital to induce the anti-viral transcriptional differences seen with WGP trained immunity

To confirm that interferon signalling was vital for inducing the anti-viral transcriptional differences I performed Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genome (KEGG) pathway analysis on the previously described RNA seq data set. As expected, trained WT BMDMs had upregulated pathways and biological processes associated with interferon signalling and anti-viral responses when compared with trained IFNAR KO BMDMs (**Figure 4.43A-B**). Interestingly, TOLL-Like receptor signalling, RIG-I-Like receptor signalling and cytosolic DNA sensing pathways were all affected by IFNAR KO (**Figure 4.43 IFN- β B**). This suggests that WGP may induce changes in the cell that leads to enhanced RNA and DNA sensing that amplifies anti-viral immune responses, and that this occurs through type I interferon signalling.

In order to explore these changes in more detail I investigated pathways that were altered at baseline, such as Influenza A and Cytosolic DNA sensing pathways, as revealed by KEGG pathway analysis. Since I saw in WT BMDMs that WGP enhanced the Influenza A pathway I decided to first investigate what genes were altered in this pathway in the absence of IFN signalling (**Figure 4.44**). Pathway analysis revealed that several genes in this pathway were significantly altered in WTs vs KO trained BMDMs (**Figure 4.44A**). The majority of these genes were associated with interferon signalling, as expected in IFNAR KOs. Genes such as *Rsad2*, which encodes for the protein viperin an ISG, were significantly higher in WT trained BMDMs. As well as this downstream signalling genes such as *Stat1* was also significantly higher in WT trained BMDMs (**Figure 4.44B**). However, genes associated with mitochondrial metabolism and signalling such as *Vdac1* were significantly decreased in WT trained BMDMs as opposed to IFNAR KO trained BMDMs. In addition, IFNAR KO trained BMDMs had significantly increased *Icam1* expression (**Figure 4.44C**). This data potentially suggests that type I interferon signalling is important in regulating metabolism and inflammatory changes associated with WGP mediated trained immunity.

I also analysed the cytosolic DNA sensing pathway that I previously observed was enhanced 6 days post WGP stimulation. Again, in the absence of interferon signalling there were significant differences between WT and IFNAR KO trained BMDMs (**Figure 4.45**). Pathway analysis showed that many of the genes again associated with interferon signalling were significantly enhanced in WT, as expected (**Figure 4.45A**). Interestingly, I did see that certain anti-viral pathways were enriched in IFNAR KO trained BMDMs. This included the RNA polymerase *PolIII* which can act as a RIG-I agonist during viral infections (**Figure 4.45A**). However, as expected the significant changes were associated with anti-viral receptor and signalling genes. I saw that the gene that encodes for RIG-I, *Ddx58*, was significantly enhanced in WT trained BMDMs as was the interferon signalling gene *Irf7* (**Figure 4.45B**). This suggests that interferon signalling is vital to enhance the anti-viral transcriptional response. However, interestingly IFNAR KO trained BMDMs had significantly increased expression of NF κ B signalling genes. There was a significant difference in the raw counts of genes such as *Ikbkg* and *Nfkb2* (**Figure 4.45C**). This again suggests that IFN signalling plays a role in enhancing the inflammatory response induced by WGP trained immunity.

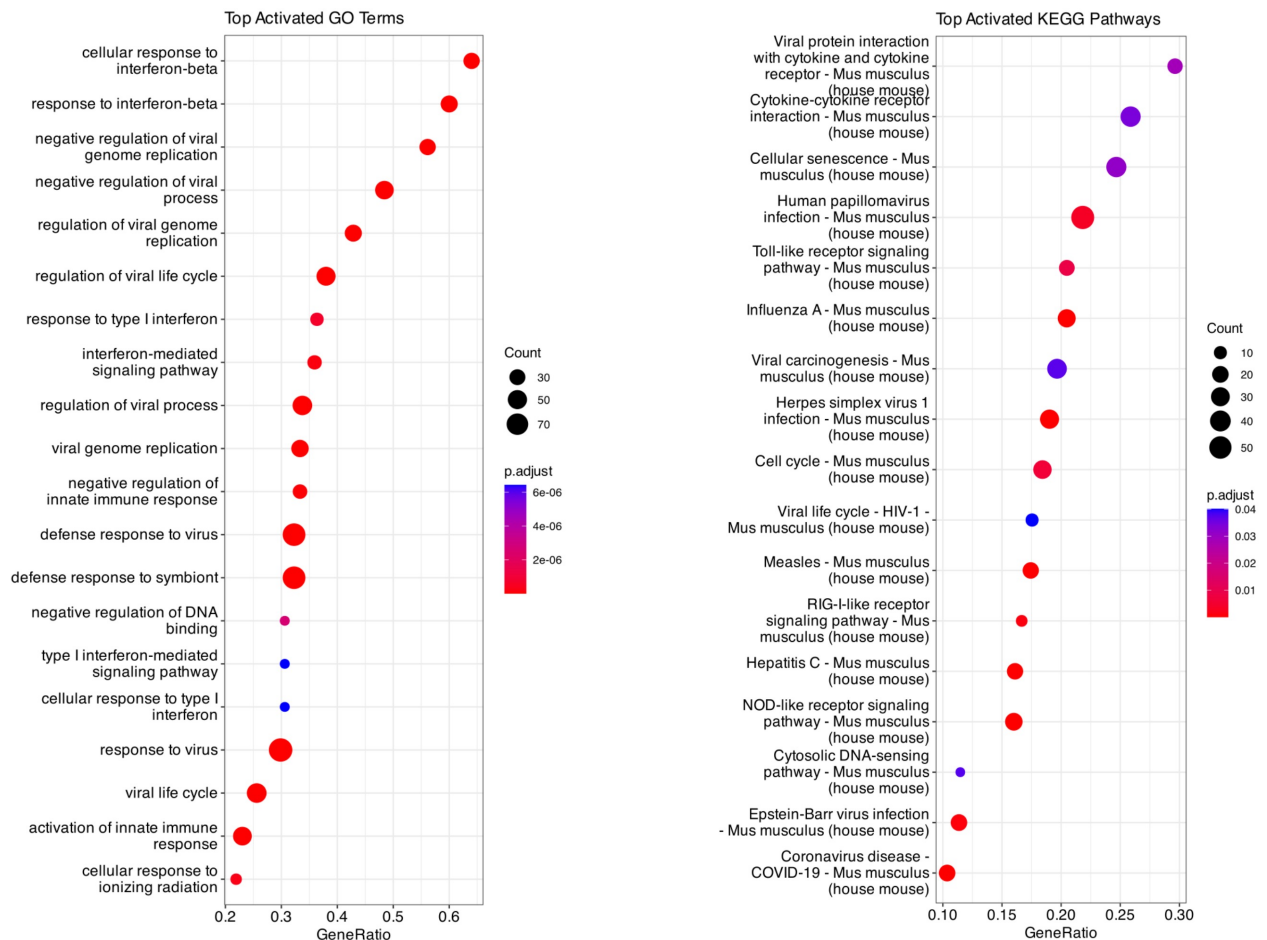
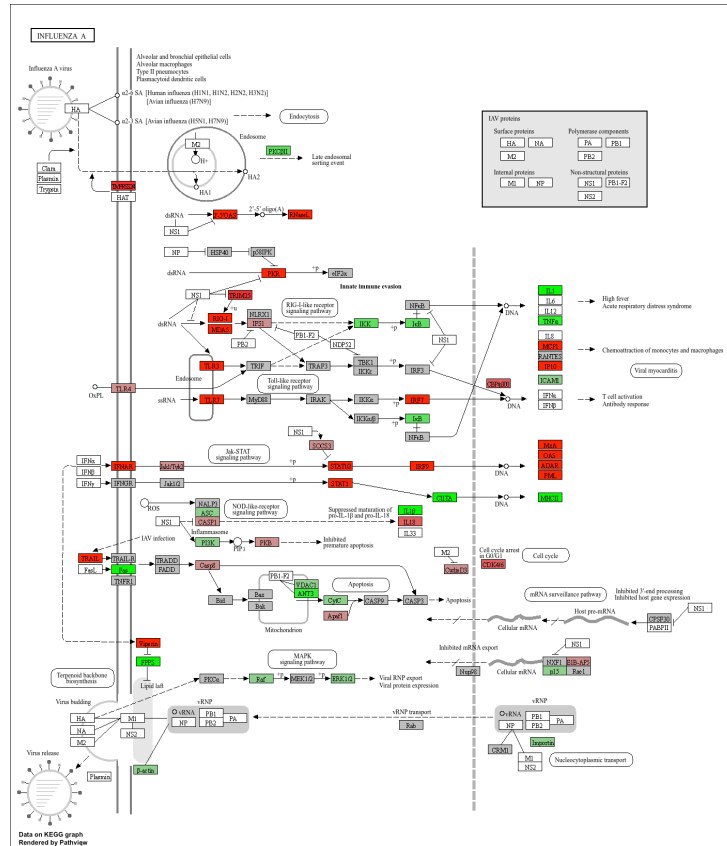


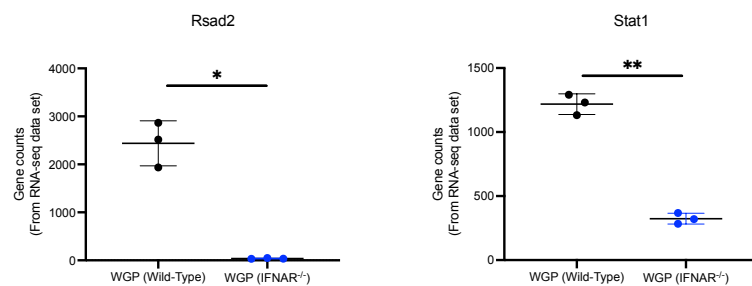
Figure 4.43 Dot-plot showing top 20 upregulated terms in GO and KEGG pathway analysis

Dot-plot representing the top activated terms involved Gene-ontology (GO) enrichment (A) or the top activated pathways as revealed by Kyoto Encyclopaedia of Genes and Genomes (KEGG) (B) in Trained WT BMDMs vs Trained IFNAR KO BMDMs (N = 3).

A



B



C

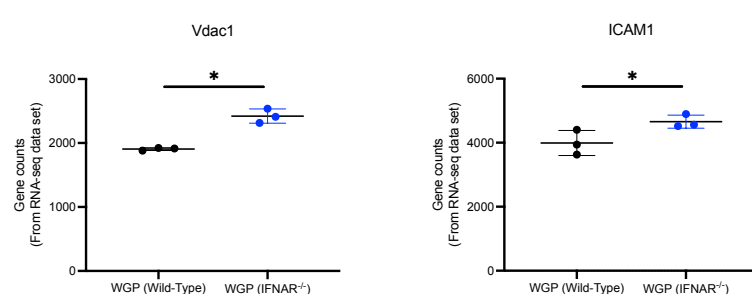
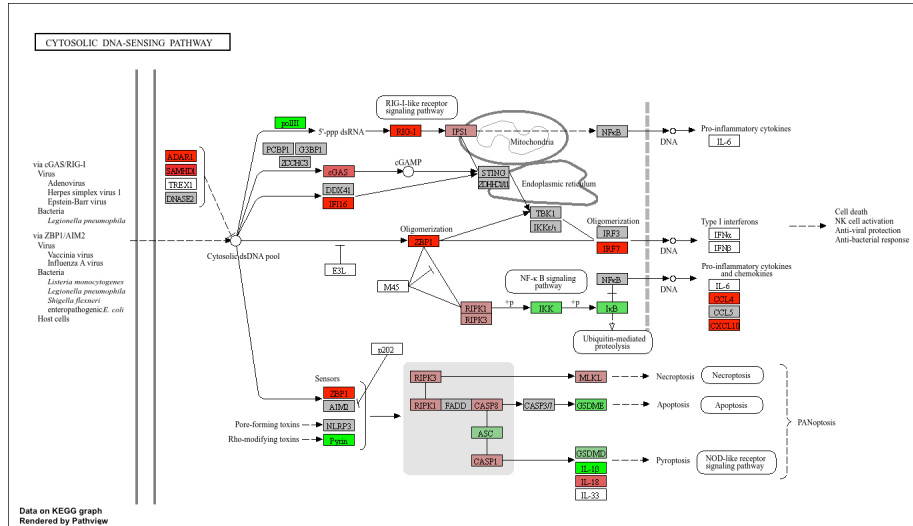


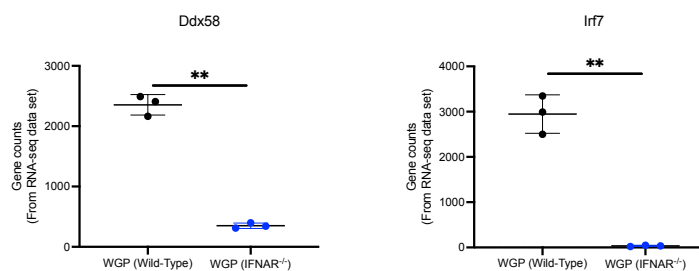
Figure 4.44 Gene changes involved in Influenza A pathway between trained BMDMs from Wild-type and IFNAR Knock-out mice

KEGG pathway analysis was performed on BMDMs from trained wild-type and IFNAR Knock-out mice. (A) Genes that are altered in Influenza A pathway. (B) Raw counts of upregulated genes taken from the pathway (*Rsad2* & *Stat1*). (C) Raw counts of downregulated genes taken from the pathway (*Vdac1* & *Icam1*). Paired student T-test. N = 3. *P-value <0.0332 & **P-value <0.0021.

A



B



C

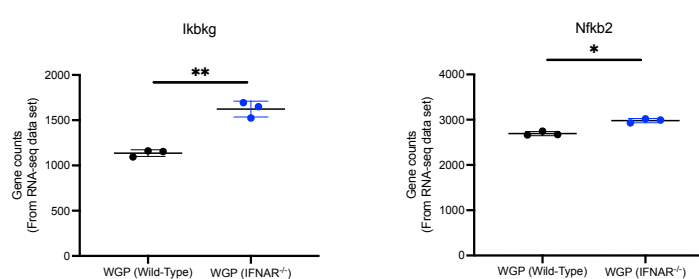


Figure 4.45 Gene changes involved in cytosolic DNA sensing pathway between trained BMDMs from Wild-type and IFNAR Knock-out mice

KEGG pathway analysis was performed on BMDMs from trained wild-type and IFNAR Knock-out mice. (A) Genes that are altered in Cytosolic DNA sensing pathway. (B) Raw counts of upregulated genes taken from the pathway (*Ddx58* & *Irf7*). (C) Raw counts of downregulated genes taken from the pathway (*Ikbkg* & *Nfkb2*). Paired student T-test. N = 3. *P-value <0.05 & **P-value <0.005.

4.2.13 Interferon signalling via IFNAR is important for enhanced pro-inflammatory trained responses to viral ligands but not bacterial ligands

Due to the data generated in the RNA sequencing data sets, I wanted to test how removing interferon signalling from BMDMs would affect their ability to be trained by WGP. To do this IFNAR KO and WT BMDMs were trained with WGP as previously described. On day 6 trained cells were stimulated with the bacterial ligands LPS and PAM, which activate TLR4 and TLR2 respectively. As expected, WT trained BMDMs enhanced their responses to both LPS and PAM with a significant increase in TNF production 4hrs post stimulation (**Figure 4.46A-B**). IFNAR KO BMDMs also display enhance TNF responses to both ligands after WGP-training. This backed up the RNA-seq data which showed that regulators of “classical/bacterial” inflammation were not affected by the lack of IFN signalling.

Next, I stimulated BMDMs with viral ligands such as pIC and TpIC, which activate TLR3 and RIG-I/MDA-5 respectively. As I have previously described BMDMs trained with WGP significantly enhanced their production of TNF in response to both ligands after 4hrs of stimulation (**Figure 4.46C-D**). However, in the absence of interferon signalling, WGP trained BMDMs could not enhance their expression of TNF. There was a significant reduction in TNF production between the WT trained BMDMs and the IFNAR KO trained BMDMs. Interestingly, untrained BMDMs had no significant differences in their ability to induce TNF between WT and IFNAR KO cells. This suggests that interferon signalling is vital for the enhanced proinflammatory responses seen in response to viral ligands in trained BMDMs, but that interferon signalling has no effect in the capacity to drive TNF in untrained cells. This data also suggests that there is some molecular specificity in signalling for non-heterologous memory responses in β -glucan induced trained immunity.

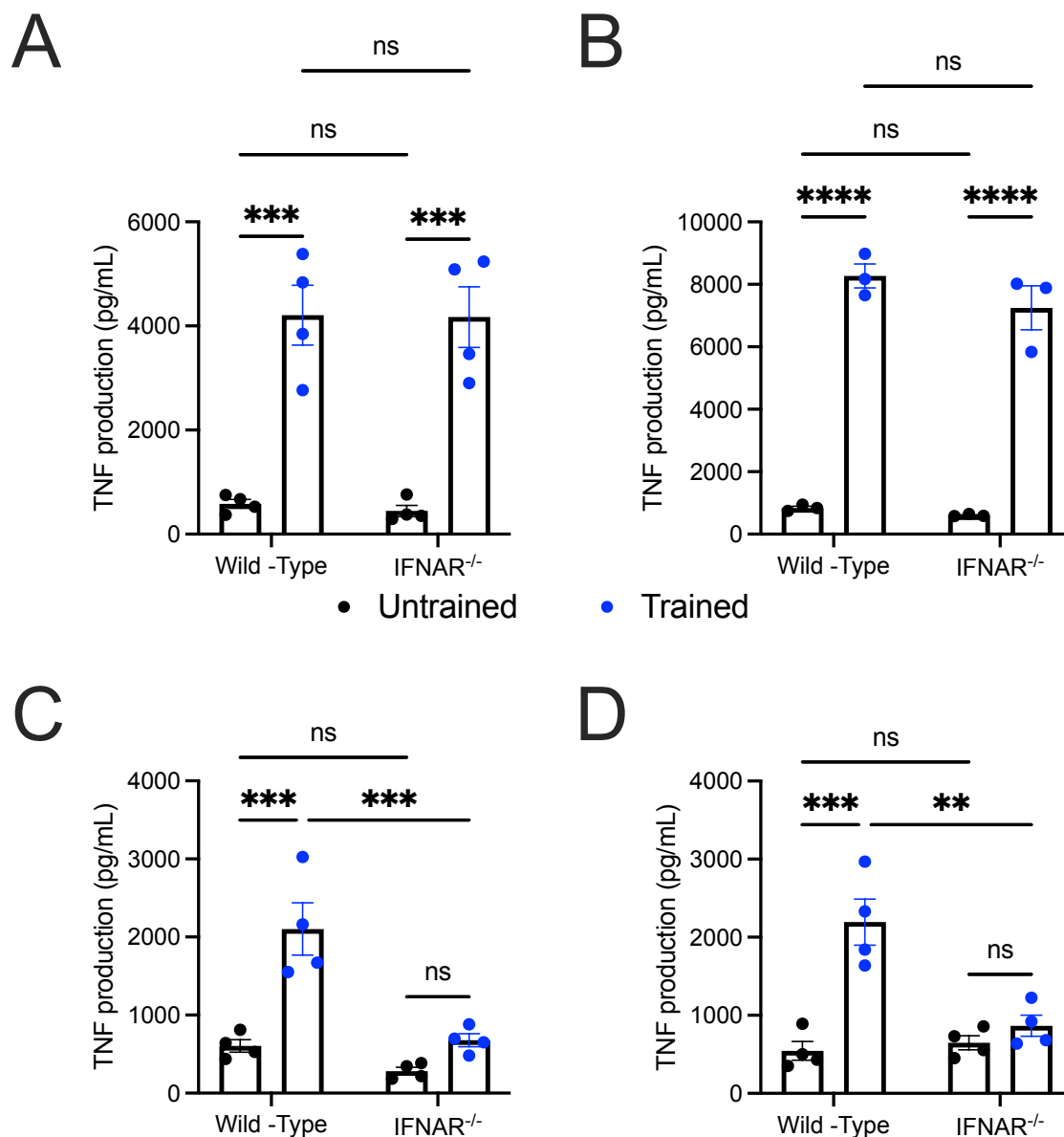


Figure 4.46 TNF responses in classical trained immunity vs interferon dependent trained immunity

BMDMs were differentiated from wild-type or IFNAR KO (-/-) mice and trained +/- WGP for a period of 6 days as previously described. BMDMs were then stimulated for a period of 4hrs with 10ng/mL LPS (A) or 10μg/mL PAM (B), 10μg/mL pIC (C) or 1μg/mL TpIC (D). Supernatant was collected and TNF production was measured via ELISA. Each dot is a representative of an individual experiment (Biological N = 4 or N = 3). Ns= Not significant, **P-value <0.005, ***P<0.0002; ****P<0.0001; (Two-way ANOVA with Šídák's multiple comparison test).

4.2.13 Interferon signalling via IFNAR is important for enhanced non-classical trained responses to viral ligands and bacterial ligands

Since I saw that IFNAR signalling was important for the enhanced TNF responses towards viral ligands, but not bacterial ligands, I wanted to test how loss of this receptor would affect non-classical training responses such as classic anti-viral cytokines IFN β and IP-10. To do this IFNAR KO and WT BMDMs were trained as previously described. On day 6 cells were stimulated for 8hrs with pIC and IFN β and IP-10 were measured. As previously shown WT trained BMDMs significantly increased IFN β and IP-10 production in response to pIC. However, in the absence of interferon signalling again trained BMDMs had a significantly reduced capacity to produce IFN β and IP-10 (**Figure 4.47A-B**). Lack of IFNAR signalling had no effect on the untrained cells capacity to produce IFN β , showing that IFN signalling did not affect the cells ability to directly produce this cytokine. However, loss of IFN signalling significantly reduced the untrained cells capacity to produce IP-10 in response to viral ligands, thus confirming that autocrine interferon signalling is required to amplify it's effects.

Since the bacterial ligand LPS can signal through canonical MyD88 as well as a TRIF-dependent mechanisms, I wanted to see if lack of IFN signalling would effect WGP's ability to enhance IFN and IP-10. Untrained or trained cells from WT or KO animals were stimulated with LPS for 8hrs. As expected, both IFN β and IP-10 were enhanced in WT BMDMs trained with WGP, however, in the absence of interferon signalling there was a significant reduction in the capacity of trained BMDMs to enhance both IFN β and IP-10 (**Figure 4.47C-D**). As expected loss of interferon signalling also significantly affected the ability of untrained BMDMs to enhance IP-10 production in response to LPS, since IP-10 is an ISG and thus requires autocrine interferon signalling to amplify it's effects. These results are interesting since classical training responses like TNF were not affected by lack of interferon signalling but non-classical training responses were. This suggest that trained immunity that impacts TLR4 signalling through MyD88 may still be in-tact, but reprogramming of ISGs and type I IFNs is lost. This again highlights the importance

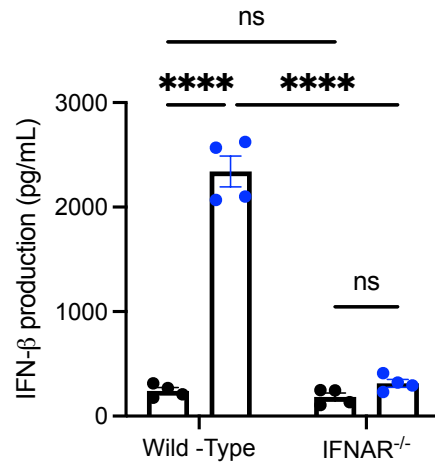
of IFNs in driving a form of non-canonical training that impacts anti-viral genes and responses.

Having seen that IFNAR deficient BMDMs fail to increase their responses in trained cells, I wanted to see if they altered protection from live viral infection. To do this I used the same training protocol as previously described and infected cells with an MOI of 5 of VSV. As previously shown, trained BMDMs had enhanced IFN β production in response to the virus (**Figure 4.48A**). In contrast, trained BMDMs from IFNAR KO animals failed to enhance their production of IFN in response to VSV. In fact, both untrained and trained BMDMs from KO animals had significantly less IFN production in comparison with WT BMDMs. These results were also replicated when IP-10 was measured (**Figure 4.48B**). Taken together this data suggests that that in the absence of IFN signalling, β -glucan alone is not sufficient to drive anti-viral responses to live viral infection.

One issue to consider is that the genetic loss of IFNAR impacts all anti-viral signalling pathways due to reduced expression of PRRs (due to tonic IFN being important in maintaining their receptor levels²¹⁸) as well as the amplification of ISGs. In order to counteract these issues I used an anti-IFNAR antibody present during the training process, but removed prior to restimulation with pIC. As shown in Fig. 4.49, there was no significant changes in IFN responses between vehicle, IgG control and anti-IFNAR antibody, showing that blocking IFNAR for 6 days has no impact on cellular responses to pIC (**Figure 4.49**). However, there was a significant reduction in the IFN response in trained cells that were in the presence of an anti-IFNAR antibody compared with the vehicle and the IgG control.

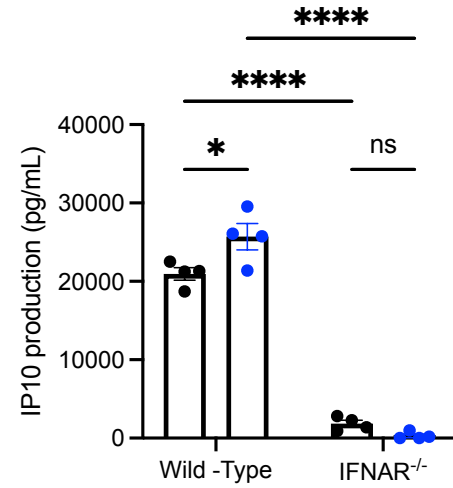
Take together, this data confirms that IFN signalling during the training process primes the cell to enhance their anti-viral potential.

A

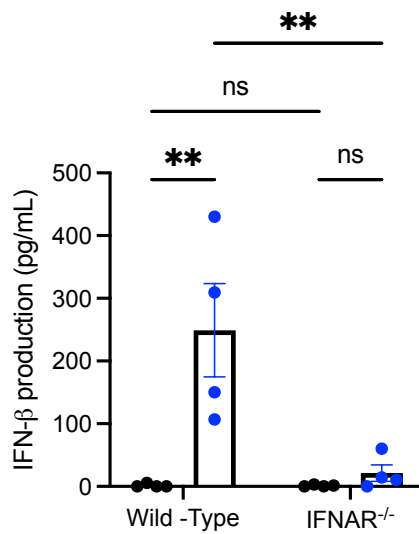


• Untrained • Trained

B



C



D

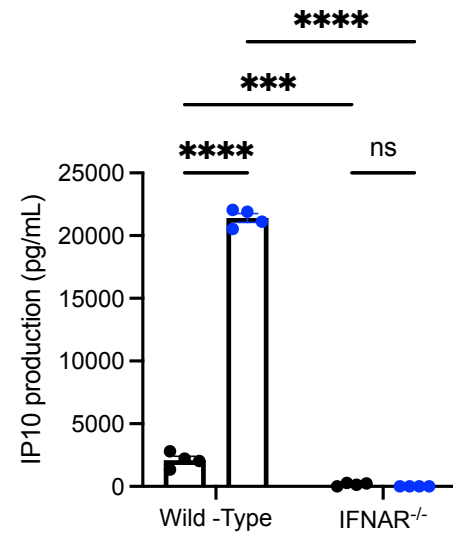


Figure 4.47 IFN signalling is vital in non-classical trained immunity

BMDMs were differentiated from wild-type or IFNAR KO (-/-) mice and trained +/- WGP for a period of 6 days as previously described. BMDMs were then stimulated for a period of 8hrs with 10 μ g/mL pIC (A-B) or 10ng/mL LPS (C-D). Supernatant was collected and IFN β or IP-10 production was measured via ELISA. Each dot is a representative of an individual experiment (Biological N = 4). Ns= Not significant, ***P<0.0002; ****P<0.0001; (Two-way ANOVA with Šídák's multiple comparison test).

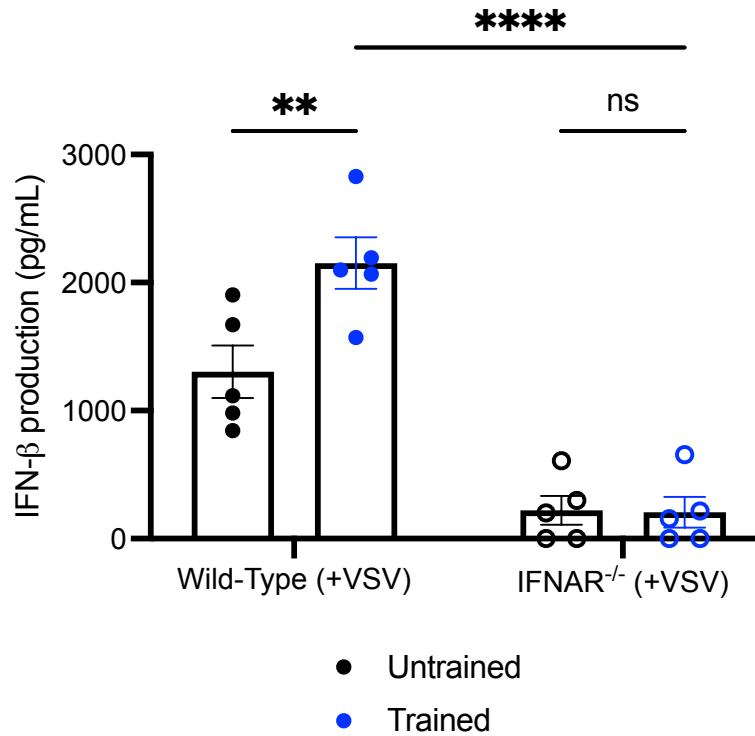


Figure 4.48 WGP mediated enhanced cytokine responses to viral infection are dependent on type I interferon signalling

BMDMs were differentiated from wild-type or IFNAR KO (-/-) mice and trained +/- WGP for a period of 6 days as previously described. BMDMs were then infected for a period of 24hrs with an MOI 5 of VSV. Supernatant was collected and IFN β production was measured via ELISA. Each dot is a representative of an individual experiment (Biological N = 5). Ns= Not significant, **P<0.002; ****P<0.0001; (Two-way ANOVA with Šídák's multiple comparison test).

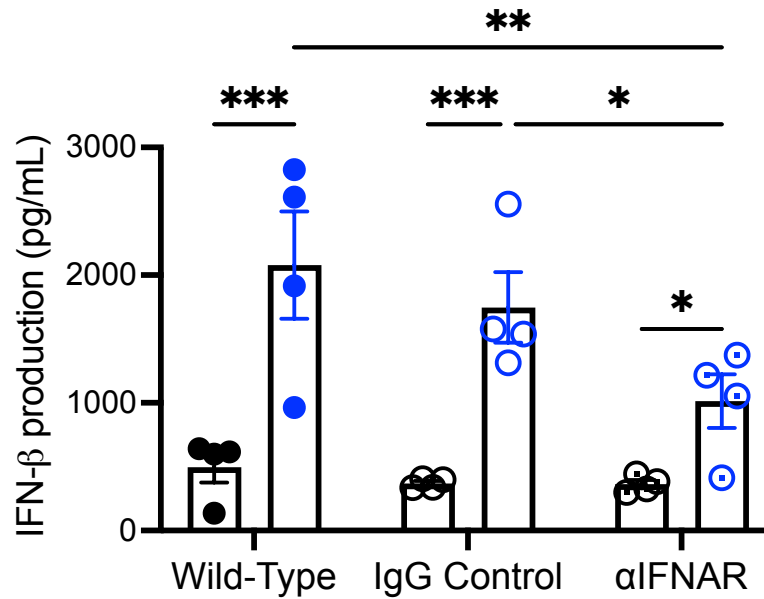


Figure 4.49 IFN signalling during the training process is vital for non-classical anti-viral trained immunity

BMDMs were differentiated from wild-type mice and trained +/- WGP for a period of 6 days as previously described. 3hrs prior to WGP stimulation BMDMs were treated with IgG isotope control or anti-IFNAR antibody. After 24hrs cells were washed and fresh antibody was added. Fresh media was added on day 3 containing fresh antibody. BMDMs were washed on day 6 and stimulated with 10µg/mL of pIC for a period of 4hrs. Supernatant was collected and IFNβ production was measured via ELISA. Each dot is a representative of an individual experiment (Biological N = 4). Ns = Not significant, *P<0.05; **P< 0.002; ***P<0.0002; (Paired Two-way ANOVA with Šídák's multiple comparison test).

4.3 Discussion

Work from this chapter built on information from the previous chapter, in that I showed that WGP mediated trained immunity was not just specific to bacterial and fungal ligands, both of which are well characterised in the literature^{219, 220}, but also effective at boosting responses to viral PRR agonists. Although trained immunity has been described in the literature to confer protection from viral infections, in both human and mouse models, little is known about the exact mechanism by which a fungal agent can confer such non-specific immunity from a viral challenge^{86, 95}. However, throughout this chapter I propose a unique mechanism by which macrophages confer cross protection against viral pathogens via type I interferon signalling. This signalling occurs throughout the training process and is vital for enhanced responses to viral agonists and viral pathogens as I have demonstrated.

The non-specific nature of trained immunity is a well-established core principal which many groups have harnessed throughout the years⁶⁶. I started this chapter by demonstrating that this is also the case for anti-viral trained immunity. I screened our trained cells versus a number of TLR and RLR agonists which are typically used to mimic viral PAMP/DAMPs in-vitro. I used TNF as a readout for these experiments, since TNF is considered a “classical” cytokine associated with trained immunity. Overall, trained cells had overall greater TNF responses to all ligands, be it cytosolic or endosomal responses, and did not depend on whether the agonist was RNA or DNA.

Next, I used a screen of PRR agonists that are well described to drive a type I interferon response in myeloid cells^{221, 222, 223}. Unlike with TNF responses I began to see some specificity with WGP mediated anti-viral training. When untrained and trained cells were stimulated with a cGAS ligand, G3-YSD, there were no significant increases in IFN- β production between the two groups. In contrast when cells were stimulated with a STING agonist, DMXAA, trained BMDMs enhanced their IFN- β production. This is suggestive of specificity in the trained response to viral ligands, potentially pointing to a mechanistic effect of priming macrophages at specific regions, either through epigenetic modifications of response genes or changes in expression of intracellular PRRs or signalling cascades.

Since I was able to modulate/activate both cytosolic and endosomal receptors with poly I:C I used this as my model viral agonist for the majority of the experiments moving forward in this thesis. I was successfully able to show that trained responses to poly I:C stimulation were not just specific to TNF and IFN- β . I demonstrated that both pro- and anti-inflammatory cytokines are heightened, with trained cells showing increased IL-6 and IL-1RA production upon poly I:C stimulation. Interestingly, even though I did not see any differences in the anti-inflammatory cytokine, IL-10, I did show that basally WGP cells produced more IL-1RA, and thus these cells are anti-inflammatory in nature. This is a vital discovery for my work since a balanced/regulated immune response is vital to confer protection from viral infection, particularly in the context of cytokine storm²²⁴. More importantly, during the COVID-19 pandemic modified IL-1RA drugs were used to treat severe disease and it has been shown that IL-1RA effects inflammatory cytokines, but importantly not viral replication or anti-viral IFNs during infection^{225, 226}. I was also able to demonstrate that WGP training enhanced IFN responses to both RNA and DNA viruses and that trained macrophages were less susceptible to viral infection. Therefore, the fact that WGP trained macrophages basally enhance their IL-1RA production, coupled with enhanced anti-viral cytokines and ISGs (IFN and IP-10) and display reduced viral susceptibility, makes WGP an attractive option for exploring anti-viral therapies.

Adding further to the literature, I was able to generate three unique transcriptomic datasets as shown in this chapter. Firstly, I showed the transcriptional differences between naïve and trained macrophages. Unlike other data sets these were left unstimulated to explore changes at baseline. Of particular interest were the increases in genes and pathways classically associated with trained immunity as I have discussed in the results section. Classically training is thought to be associated with an effective return to baseline. Our group have shown that WGP trained cells do not show increased cytokine responses unless stimulated with a secondary ligand, thus we thought these cells return to baseline⁹⁹. However, the RNA-seq data has revealed that these cells have an altered transcriptional landscape with changes in the expression of various cytokines and anti-pathogen signalling pathways. However, these changes could be due to the epigenetic state of the cells,

something that I wish to explore further in the future. Interestingly there was also a novel pathway involved in cytosolic nucleic acid sensing as well as anti-viral pathways. Groups have shown in-vivo that viruses can induce localized trained immunity and others have shown that enhanced anti-viral responses are due to changes in cellular receptor responses^{93, 95, 211}. However, the mechanism behind these changes are poorly defined. My transcriptional data set, particularly cytosolic nucleic acid sensing, may point to a potential mechanism linking enhanced anti-viral changes, IFN signalling and metabolism, which I shall expand upon in my next chapter. Secondly, I have expanded on literature of restimulated cells that have been trained. I showed that some of the most highly upregulated pathways in trained cells that were stimulated with poly I:C were involved with cellular metabolism, particularly in amino acid transport, including glutamine, as well as in oxidative phosphorylation. I have shown the importance of oxidative metabolism for trained immunity in the previous chapter, however, I expand on it here showing that transcriptionally trained cells express higher TCA and OxPhos genes than untrained cells when they are stimulated with poly I:C. It has been shown that RLR-mediated anti-viral responses are dependent on OxPhos in certain cell types and therefore it is interesting to postulate whether trained cells have enhanced responses to viruses due to their enhanced OxPhos during viral ligand antagonism²²⁷.

Finally, I was able to identify that the enhanced anti-viral transcriptome was dependent on IFN-signalling. I showed that many of what I refer to as “classical” trained genes and pathways were not affected by the loss of IFN signalling in IFNAR deficient macrophages. However, the “non-classical” anti-viral pathways were greatly affected, in particular, cytosolic nucleic acid sensing pathways. I mentioned in the intro to these results that a major limitation to this was that I was comparing WT to KO cells without taking into account the loss of the genotype. Although this is a worry, which I am currently focusing on for my studies, I have been able to show that anti-IFNAR antibodies (removed prior to pIC stimulation) recapitulate the effects of KOs. Thus these anti-viral responses are still dependent on IFN signalling through IFNAR. The literature focuses on cytokines such as IL-1 β and type II IFN, IFN- γ , as drivers of trained immunity^{34, 228}. However, I provide evidence in this chapter for a role of type I IFNs in priming cells for specific anti-viral responses. Interestingly,

when we trained cells in-vitro with WGP in wild-type vs IFNAR deficient cells we saw that loss of IFNAR had no effect on the enhanced pro-inflammatory responses to bacterial ligands, however, enhanced TNF responses were lost in poly I:C stimulated cells. This is suggestive of an unique IFN dependent training mechanism which I refer to as non-classical or Interferon-signalling Dependent innate Training (IDT). Without the ability to drive this novel pathway macrophages are incapable of enhancing IFN and ISG responses to both bacterial and viral ligands as well as live viral infections. Finally, I show that this pathway is independent of an altered signalling cascade. Removal of IFNAR from cells will alter their ability to respond to viral ligand stimulation since IFNAR is required for maintaining receptors such as RIG-I and MDA5 as well as downstream cascades vital for enhanced IFN responses²²⁹. Therefore, to confirm that IDT was dependent on IFN signalling and not changes in receptor or downstream signalling cascades I used an anti-IFNAR antibody only present during the training phase and not present during restimulation. Loss of IFN signalling during the training phase significantly reduced the ability of trained BMDMs to enhance their responses to poly I:C and thus confirmed the need for IFN to induce this novel training pathway. One limitation to these studies, however, was that I didn't measure changes in the protein expression of anti-viral receptors. In the transcriptional data there are changes in the expression of genes associated with anti-viral signalling, which can include receptors such as MDA-5 and RIG-I. One theory that I didn't explore, but I will be investigating in the future, was that changes in the protein expression due to IFN signalling induced by WGP would prime macrophages to be more sensitive to anti-viral responses.

The identification of this novel pathway provides an attractive area to research in the context of disease. It has been well documented that in certain autoimmune and inflammatory diseases that type I IFN play a role in driving inflammation and disease progression^{230, 231, 232}. However, more recently this type I interferon signature has been associated with a maladaptive trained phenotype in monocytes and macrophages in Primary Sjögren's syndrome²¹³. Since the field of trained immunity often looks at ways of blocking this pathway to show the mechanistic cogs involved in training there is a potential for development of therapies to combat this disease. Therefore, it is interesting to speculate that IDT may play a role in both health and disease and that further research into this pathway could unlock novel therapies for

patients suffering with diseases induced by increased type I IFN levels. It is also important to note that since type I interferon itself can induce trained immunity that elevated levels of IFN in the circulation could be a major driver of various inflammatory conditions (as mentioned above) and thus a causative agent of disease progression.

One limitation of the work in this chapter is the utilisation of murine bone marrow derived macrophages. BMDMs are typically grown in culture for 6 days in-vitro and thus may not fully represent in-vivo macrophages. However, they are a very easy cell type to model macrophage responses to ligands and have been used to model metabolic and cell signalling responses to ligands such as LPS and viral infections previously^{233, 234, 235}. Another limitation of this chapter was again to measure whether this pathway was linked with specific epigenetic modifications. It has been shown that interferon can drive specific epigenetic modifications, particularly disposition of H3.3 on replacement histones and H3K36me3¹⁸⁴. The authors describe that certain ISGs can be epigenetically primed and thus form a “memory” phenotype, which they refer to as memory ISGs, whereas other ISGs are not epigenetically primed. It would be interesting moving forward with my work to investigate if a similar phenomenon happens with IDT post WGP training and if patients from interferon diseases also have similar epigenomes.

The work in this chapter found that WGP was capable of driving a unique type I interferon-signalling dependent training pathway that enhanced anti-viral responses and also reduced macrophage susceptibility to viral infections. The identification of IDT in this chapter could have significant impact in the study of diseases that induce type I IFNs as well as in the development of therapies that block IFN. Further insights into the epigenetic regulation of this pathway could yield enhanced therapeutic strategies not only for the development of therapies for patients with high IFN levels but also in developing drugs to combat emerging viral pathogens with pandemic potential.

Chapter 5. WGP β -glucan drives type I interferon via immunometabolic reprogramming

5. 1 Introduction

Immunometabolic rewiring is a core component of how innate immune cells undergo innate immune training²³⁶. It has long been known that metabolism can impact the immune response. Early studies showed the effects of malnutrition on the immune response particularly in the context of obesity where over-nutrition could lead to enhanced proinflammatory cytokine production²³⁷. More recently, work has linked the metabolic cellular responses with cellular effector functions, highlighting the dynamic link between metabolism & immune function^{233, 238}.

In the context of trained immunity cellular metabolism has been described as a way by which scientists have been able to link the changing metabolic landscape with epigenetic changes. A pioneering study by Arts *et al*, demonstrated the impact of accumulating metabolites on β -glucan induced training can have on the epigenetic landscape of cells²³⁹. This research linked metabolic changes to the functional output of trained immunity. It has also been shown that type I IFNs can induce immunological memory through epigenetic regulation¹⁶⁴. However as of yet no one has shown any link between fungal ligand induced trained immunity, metabolism and type I IFNs. Similarly, very little is known about the impact the altered metabolic state of innate cells might have directly on anti-viral responses.

Other groups have shown that recognition of fungal ligands through Dectin-1 can drive a type I IFN response¹⁶⁸. Therefore, I hypothesized that WGP induced early interferon would drive metabolic responses and influence macrophage function to induce IDT. Alternatively, I hypothesized that metabolic responses triggered by WGP would induce an immunometabolic landscape that would facilitate IDT and enhanced anti-viral activity. This could occur either through IFNs directly impacting epigenetic modifications or metabolic alterations producing a cell more favourable for anti-viral activity.

5.2 Results

5.2.1 WGP drives type I interferon signature at early timepoints in BMDMs

Since I have previously shown a unique role for type I IFN in driving IDT, specifically in conferring non-specific memory to viral infections I next hypothesized that WGP stimulation of BMDMs drives a type I IFN signature. To test this hypothesis, I first stimulated BMDMs with WGP or LPS (positive control) for shorter time-points (>24hrs). As expected, stimulation with LPS drove an increase in the mRNA expression of *Ifn- β* (**Fig 5.01A**). LPS exposure drove heightened expression at very early timepoints (2hrs to 4hrs) with a drop off by 8hrs to 24hrs. Similarly, WGP was also able to increase the expression of *Ifn- β* . However, WGP did not induce as potent an IFN response as LPS and had very different *Ifn- β* kinetics. This response was more gradual and peaked with a 2-fold increase at 8hrs, before returning towards baseline. Similarly, I investigated the changes in the expression of the ISG IP-10 (**Fig 5.01B**). Like *Ifn- β* , *Ip-10*, which has been shown to be induced downstream of IFN signalling as well as NF- κ B signalling^{240, 241}, also rapidly peaked in LPS exposed BMDMs. By 24hrs the *Ip-10* response was returning towards baseline, although was still highly expressed. In contrast, WGP exposed cells had a more gradual *Ip-10* expression, with levels again peaking at 8hrs, like the *Ifn- β* trace. Again, by 24hrs *Ip-10* expression levels returned to baseline.

Since I saw similar traces for *Ifn- β* and *Ip-10* expression I wanted to investigate whether these increases were dependent on an IFN feedback loop. To test this, wild-type and IFNAR-KO BMDMs were treated with or without WGP for 8hrs (for mRNA) and 24hrs (for protein). As before, 8hrs of treatment with WGP enhanced the expression of *Ip-10* mRNA (**Fig 5.02A**). However, in the absence of IFN signalling there was no significant difference between media and WGP treated cells. There was also a significant reduction of *Ip-10* mRNA between wild-type WGP treated and IFNAR-KO WGP treated BMDMs. I also examined if the protein expression was altered between WGP treated wild-type and IFNAR-KO BMDMs (**Fig 5.02B**). 24hrs post WGP treatment there was a significant increase in IP-10

production in WGP treated cells. In-contrast this effect was lost in the IFNAR-KO. Taken together this data highlights that exposure to WGP induces an IFN signature in BMDMs and that this early induction of IFN signalling primes BMDMs ISG expression.

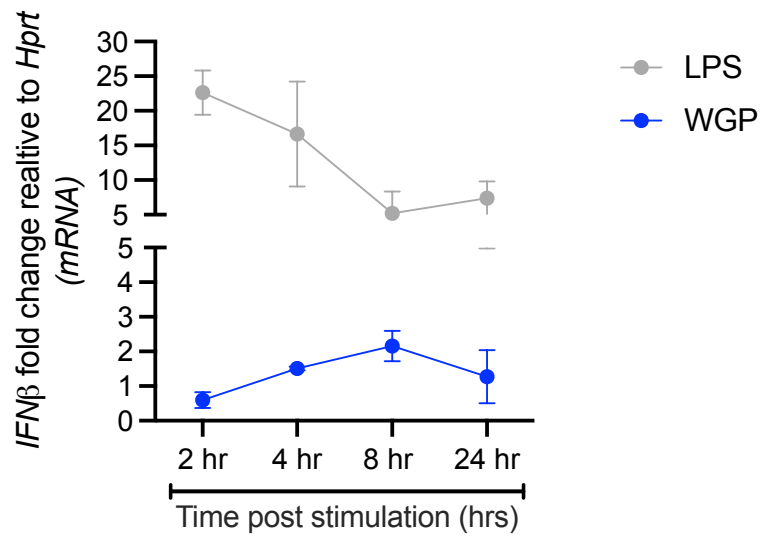
Having seen these previous results I wanted to investigate if other ISGs were sensitive to WGP stimulation. Again, using qPCR, I investigated changes in the expression of *Isg15*, *Usp18* and *Irg1* (**Fig 5.03A-C**). Similar to *Ip-10* expression there was a minor, although significant increase in the expression of *Isg15*. Interestingly, the expression levels of *Usp18* were not significantly altered with WGP stimulation. However, the expression of the metabolic ISG *Irg1* was significantly increased. This data indicates that ISG expression is altered upon WGP stimulation and that there is some specificity in the ISG response.

Since it has been shown in DCs that IFN responses to β -glucan occur independently of IRF3 and IRF7 signalling¹⁶⁸, I decided to measure the expression of two other interferon response factors *Irf1* and *Irf5*, two regulators of IFN signalling. Interestingly, 2hrs post WGP stimulation both *Irf1* and *Irf5* expression levels were increased relative to the media control (**Fig 5.04A**). 4hrs after WGP stimulation *Irf5* expression was further amplified, however, *Irf1* expression was reduced below baseline levels. However, this trend was again inversed again 8hrs after WGP stimulation. *Irf1* levels were enhanced whereas *Irf5* levels fell below baseline. By 24hrs both *Irf1* and *Irf5* expression levels returned to that seen in the media control. This data suggests that early induction of both *Irf1* and *Irf5* may be important for IFN signalling and that throughout the first 24hrs there are various regulatory factors that alter mRNA expression of *Irf1* and *Irf5*.

It was important to investigate whether the change in *Irf1* expression occurred independently of IFN signalling, since IRF1 has been shown to enhance ISG responses independently of IFNAR. Wild-type and IFNAR-KO BMDMs were treated with or without WGP for 8hrs (**Fig 5.04B**). As expected in wild-type BMDMs there was a significant increase in *Irf1* expression. *Irf1* levels were reduced in the media control in the IFNAR-KOs compared with the media wild-type. However, WGP

treated IFNAR-KO BMDMs had significantly increased *Irf1* expression, however, this was lower than WGP treated wild-types showing that WGP induced *Irf1* expression can be induced in both an IFN dependent and IFN independent manner.

A



B

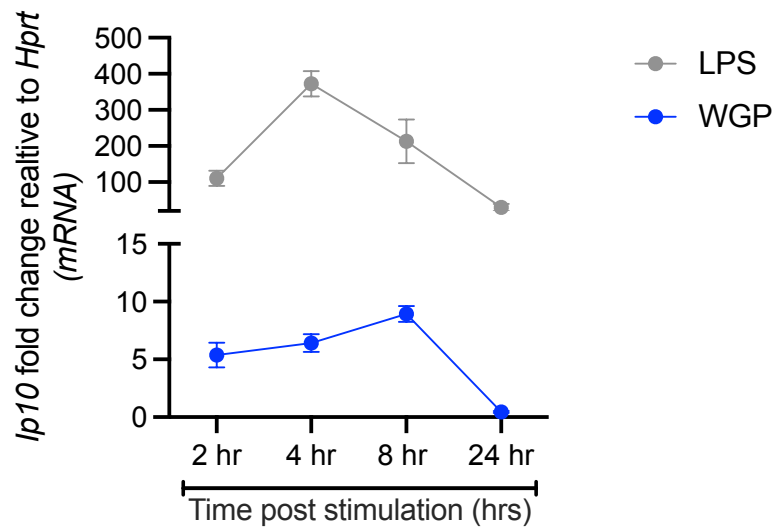
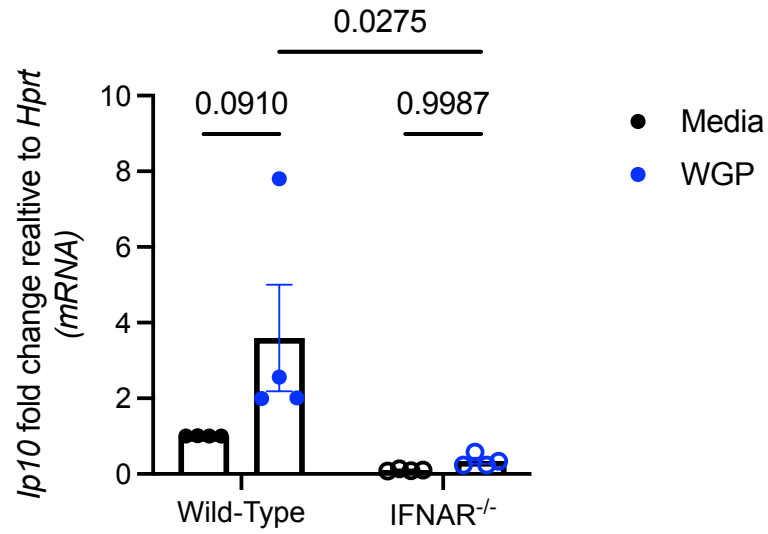


Figure 5.1 Early WGP exposure drives IFN and ISG signalling in BMDMs

BMDMs were treated with 100ug/mL WGP or 100ng/mL LPS control at the different timepoints shown on the graphs. Cell lysate was taken for RNA extraction and purification at these various timepoints. qPCR was performed according to material & methods section. X-Y graph is representative of changes in mRNA expression to media control. (A) changes in mRNA expression of *IFN-β* and (B) *IP-10*. (N = 3 (A) and 4 (B)).

A



B

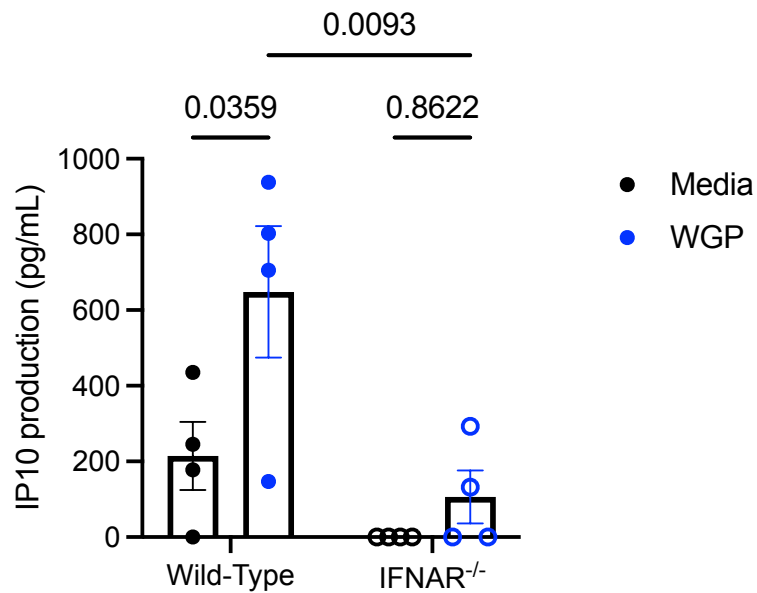


Figure 5.2 Changes in IP-10 expression at early timepoints upon WGP exposure in wild-type vs IFNAR-KO BMDMs

BMDMs were treated 100ug/mL WGP media control for a period of 8hrs (A) or 24hrs (B). Cells were washed and cell lysate was taken for RNA extraction and purification. qPCR was performed according to material & methods section for (A). After 24hrs media was taken from cells and IP-10 ELISA was performed according to the manufacturer's instructions (B). Each dot is a representative of an individual experiment (Biological N = 4). Exact P-values shown. (Two-way ANOVA with Šidák's multiple comparison test).

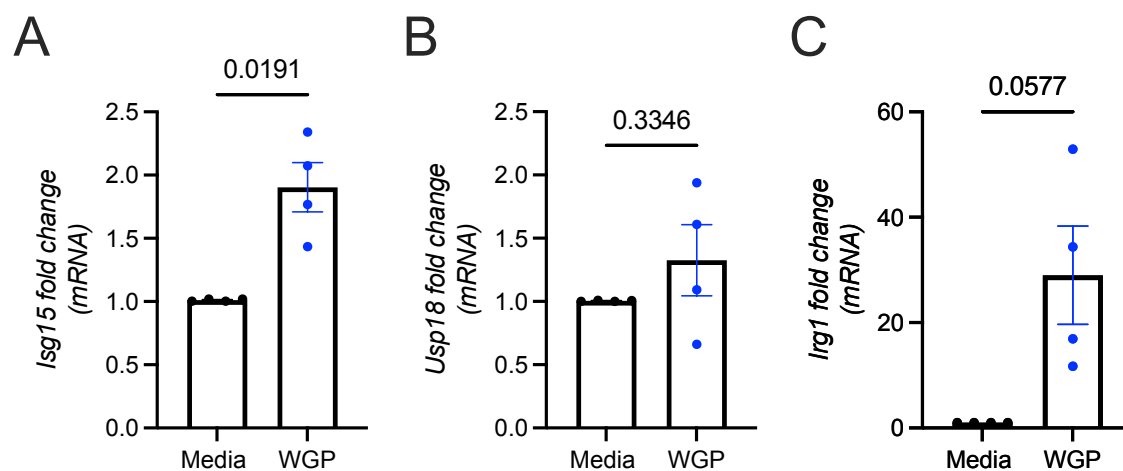
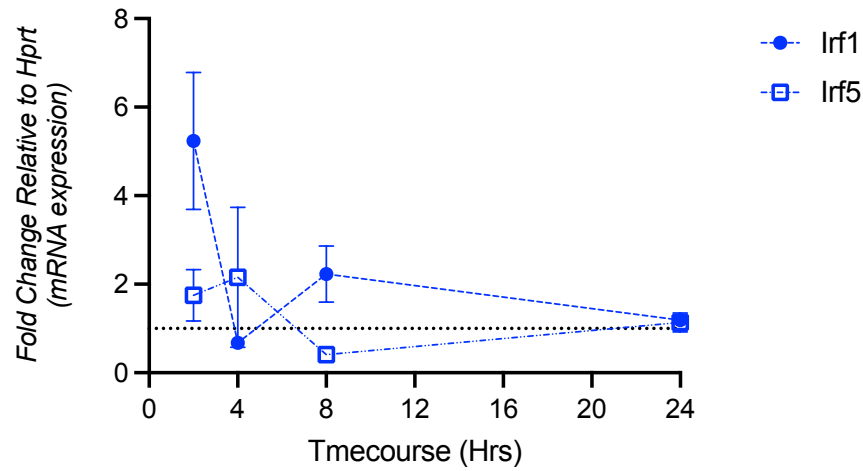


Figure 5.3 WGP induces changes in interferon-stimulated gene expression levels 8hrs post stimulation in BMDMs

BMDMs were treated 100ug/mL WGP media control for a period of 8hrs. Cells were washed and cell lysate was taken for RNA extraction and purification. qPCR was performed according to material & methods section. Quantification of *Isg15* (A), *Usp18* (B) and *Irg1* (C). Each dot is a representative of an individual experiment (Biological N = 4). Exact P-values shown (Paired student T-test).

A



B

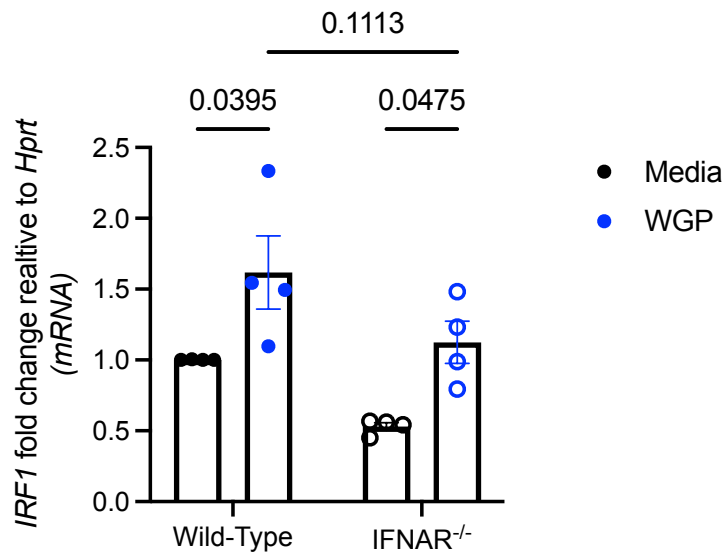


Figure 5.4 WGP drives early and differential expression of Irf5 independent of IFN signalling

BMDMs were treated 100ug/mL WGP at different timepoints up to 24hrs. Cells were washed and cell lysate was taken for RNA extraction and purification. qPCR was performed according to material & methods section. (A) X-Y graph is representative of changes in mRNA expression to media control (0hrs). (N = 4). (B) Wild-type or IFNAR-KO BMDMs were treated with WGP or media control for 8hrs. Cells were washed and cell lysate was taken for RNA extraction and purification. qPCR was performed according to material & methods section. (N = 4); Exact P-values shown. (Two-way ANOVA with Šídák's multiple comparison test).

5.2.2 WGP metabolic rewiring occurs independently from type I interferon signalling

Since I had seen that WGP induces early type I interferon signalling I wanted to investigate whether this early signal induced the metabolic changes seen in earlier chapters. I have previously shown that metabolic reprogramming peaks 3 days post WGP stimulation I decided to investigate whether this was dependent on type I interferon. To do this I trained wild-type (WT) or IFNAR-KO (KO) BMDMs as previously described and ran extracellular flux analysis on these cells 72hrs post WGP stimulation.

First, I investigated changes in extracellular acidification rate (ECAR) a readout for glycolysis (**Fig 5.05**). As can be seen on the trace, stimulation with WGP induces changes in both WT and KO BMDMs relative to controls (**Fig 5.05A**). When this was further investigated it was seen that WGP stimulation led to increased basal glycolysis in both WT and KO BMDMs 3 days post stimulation, with no significant difference across genotypes (**Fig 5.05B**). Similarly, type I interferon signalling was not required to maintain baseline glycolysis, since there were no significant changes between media treated WT and KO BMDMs. Next, I investigated changes in glycolytic capacity (**Fig 5.05C**). WGP induced significant changes in KO BMDMs alongside a non-significant increase, in the WT cells (p-value = 0.11). However, interestingly I showed that type I interferon signalling was important for maintaining glycolytic capacity, since there was a significant reduction in this readout in media treated KO cells. Similarly, there was a trend towards a significant reduction between the WT WGP treated and KO WGP treated (p-value = 0.058), suggesting that baseline IFN signalling plays a role in this enhancement. There was no significant change in glycolytic reserve between media and WGP treated WT or KO cells, showing that WGP does not enhance the cells' ability to utilise glycolysis for ATP production (**Fig 5.05D**). However, both sets of KO cells had reduced glycolytic reserve relative to their WT counterparts, again suggesting that interferon signalling plays a role in glycolytic metabolism. Finally, in order to make sure that changes in ECAR were dependent in changes in glycolysis I investigated the non-glycolytic acidification rate (**Fig 5.05E**). WT BMDMs had no statistical changes between media or WGP treated cells. However, in contrast WGP treated KO BMDMs had an

increase relative to the media treated control. However, it is important to note here that media treated KO BMDMs had overall lower non-acidification rates relative to the WT media treated cells and therefore this statistical increase was just due to a lower baseline level. This was also shown since WGP treated cells had no changes between WT or KO cells. Taken together this data indicates that overall interferon signalling does not play a role in WGP mediated glycolytic reprogramming. However, it does indicate that type I interferon signalling might be important in maintaining baseline glycolytic changes.

Since I have shown previously in chapter 3 that both glycolysis and oxidative metabolism are affected by WGP stimulation I next wanted to investigate changes in OxPhos in the context of IFN signalling (**Fig 5.06**). Again, the trace showing oxygen consumption rate (OCR) highlighted that WGP enhanced the OxPhos metabolism of BMDMs in both WT and KO cells (**Fig 5.06A**). Upon closer investigation as I have previously shown, WGP significantly increased basal OxPhos in WT BMDMs (**Fig 5.06B**). Similarly, WGP enhanced basal OxPhos in KO BMDMs as well. There were no significant differences in basal OxPhos between WGP treated WT and WGP treated KO BMDMs (p-value = 0.93). However, similar to glycolytic metabolism the inability to signal via IFNAR significantly reduced the cells basal OxPhos. Next, I showed that WGP enhanced the max respiratory rate of BMDMs in both WT and KO cells (**Fig 5.06C**). However, there was a significant reduction between both WGP treated WT and WGP treated KO BMDMs as well as between media treated WT and KO cells. Interestingly, WGP treated KO cells could only reach similar levels to media treated WT cells, showing that type I interferon might be important in reaching the cells full respiration rate. Since I saw that WGP treated cells didn't rely on glycolysis for ATP production I wanted to see if they used mitochondrial metabolism for their ATP reserves (**Fig 5.06D**). WGP treated WT and KO cells had a significantly enhanced ability to generate ATP via OxPhos. Interestingly, baseline ATP levels seemed to be dependent on type I interferon signalling, with a significant reduction between media treated WT and media treated KO BMDMs. Finally, I showed here that the spare respiratory capacity (SRC) was increased in BMDMs in both WT and KOs after WGP treatment (**Fig 5.06E**). Both media and WGP treated KO BMDMs had a significantly reduced SRC, with media treated cells having virtually zero SRC. Taken together this data indicates that WGP can enhance

mitochondrial metabolism independently of type I interferon signalling. However, another result from these experiments demonstrates that the maintenance of baseline metabolic processes - both glycolytic and mitochondrial metabolism - in BMDMs are dependent on IFNAR signalling.

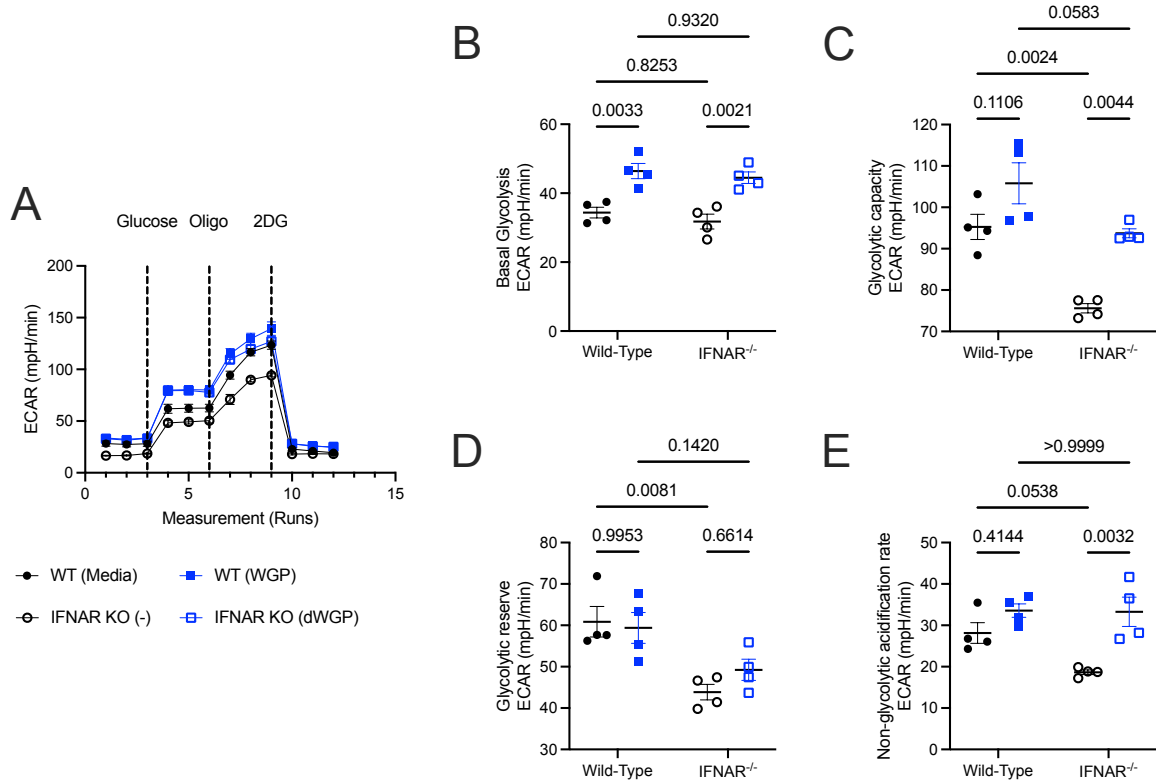


Figure 5.5 WGP drives glycolytic reprogramming independent of IFN signalling

Wild-type or IFNAR KO BMDMs were treated with 100ug/mL of WGP for 24hrs. Cells were washed and fresh media was added. Cells were rested for 48hrs. On day 3 post WGP stimulation glycolytic flux analysis was performed via Seahorse. (A) Overall glycolytic trace showing changes in glycolysis after various inhibitor treatments. (B) Basal glycolysis of wild-type and IFNAR KO BMDMs either untreated or treated with WGP. (C) Glycolytic capacity of wild-type and IFNAR KO BMDMs either untreated or treated with WGP. (D) Glycolytic reserve of wild-type and IFNAR KO BMDMs either untreated or treated with WGP. (E) Non-glycolytic acidification rate of wild-type and IFNAR KO BMDMs either untreated or treated with WGP. (N = 4); Exact P values shown. (Two-way ANOVA with Šidák's multiple comparison test).

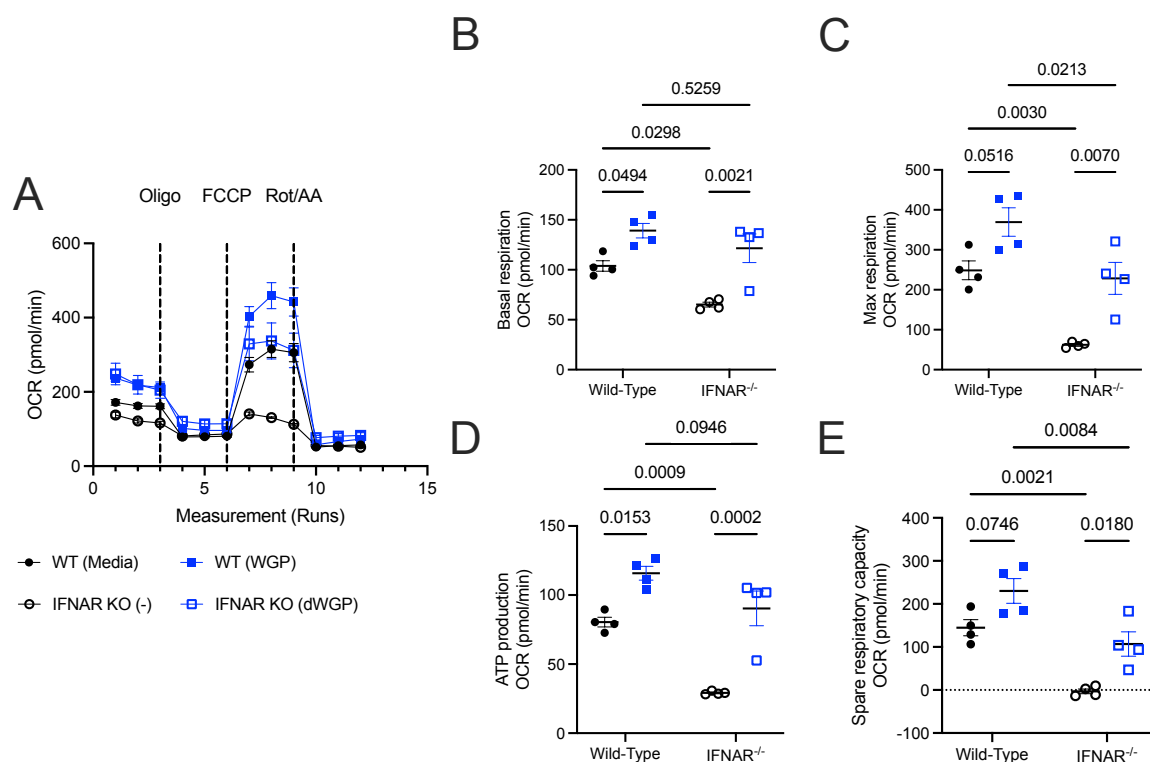


Figure 5.6 WGP drives oxidative reprogramming independent of IFN signalling

Wild-type or IFNAR KO BMDMs were treated with 100ug/mL of WGP for 24hrs. Cells were washed and fresh media was added. Cells were rested for 48hrs. On day 3 post WGP stimulation oxidative flux analysis was performed via Seahorse. (A) Overall oxidative trace showing changes in oxygen consumption rate after various inhibitor treatments. (B) Basal OxPhos respiration of wild-type and IFNAR KO BMDMs either untreated or treated with WGP. (C) Max respiration of wild-type and IFNAR KO BMDMs either untreated or treated with WGP. (D) ATP production of wild-type and IFNAR KO BMDMs either untreated or treated with WGP. (E) Spare respiratory capacity of wild-type and IFNAR KO BMDMs either untreated or treated with WGP. (N = 4); Exact P values shown. (Two-way ANOVA with Šídák's multiple comparison test).

5.2.3 WGP induces changes in glucose uptake and downstream flux, but not glycolytically linked metabolite abundance

To further investigate metabolic changes induced by WGP trained immunity I investigated changes in metabolic signalling and metabolite accumulation via carbon 13 labelled glucose isotope tracing 3 days post WGP stimulation in BMDMs. A set concentration of norvaline was spiked onto each sample to use as an internal control and normalisation of the results. The first group of metabolites I analysed were from lower glycolysis. I have previously shown that WGP stimulation leads to increased extracellular lactate production and glycolysis as measured by Seahorse as well as changes in glycolytic gene expression by day 3. Isotope tracing using universally labelled carbon 13 labelled glucose enables the ability to measure a number of glycolytic metabolites including phosphoenolpyruvate (PEP), pyruvate, lactate, as well as the amino acid, alanine, derived from glycolysis (**Fig 5.07A**). However, in order to validate the quality of my samples I measured the difference in glucose uptake between media control and day 3 WGP BMDMs (**Fig 5.07B**). To do this the total concentration of glucose was first measured in the media that I use in cell culture. Percentage glucose consumption was defined as the % total of glucose in the media minus the % of glucose after treatment. As a control I pretreated BMDMs with 2DG which inhibits glycolysis via the inhibition of hexokinase and glucose 6 phosphate isomerase. Three days post WGP stimulation there was a significant consumption of glucose by WGP treated cells compared to the control. There was a slight increase in the consumption of glucose in WGP treated BMDMs that had been pretreated with 2DG, however, this did not reach statistical significance. There was however, a reduction in the consumption of glucose in both media and WGP treated BMDMs that had been pretreated with 2DG, although again this did not meet statistical significance.

Next, using GC-MS I measured the relative abundance of metabolites I mentioned previously (**Fig 5.07C-F**). There was no change in the total levels of PEP, pyruvate, alanine or lactate, unimpeded glycolysis in WGP-trained cells, which would prevent accumulation of these metabolites. Therefore, I next decided to analyse the relative ratio of the different metabolites which helps to identify if there are changes in

pathway activity, metabolite bottle necking, as well as helping to link cellular function to metabolic state. First, I measured the ratio of lactate to pyruvate which helps to establish whether cells have switched their pyruvate stores away from TCA cycle fuelling to lactate generation, a hallmark of aerobic glycolysis (**Fig 5.07G**). There was no significant differences suggesting that the cellular redox state is maintained. Next, I measured the ratio of alanine to lactate (**Fig 5.07H**). Again, I did not see any significant changes. This suggests that both pyruvate reduction (towards lactate) and transamination are in equilibrium. Next I measured the ratio of PEP to pyruvate (**Fig 5.07I**). Again there was no change in this ratio. This is an important result as it suggests that pyruvate is actively being used, either via reduction, transamination or the TCA cycle and that there is no backup of PEP which would be suggestive of utilising glucose for gluconeogenesis. Finally, I measured the ratio of alanine to pyruvate (**Fig 5.07J**). There was a significant increase in this ratio, although it was a small increase (1.3 to 1.5 fold). This may suggest that there is alteration of the transamination enzyme alanine transaminase (ALT). This is an interesting finding since this enzyme requires glutamate stores to function. Therefore, this could indicate that WGP induces an increase in the uptake of both glutamine as well as glucose to fuel cellular metabolism.

Having seen that there were no changes in total metabolite abundance during lower glycolysis I decided to investigate whether flux was altered. To do this I measured the relative abundance of the various isotopologues of each metabolite. This is the amount of tracer found at the different derivatives of glucose. For lower glycolysis there are four main parts of the carbon backbone that can be derived from glucose (**Fig 5.08A**). M0 is where there is zero contribution of the tracer (glucose) to the metabolite. M1 and M2 means that there is partial incorporation of tracer into these metabolites. This can occur due to incorporation of unlabelled carbons from another fuel source (glutamine), TCA scrambling or through recycling pathways. Finally, this means that M3 labelling is total incorporation of the tracer by the cells.

The first metabolite I measured was phosphoenolpyruvate (**Fig 5.08B**). WGP caused a change in the isotopology of glucose derived carbons. It caused a significant reduction in M0 with a significant increase in M3, indicating that although there was no increase in total PEP abundance (bottleneck), there was a significant

contribution of glucose for generating PEP, indicating that glycolytic flux may be increasing. I next measured pyruvate (**Fig 5.08C**) and saw that there was no significant changes in labelling, suggesting that pyruvate is rapidly used by the cells. To investigate this further I measured the changes in reduction (lactate) and transamination (alanine) (**Fig 5.08D-E**). There was no significant differences in isotopology of lactate, but since we see that WGP enhances extracellular lactate it is safe to suggest that there is an adaptation in cellular release or transport of lactate out of the cell, potentially through changes in monocarboxylate transporters. However, I did see a significant reduction in M0 and a significant increase in M3 for glucose derived alanine labelling, suggesting that WGP causes a switch in the fuel source for alanine generation.

I also measured changes in ratios of the total M3 labelled metabolites. I first measured the ratio of pyruvate (end product of glycolysis) to PEP (the direct output from glycolysis) (**Fig 5.08F**). Here I saw a significant reduction in this ratio in WGP treated BMDMs, which was expected since glucose derived carbons were increased in PEP and were unchanged in pyruvate. Interestingly, there were no significant changes in the ratios of lactate to pyruvate (**Fig 5.08G**), alanine to pyruvate (**Fig 5.08H**) or lactate to alanine (**Fig 5.08I**). Taken together this data suggests that by day 3 WGP training does not cause changes in metabolite abundance during glycolysis, but does effect the source of carbons (increase in M3 alanine) as well as glycolytic flux (increase M3 PEP).

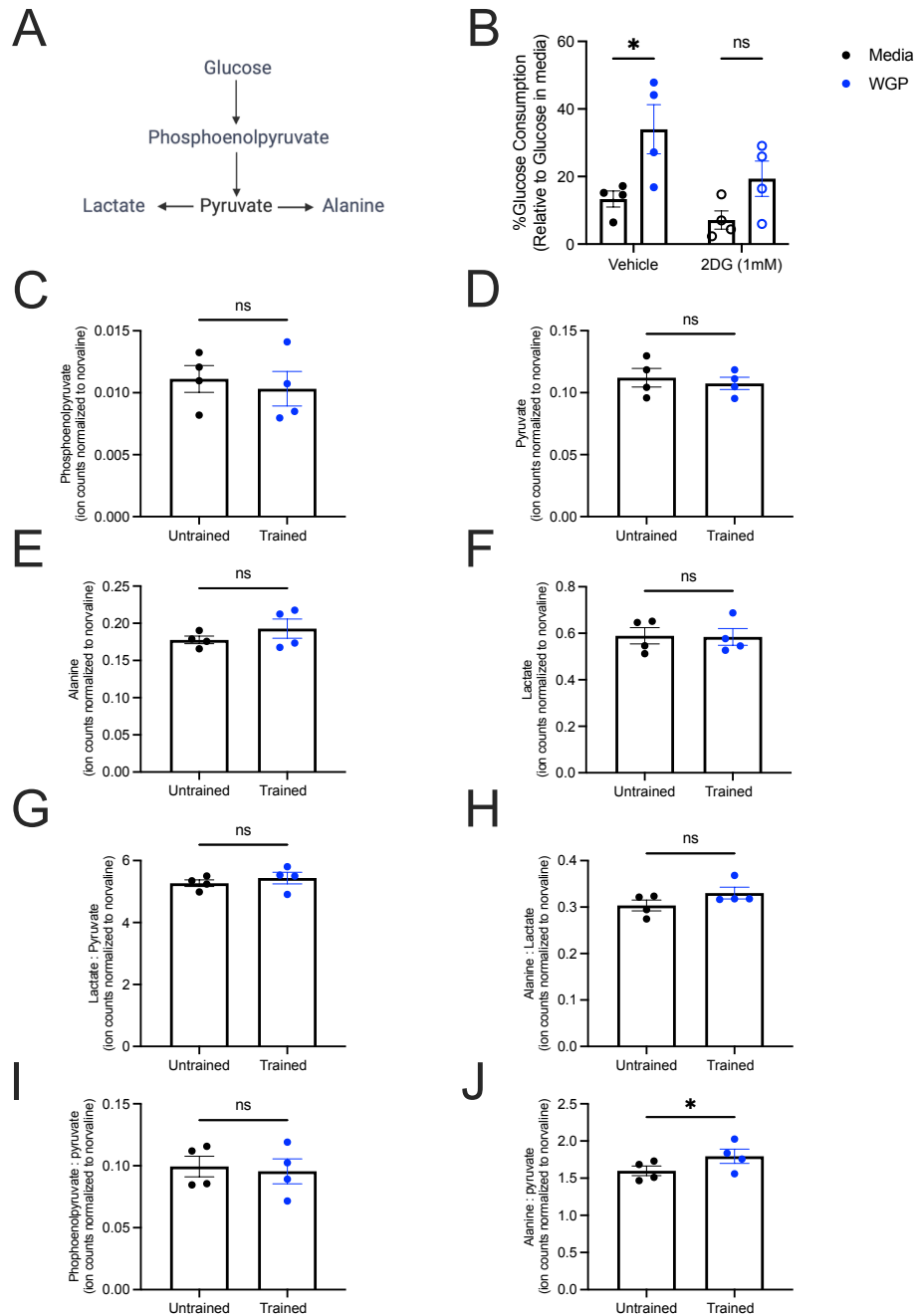


Figure 5.7 Glycolytic metabolite abundance is unchanged day 3 post WGP stimulation

BMDMs were treated with 100ug/mL of WGP for 24hrs. For glucose uptake assay, cells were pretreated for 1hr with 1mM 2DG (A). After 24hrs cells were washed and fresh media was added. Cells were rested for 48hrs. On day 3 post WGP stimulation cell media was removed for analysis of glucose uptake and cells were lifted, counted and replated. Freshly prepared media containing universally 13 labelled glucose was added to each sample for 8hrs. After 8hrs cells were lysed and normalised with Norvaline. Cell lysate was prepared as previously described and metabolites were measured by GC-MS (B). Each dot is a representative of an individual experiment (Biological N = 4). NS = P value >0.05, *P-value <0.05. (Paired t-test).

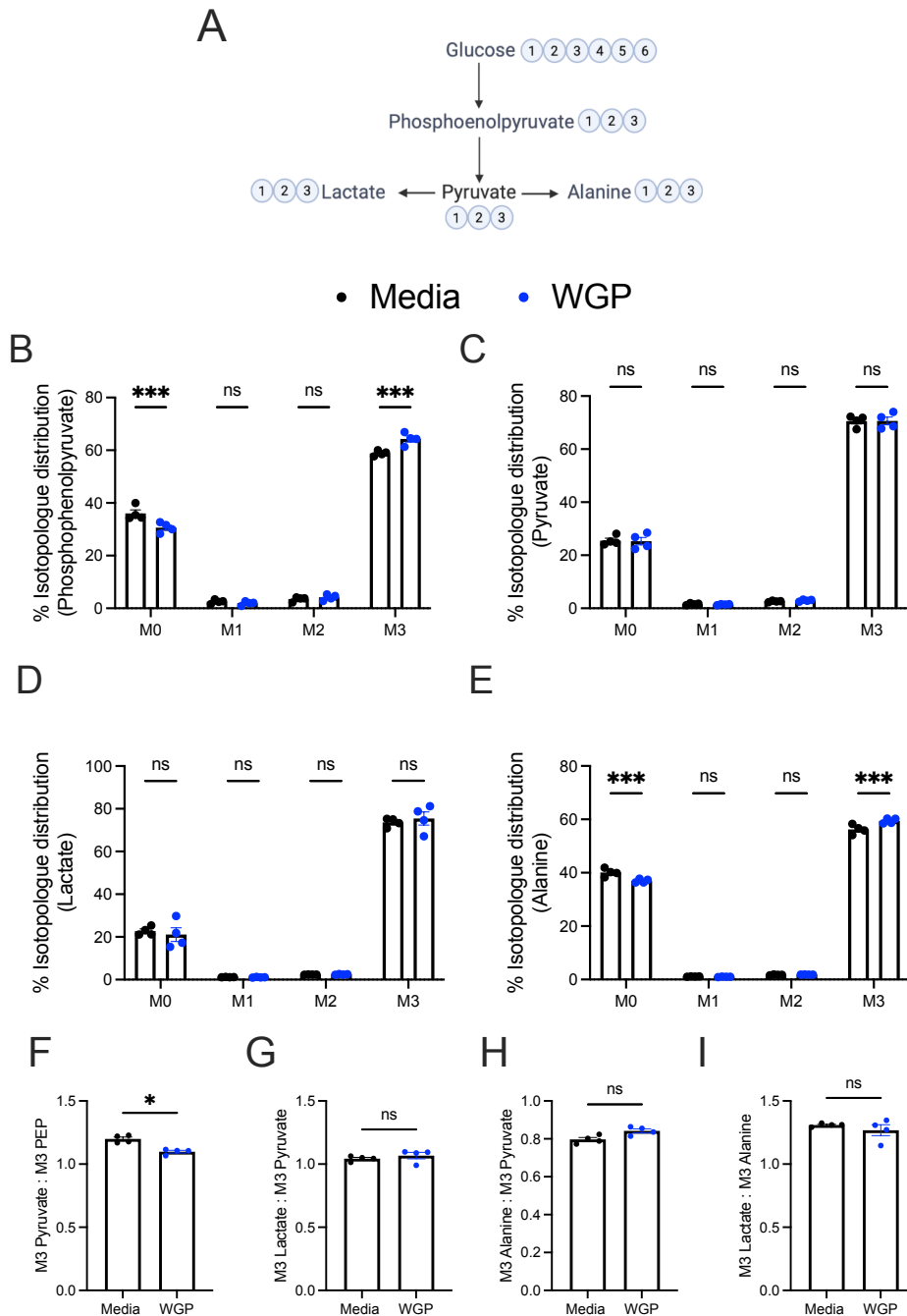


Figure 5.8 Glycolytic flux and labelling is altered day 3 post WGP stimulation

BMDMs were treated with 100ug/mL of WGP for 24hrs. Cells were washed and fresh media was added. Cells were rested for 48hrs. On day 3 post WGP stimulation cells were lifted, counted and replated. Freshly prepared media containing universally 13 labelled glucose was added to each sample for 8hrs. After 8hrs cells were lysed and normalised with Norvaline. Cell lysate was prepared as previously described and labelling of metabolites were measured by GC-MS. Each dot is a representative of an individual experiment (Biological N = 4). NS = P value >0.05, *P-value <0.05, ***P-value <0.0005. (Two-way ANOVA with Šídák's multiple comparison test (B-E) Paired t-test were one comparison was made (F-I)).

5.2.4 WGP induces changes in the abundance of core TCA-linked metabolites as well as flux from glucose derivatives

Even though WGP did not affect glycolytic metabolite abundance the effects on both glycolytic flux and oxidative metabolism led me to investigate whether TCA cycle metabolites were altered. Again, a carbon 13 labelled glucose tracer was used to investigate these metabolites (**Fig 5.09A**). I first measured the relative abundance of citrate in media treated vs WGP treated BMDMs on day 3 (**Fig 5.09B**). Similar to what was seen with the glycolytic metabolites, I did not see any changes in the relative abundance of citrate. The next metabolite I measured, itaconate, is very important in immunometabolic signalling (**Fig 5.09C**). Interestingly, 3 days post WGP stimulation I saw a significant increase in the relative abundance of itaconate. This nicely mirrored the increase in the gene *Irg1*, (seen earlier in **Fig 3.33B**), which encodes for the enzyme aconitate decarboxylase 1 (*Acod1*). Next, I measured the relative abundance of α -Ketoglutarate (**Fig 5.09D**). Similar to citrate, there was no significant increase in the abundance of this metabolite. There was, however, a significant increase in the relative abundance of succinate, another key immunometabolic signalling metabolite (**Fig 5.09E**). This corresponds with up-regulation of SDHB, (seen earlier in **Fig 3.33D**). Similarly, I also saw a trend towards a significant increase in another key TCA metabolite, fumarate (**Fig 5.09F**, p-value = 0.095). Again, this was interesting since previous data I have shown, the enzyme fumarate hydratase (*Fh*), which converts fumarate to malate, was significantly downregulated (seen earlier in **Fig 3.33E**). Finally, I saw no significant differences in the relative abundance of malate (**Fig 5.09G**).

To further appreciate these changes in metabolite abundance, I next analysed the ratios of metabolites that give important information on the metabolic profile of our cells. I first analysed the ability of 3 day treated BMDMs to produce itaconate from citrate (**Fig 5.09H**). This important ratio is used to indicate the enzymatic activity of ACOD1. I saw that there was a significant increase in the itaconate to citrate ratio, indicating increased itaconate production from citrate stores. Itaconate has a direct impact on succinate pools since itaconate has been shown to inhibit SDH. Therefore, I analysed the ratio of itaconate to succinate levels (**Fig 5.09I**). I saw that

this ratio was increased which suggests that in BMDMs 3-days post WGP treatment, there is a bias towards itaconate generation and away from accumulation of lower TCA cycle metabolites such as succinate. Interestingly however, I also saw an increase in the ratio of α -Ketoglutarate to citrate abundance (**Fig 5.09J**). Finally, I saw that there were significant changes in the succinate to citrate ratio as well (**Fig 5.09K**). Overall, this data indicates that 72hrs post WGP stimulation macrophages begin to accumulate key metabolites in the TCA cycle and with changes in the α -Ketoglutarate to citrate abundance as well as lower isotopologues increasing for α -Ketoglutarate may suggest changes in glutamine uptake. This data also provided me with evidence to suggest that there may be a break at SDH, as evident by increased succinate accumulation.

Next, I wanted to see if there were changes in flux of the TCA cycle and whether the abundance of metabolites were derived from glycolysis. To do this I tracked the various glucose-derived carbons that were imported into the TCA via pyruvate, which has three carbons derived from glucose uptake (**Fig 5.10A**). The first metabolite I measured was citrate (**Fig 5.10B**). I saw a significant reduction in the unlabelled M0 fraction after WGP stimulation, as well as a small but non-significant reduction in the M1 fraction. This suggests that another fuel source is not impacting carbon flux here and that it is more than likely pyruvate that has been generated via glycolysis. However, I noted an increase in the M4 and M5 fractions although neither of these increases reached statistical significance. M4 and M5 isotopologues are donated from M1 and M2 of acetyl-CoA and therefore increases in these isotopologues means increased flux from glucose.

I next measured the metabolite itaconate (**Fig 5.10C**). I showed before that the abundance of itaconate is increased, however, overall there were no statistical changes in the isotopologues of this metabolite. However, even though it failed to reach statistical significance there was a slight increase in the M3 and M4 fraction of itaconate after WGP stimulation. I next measured α -ketoglutarate (**Fig 5.10D**). There had been a non-significant increase in the abundance of this metabolite, however, I saw that there were changes in the isotopology. Firstly, I saw that there was a trend increase, although not significant, in the M0 fraction. This suggests that

there is some dilution of the tracer again potentially indicating that carbon may also be fuelled via glutaminolysis. I also saw that WGP induced a trend increase in the M2 fraction and interestingly, a significantly increase in the M4 fraction of α -ketoglutarate. Taken together this data suggests that although there was some dilution, potentially due to glutaminolysis, the increases in M2 and M4 suggest deeper oxidation of α -ketoglutarate stemming from increased flux from pyruvate.

Finally, there was a significant reduction in unlabelled M0 isotopologues for succinate (**Fig 5.10E**), fumarate (**Fig 5.10F**) and malate (**Fig 5.10G**). With no significant changes for the other isotopologues in these metabolites. This suggests that for these metabolites there was a greater incorporation of glucose derived carbon although no significant increase in overall flux, since M3 and M4 were not significantly altered.

Having seen these changes, I next analysed the ratio of fractions of various metabolites. This helps to identify bottlenecks and metabolic shifts that are occurring in the cells. To better understand the efficiency within which glycolysis was contributing to TCA cycle I measured the ratio of the M4 citrate to M3 pyruvate fractions (**Fig 5.11A**). As I have previously shown, the M3 carbon of pyruvate corresponds to the M4 carbon of citrate. WGP led to an increase in this ratio, due to more citrate M4 then pyruvate M3. To further understand our glycolytic data, I next analysed the ratio of pyruvate M3 to α -ketoglutarate M4 levels (**Fig 5.11B**). This would help me understand whether pyruvate was favoured for TCA cycle flux. Although not reaching significance there was a trend towards a reduction in this ratio, reflective of the reduction in the M3 pyruvate levels by WGP and an increase in the M4 α -ketoglutarate levels. Next, I wanted to check the efficiency of the TCA cycle in WGP treated BMDMs. To do this I analysed the ratio of M2 succinate to M3 pyruvate (**Fig 5.11C**). I saw that WGP significantly increased the ratio, due to higher M2 succinate levels and lower M3 pyruvate levels. Finally, since I have seen an increase in itaconate accumulation which acts as an SDH inhibitor I wanted to see if SDH activity may be affected. To do this I measured the ratio of M2 fumarate to M2 succinate, since fumarate is generated after succinate via SDH (**Fig 5.11D**). Interestingly, I saw that there was a trend reduction in this ratio meaning that

succinate flux was out competing fumarate flux. Taken together this data indicates that TCA cycling is rewired 3 days post WGP stimulation and that these metabolites are generated via glucose uptake and flux down through pyruvate and into the TCA cycle.

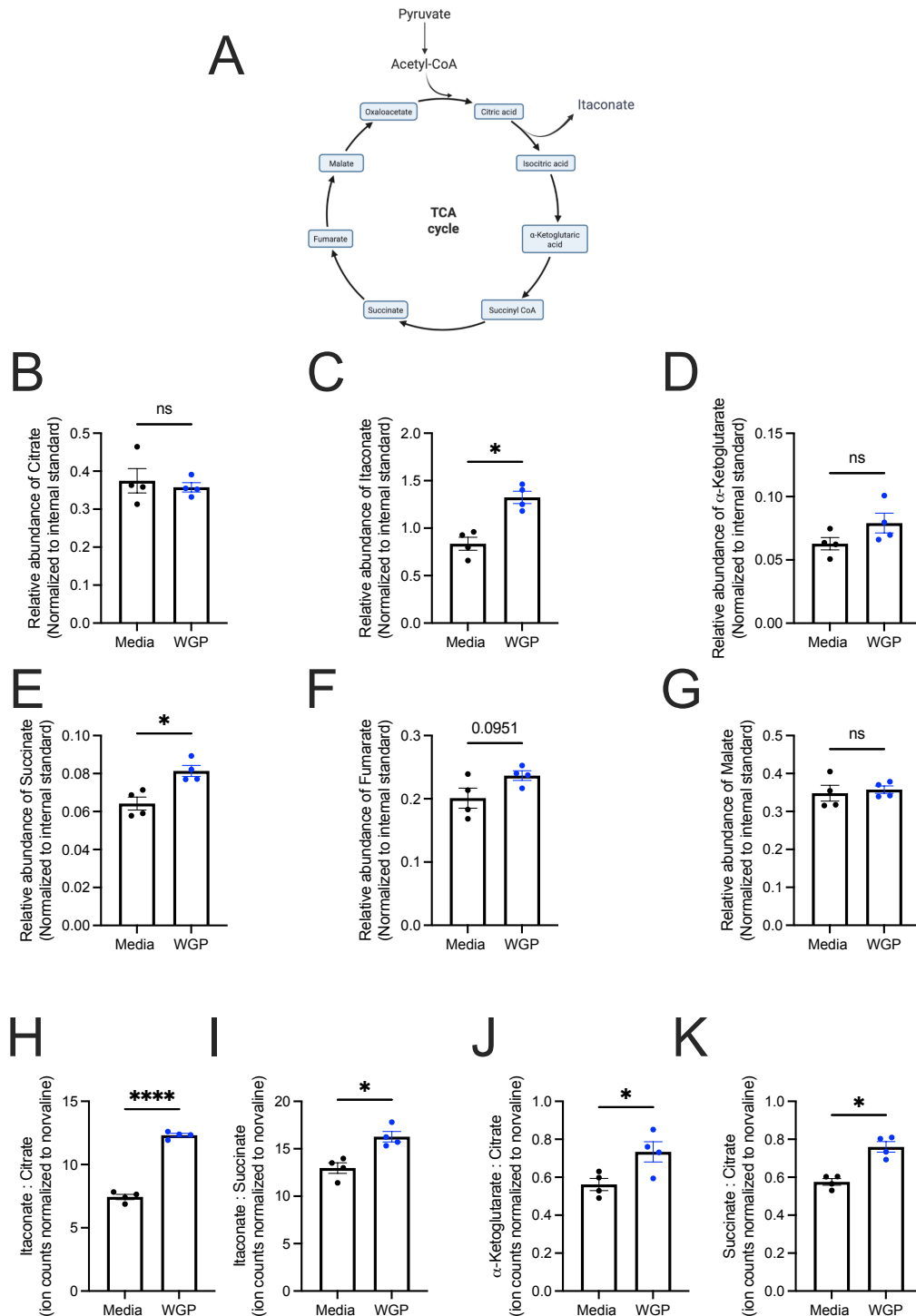


Figure 5.9 Core TCA cycle metabolite abundance is altered day 3 post WGP stimulation

BMDMs were treated with 100ug/mL of WGP for 24hrs. Cells were washed and fresh media was added. Cells were rested for 48hrs. On day 3 post WGP stimulation cells were lifted, counted and replated. Freshly prepared media containing universally 13 labelled glucose was added to each sample for 8hrs. After 8hrs cells were lysed and normalised with Norvaline. Cell lysate was prepared as previously described and metabolites were measured by GC-MS. Each dot is a representative of

an individual experiment (Biological N = 4). NS = P value >0.05, *P-value <0.05, ****P-value <0.0001. (Paired t-test).

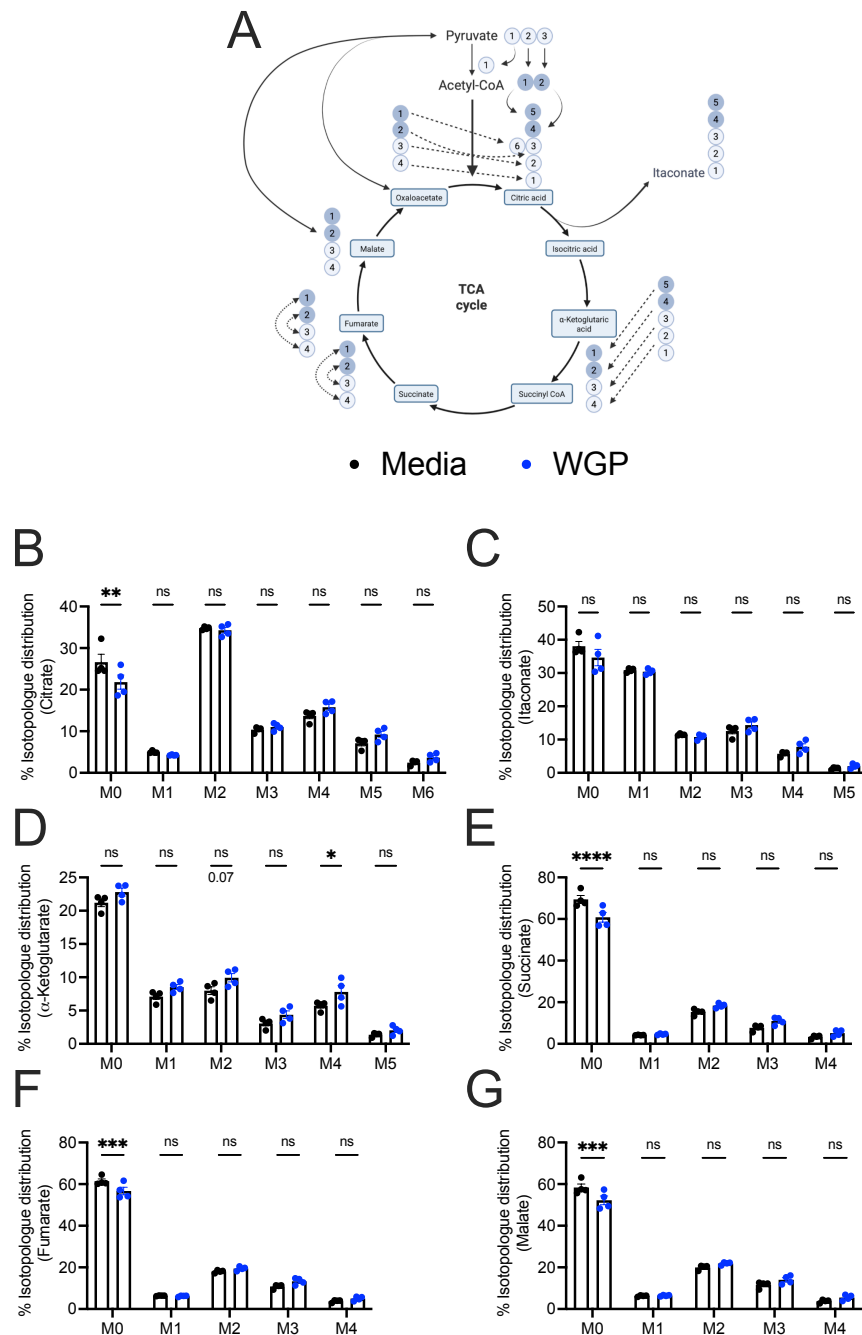


Figure 5.10 TCA flux is altered day 3 post WGP stimulation

BMDMs were treated with 100ug/mL of WGP for 24hrs. Cells were washed and fresh media was added. Cells were rested for 48hrs. On day 3 post WGP stimulation cells were lifted, counted and replated. Freshly prepared media containing universally 13 labelled glucose was added to each sample for 8hrs. After 8hrs cells were lysed and normalised with Norvaline. Cell lysate was prepared as previously described and metabolites were measured by GC-MS. Each dot is a representative of

an individual experiment (Biological N = 4). NS = P value >0.05, *P-value <0.05, ****P-value <0.0001.
(Two-way ANOVA with Šídák's multiple comparison test).

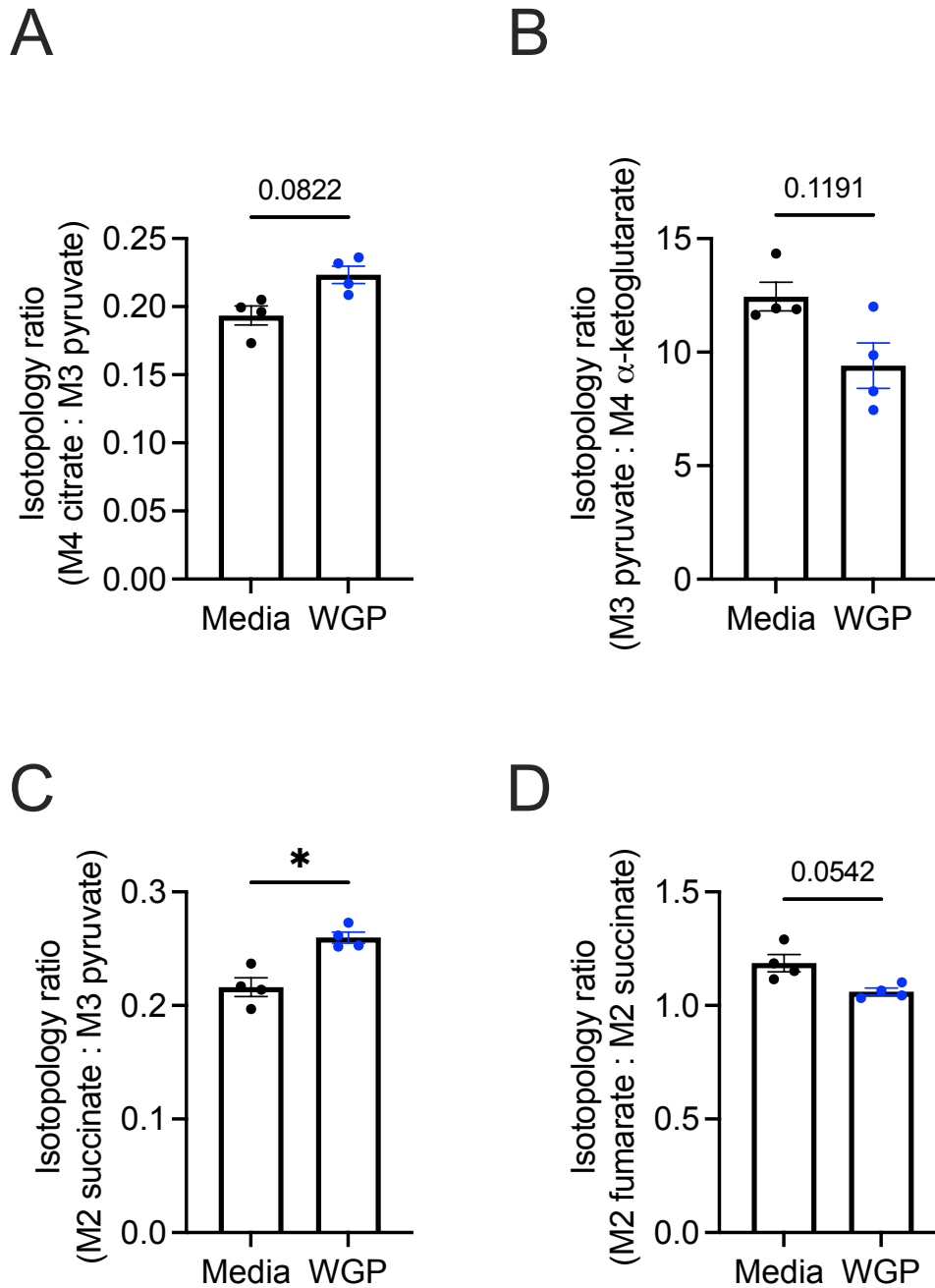


Figure 5.11 TCA flux ratios are altered day 3 post WGP stimulation

BMDMs were treated with 100ug/mL of WGP for 24hrs. Cells were washed and fresh media was added. Cells were rested for 48hrs. On day 3 post WGP stimulation cells were lifted, counted and replated. Freshly prepared media containing universally 13 labelled glucose was added to each sample for 8hrs. After 8hrs cells were lysed and normalised with Norvaline. Cell lysate was prepared as previously described and metabolites were measured by GC-MS. Each dot is a representative of an individual experiment (Biological N = 4). NS = P value >0.05, *P-value <0.05, ****P-value <0.0001. (Two-way ANOVA with Šídák's multiple comparison test).

5.2.5 WGP induced IDT is dependent on oxidative phosphorylation

Type I interferon signalling was not required to induce metabolic reprogramming seen post WGP stimulation. However, I have previously shown that this metabolic reprogramming was important for driving anti-bacterial trained responses. Therefore, even though type I interferon didn't effect metabolic responses I wanted to investigate whether metabolism was important for the anti-viral trained responses seen with WGP stimulation.

Since I have previously shown that WGP induced trained immunity depended on OxPhos and with the data above showing the importance of TCA cycling I therefore wanted to determine this was also the case for the anti-viral affects seen. To do this I inhibited various parts of the electron transport chain (ETC) (**Fig 5.12**). I first inhibited complex V of the ETC, which causes arrest of ETC and as a result of NADH build-up, slows TCA cycling. To do this BMDMs were treated with oligomycin for 24hrs 3 days after WGP had been added since this time-point aligned with maximal metabolic changes. After 24hrs incubation, vehicle or oligomycin was washed off and the standard training protocol was followed. As shown (**Fig 5.12A**) inhibition of complex V via oligomycin significantly reduced the enhanced TNF responses to pIC induced by WGP training, with oligomycin trained cells failing to increase TNF with WGP training As well as this, inhibition of complex V alone had no significant effect on anti-viral responses since there was no change between the vehicle untrained and oligomycin treated untrained group. This suggests that reduced responses was fully due to inhibition of anti-viral training and not just through acute inhibition of anti-viral signalling via metabolism. Next I inhibited complex III of the ETC via antimycin A (**Fig 5.12B**). Again, this can impact TCA cycling and the accumulation of metabolites due to NADH accumulation. Again, inhibition caused a significant reduction in the enhanced TNF response to pIC by WGP, with no significant changes between the antimycin A treated untrained and trained. Although there was more TNF production in the antimycin untrained treated group, this was not significantly enhanced versus the vehicle untrained control. Finally, I inhibited complex I using rotenone (**Fig 5.12C**). Just like the other two inhibitors, inhibition of complex I led to a significant reduction in the enhanced TNF trained response to

pIC. However, the TNF response in trained cells seemed to rely wholly on complex I signalling, as inhibition of this enzyme didn't just reduce TNF to untrained levels, but completely abolished the ability of trained cells specifically to respond to pIC. Taken together this data suggests that the anti-viral effects of WGP are dependent on metabolic changes associated with ETC and TCA cycle.

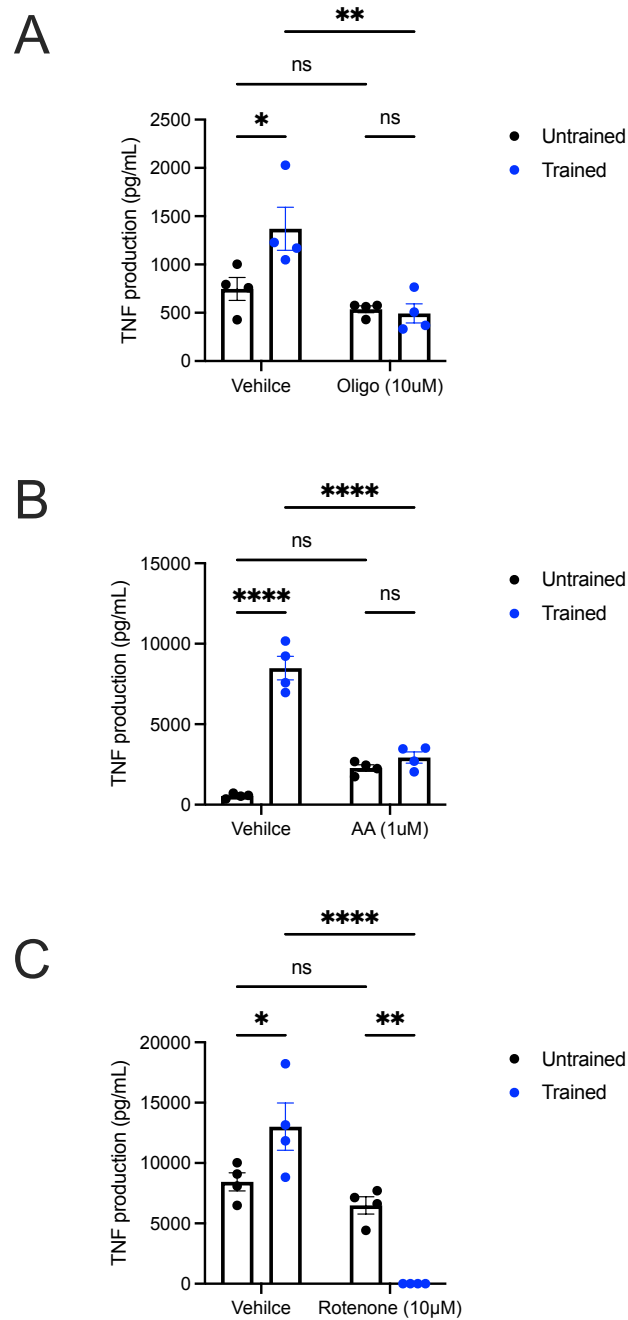


Figure 5.12 Anti-viral trained immunity is dependent on OxPhos signalling

BMDMs were treated with 100ug/mL of WGP for 24hrs. Cells were washed and fresh media was added. Cells were rested for 48hrs. On day 3 post WGP stimulation various inhibitors of the ETC were added for 24hrs: (A) oligomycin (10 μ), (B) Rotenone (10 μ) and (C) Antimycin A (110 μ M). After 24hrs inhibitors were washed off and cells were allowed to rest for 2 days. On day 6 cells were stimulated with poly I:C (10 μ g/mL) for 8hrs. Each dot is a representative of an individual experiment (Biological N = 4). NS: P value >0.05, *P-value <0.05, **P<0.0078; ***P=0.0001; ****P<0.0001; (Two-way ANOVA with Šídák's multiple comparison test).

5.2.6 WGP induced IDT is independent of itaconate accumulation and itaconate signalling

Recently, it has been shown that metabolites can do more than just act as fuel sources by cells but actually prime various immunological responses²⁴². However, in the field of trained immunity not much has been investigated in the role these metabolites may have in priming responses independent of epigenetic changes. Since I have shown that oxidative phosphorylation and TCA cycling is vital for WGP induced antiviral training, I hypothesized that the training effects may be due to accumulation of TCA metabolites previously detected. To test this hypothesis, I first determined if exogenous itaconate and its' derivatives (4-octyl itaconate/4-OI) could prime BMDMs to induce the enhanced anti-viral effects I see with WGP/or in the absence of WGP-training. It has been previously shown that exogenous itaconate could boost IFN production in response to bacterial ligands, through mitochondrial reprogramming and nucleic acid release²⁴³. Therefore, I wanted to investigate this from an anti-viral prospective. Upon 3hr pre-treatment with exogenous itaconate BMDMs, had significantly enhanced type I interferon responses as well as a trend towards a significant increase in the induction of the ISG IP-10 after 24hrs stimulation with pIC relative to the non-pretreated pIC control (**Fig 5.13A-B**). This is consistent with published data and suggests an immunomodulatory role for these metabolites, beyond simply fueling metabolic reactions²⁴³.

Having seen that exogenous itaconate was capable of driving an increase in IFN β and IP-10, I next investigated whether the itaconate derivative (4-OI) could also show similar results. It has previously been shown that 4-OI blocks type I interferon and inhibits coagulation in response to bacterial stimulation²⁴⁴. Therefore, I decided to investigate whether this also occurred in the context of anti-viral immunity. Similar to the itaconate experiment (**Fig. 5.13**), BMDMs were pre-treated for 3hrs with 4-OI or vehicle control and then stimulated with pIC for 24hrs. Unlike exogenous itaconate, 4-OI pre-treatment led to a significant reduction in both IFN β and IP-10 production (**Fig 5.14A-B**). Taken together this data highlights the importance of testing different derivatives of metabolites since they can have contradicting immunological effects.

Next, I investigated further the role of itaconate in viral immunity. Itaconate is generated via the conversion of Cis-aconitate to itaconate via aconitate decarboxylase 1 (ACOD1). Since I saw that exogenous itaconate could enhance IFN and IP-10 production, I wanted to see if endogenous itaconate had a similar effect. It has been shown that itaconates' naturally occurring isomer, citraconate, is capable of inhibiting ACOD1 activity and thus prevents itaconate synthesis²⁴⁵. Therefore, I pre-treated BMDMs with vehicle or citraconate for 3hrs and stimulated BMDMs for 24hrs with pIC. Inhibition of endogenous itaconate resulted in a loss of BMDMs ability to induce type I interferon responses (**Fig 5.15A**). However, interestingly there was no significant difference in the cells ability to induce IP-10 (**Fig 5.15B**). This data indicates that endogenous itaconate is important in the acute type I interferon response to TLR-3 agonists.

Since it has been shown that both exogenous and endogenous itaconate functionally inhibits SDH activity during LPS stimulation, I wanted to investigate whether this functional role of itaconate could be the cause of the type I interferon increase upon pIC stimulation²⁴⁶. To do this I used a pharmacological inhibitor of SDH known as apamin A5 (AA5). Inhibition of SDH by AA5 led to a significant reduction in both the IFN β and IP-10 responses of BMDMs to pIC stimulation (**Fig 5.16A-B**). Taken together this data indicates that unlike in bacterial responses, the metabolic reprogramming of SDH by itaconate is not the mechanism via which macrophages enhance type I interferon responses to TLR3 ligands. The results from this coupled with the citraconate experiments highlight that in the context of viral immunity itaconate generation through ACOD1 is important for type I interferon responses but itaconate may play a different role here other than inhibiting SDH, since functioning SDH is required for anti-viral responses.

Since I have shown that itaconate can boost interferon responses to TLR3 ligands like pIC I wanted to investigate whether accumulation of itaconate by BMDMs during the training phase could lead to the significant interferon responses I see in my WGP trained macrophages. To do this I used BMDMs from mice deficient in the immunoresponse gene 1 (IRG1^{-/-}), which encodes for the enzyme ACOD1. BMDMs

from aged and sex matched wild type mice were used as a control. I first investigated whether classical training was affected by IRG1 deficiency. Both wild type and knock out BMDMs were trained as previously described. WGP-Trained wild type BMDMs had a significant increase in TNF compared to the untrained wild type BMDMs in response to pIC stimulation as previously shown (**Fig 5.17A**). Interestingly, in response to pIC trained knock out BMDMs could also significantly enhance TNF responses. There was no significant difference between trained wild type and trained knock out BMDMs. Next, I tested whether IRG1 deficiency affected trained interferon responses (**Fig 5.17B**). Like the TNF responses, WGP-trained wild type BMDMs had a significant increase in IFN β compared to the untrained wild type BMDMs in response to pIC stimulation as previously shown. Similarly, IRG1 knock out trained BMDMs displayed enhanced interferon responses versus the untrained control. There were no significant differences between trained wild type BMDM responses and trained knock out BMDM responses. Neither were there any significant differences in interferon responses between wild type untrained BMDMs and knock out untrained BMDM responses.

As mentioned, it has been shown that derivatives of itaconate can drive trained immunity with enhanced inflammatory response to bacterial ligands¹²². However, I wanted to see if exogenous itaconate could do the same for viral ligands. To do this 10mM itaconate was added to BMDMs with WGP used as a positive control for 24hrs before washing off the stimulus. The standard training protocol was adhered to, and cells were restimulated on day 6 with pIC for 24hrs. TNF and IFN responses were then measured (**Fig 5.18A-B**). As expected WGP training led to significantly more TNF and IFN production in response to pIC. However, itaconate alone could not drive a significant increase in either TNF or IFN in response to pIC. This indicates that potentially derivatives of itaconate are required to induce trained responses in cells. However, I next investigated whether itaconate could have a synergistic trained effect with WGP (**Fig 5.19A-B**). A similar training protocol was used as previously described, however, for this experiment itaconate was added to the cells at different concentrations 3hrs prior to WGP stimulation. Interestingly, in this context exogenous itaconate had a synergistic effect with WGP with 100 μ M

itaconate significantly enhancing the WGP training effect in response to pIC with both increased TNF and IFN (**Fig 5.19A-B**).

Taken together this data indicates that although exogenous itaconate can alter pIC driven interferon responses, in the context of WGP induced anti-viral trained immunity itaconate accumulation may be redundant for the overall anti-viral responses. This data also shows that in the context of anti-viral immunity the inhibitory role of itaconate on the TCA cycle and ETC is anti-inflammatory, reducing IFN responses. However, although itaconate alone was insufficient in driving the trained response exogenous itaconate was capable of synergising and enhancing this response which may be important in clinical applications.

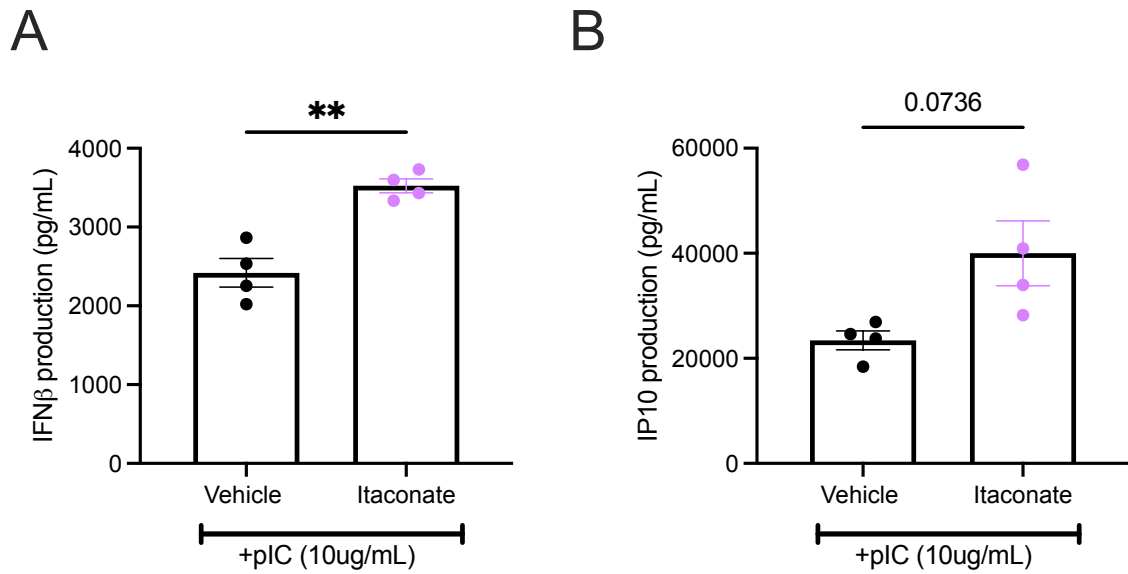
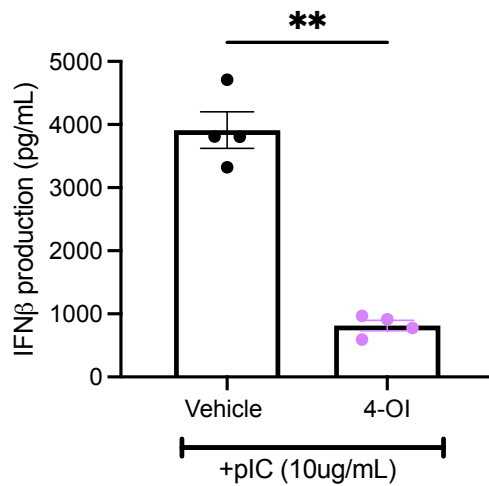


Figure 5.13 Exogenous Itaconate enhances anti-viral IFN and ISG (IP-10) production

BMDMs were treated with 10mM itaconate for a period of 3hrs prior to stimulation. After 3 hours, cells were stimulated for 24hrs with pIC. IFN β (A) or IP-10 (B) production was measured after 24hrs in pIC stimulated BMDMs pretreated with itaconate. Cytokine levels were measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). NS: P value >0.05, **P<0.001; (Paired Student T-test).

A



B

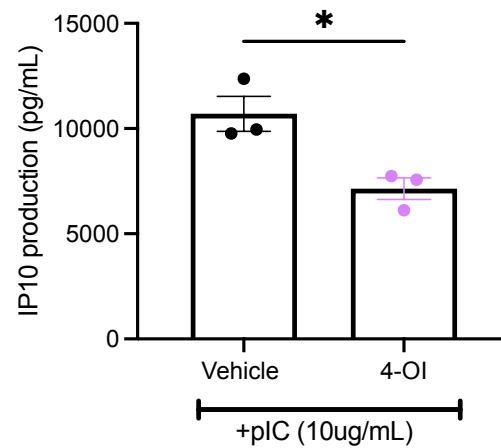


Figure 5.14 The itaconate derivative 4-OI reduces anti-viral IFN and ISG (IP-10) production

BMDMs were treated with 250 μ M 4-OI for a period of 3hrs prior to stimulation. After 3 hours, cells were stimulated for 24hrs with pIC. IFN β (A) or IP-10 (B) production was measured after 24hrs in pIC stimulated BMDMs pretreated with itaconate. Cytokine levels were measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). NS: P value >0.05, *P<0.05; **P<0.001; (Paired Student T-test).

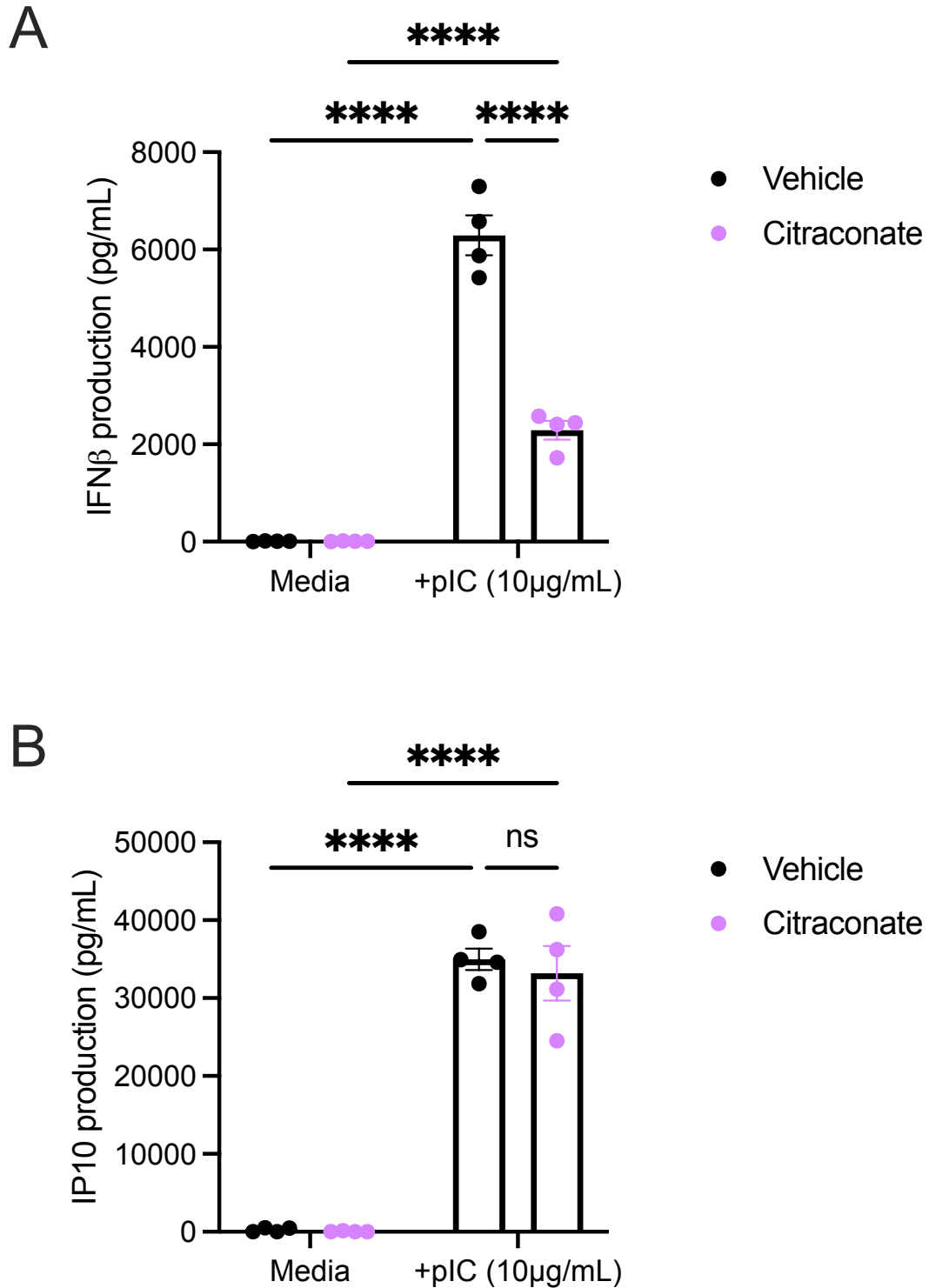


Figure 5.15 Inhibition of ACOD1 by citraconate disrupts IFN but not IP-10 production in response to pIC

BMDMs were treated with 10 μ M Citraconate for a period of 3hrs prior to stimulation. After 3 hours cells were stimulated for 24hrs with pIC. IFN β (A) and IP-10 (B) production was measured after 24hrs in pIC stimulated BMDMs. Cytokines were measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). NS: P value >0.05, ****P<0.0001; (Two-Way ANOVA with Šídák's multiple comparisons test).

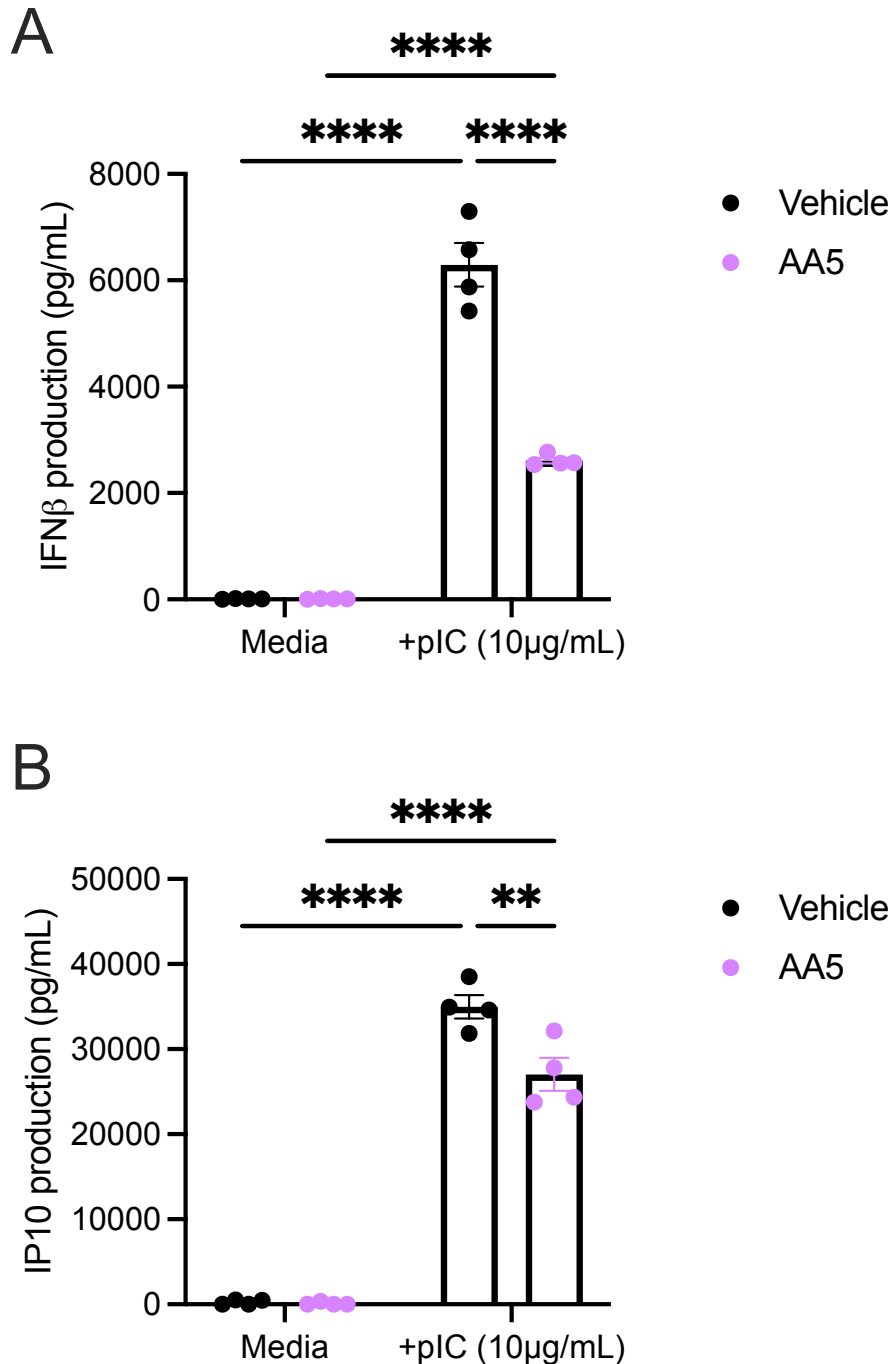
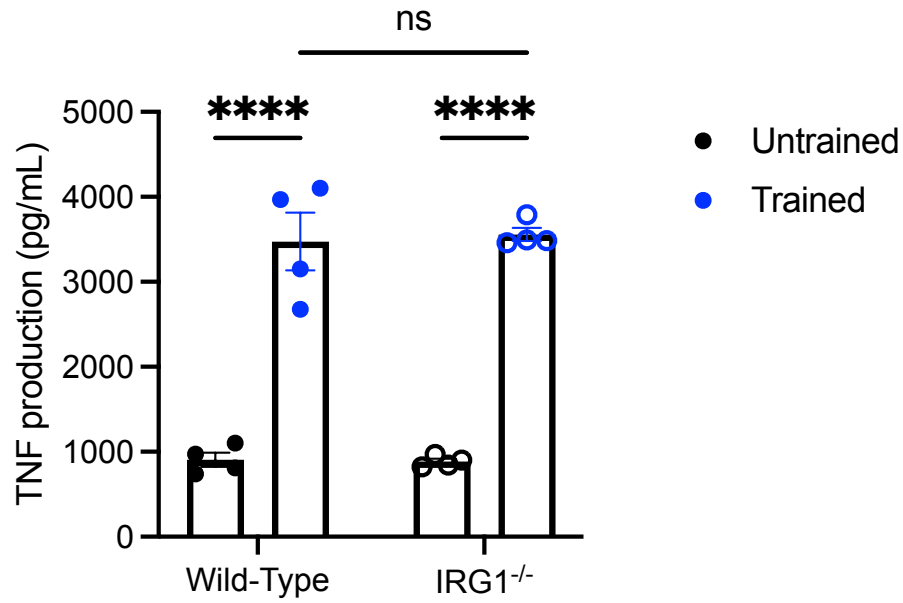


Figure 5.16 Inhibition of SDH disrupts both type 1 interferon and ISG production in response to pIC

BMDMs were treated with 10 μ M Apenin A5 (AA5) for a period of 3hrs prior to stimulation. After 3 hours cells were stimulated for 24hrs with pIC. IFN β (A) and IP-10 (B) production was measured after 24hrs in pIC stimulated BMDMs. Cytokines were measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). NS: P value >0.05, **P<0.01; ****P<0.0001; (Two-Way ANOVA with Šídák's multiple comparisons test).

A



B

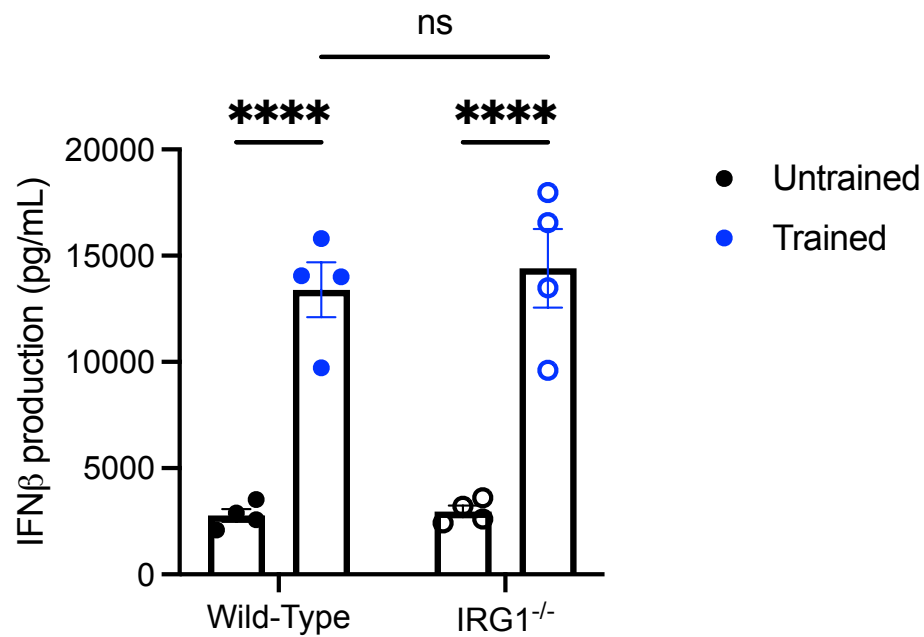


Figure 5.17 Loss of IRG1 does not affect BMDMs ability to undergo IDT

Wild-type and IRG1 KO BMDMs were treated with 100μg/mL WGP for a period of 24hrs. After 24hrs cells were washed and treated the same as previously described. On day 6 cells were stimulated with pIC for a period of 8hrs and TNF (A) or IFNβ (B) was measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). NS: P value >0.05, ****P<0.0001; (Two-Way ANOVA with Šídák's multiple comparisons test).

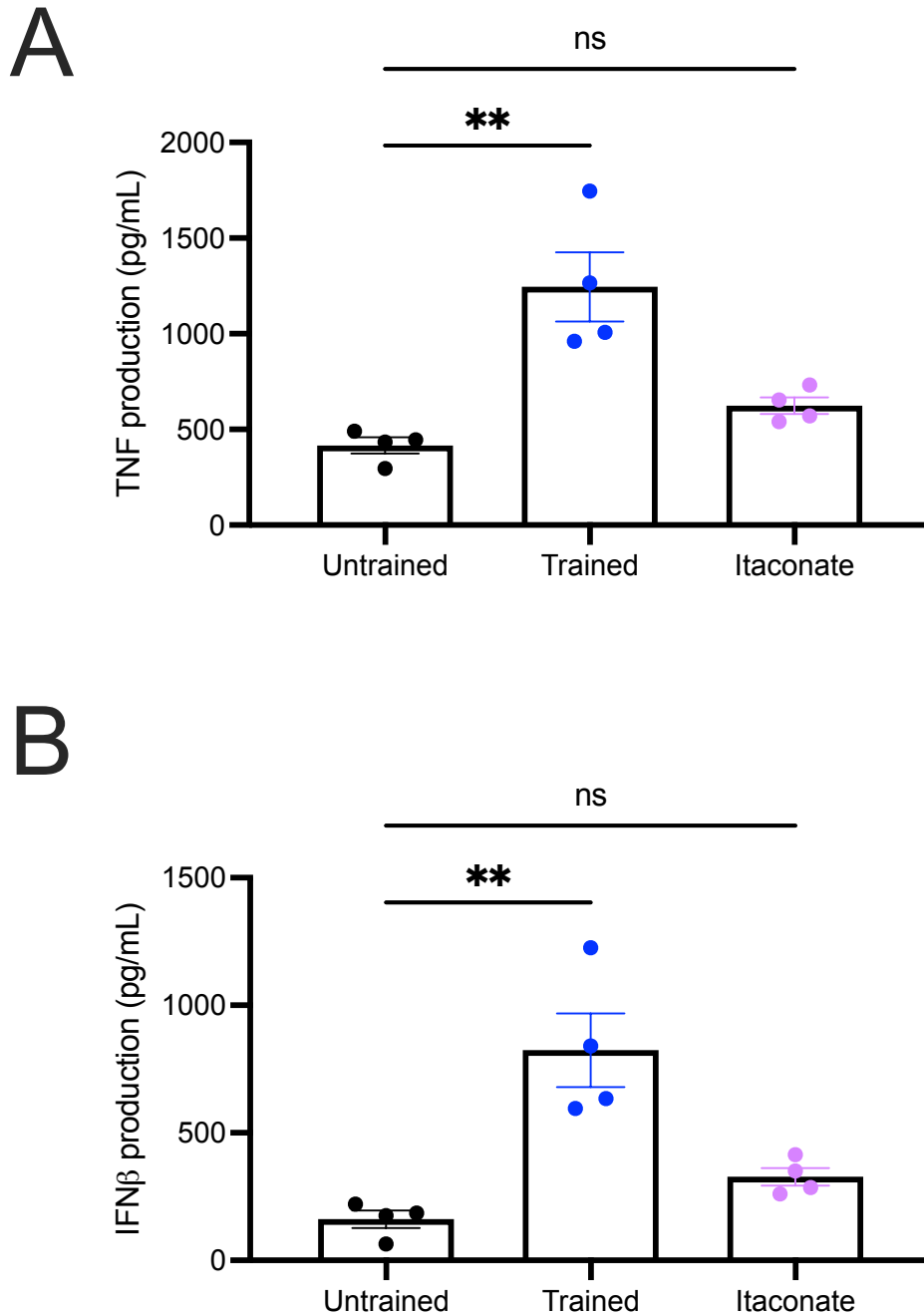


Figure 5.18 Exogenous itaconate cannot induce trained immunity alone

BMDMs were treated with 10mM itaconate or 100 μ g/mL WGP for a period of 24hrs. After 24hrs cells were washed and allowed to rest according to the training protocol previously described. On day 6 cells were stimulated for 24hrs with 10 μ g/mL pIC. TNF (A) or IFN β (B) production was measured after 24hrs. Cytokine levels were measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). NS: P value >0.05, **P<0.01; (RM-One way ANOVA with Holm-Šidák's multiple comparisons test).

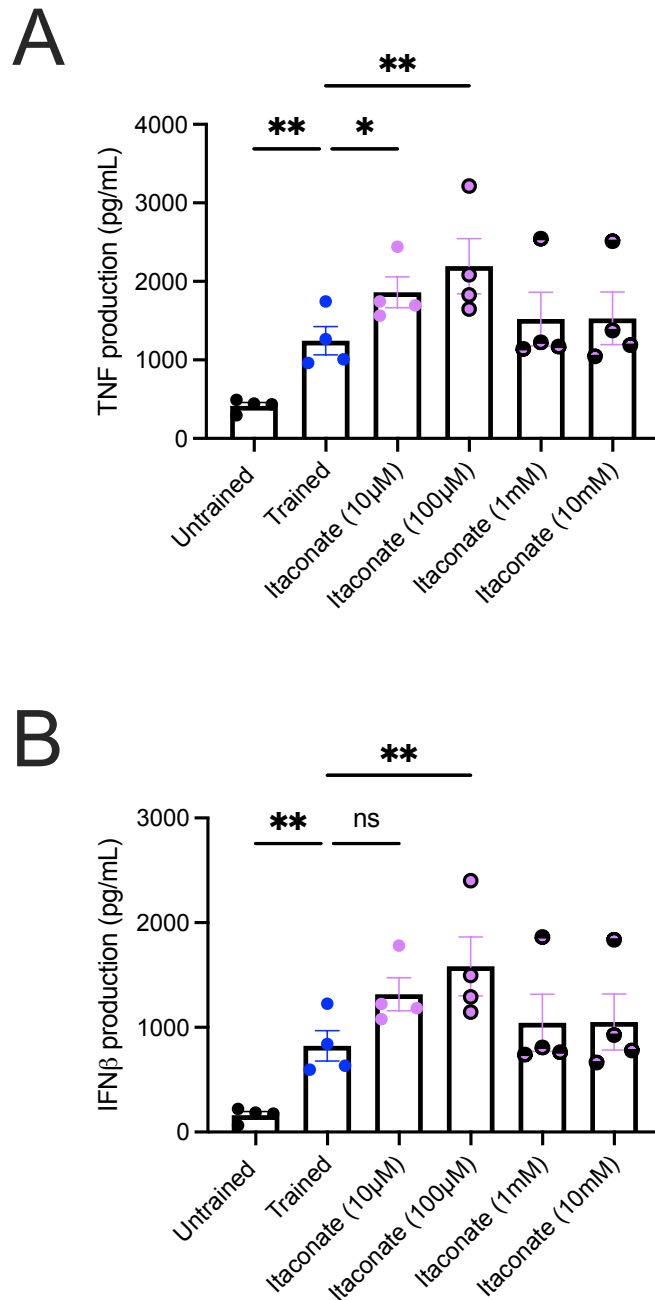


Figure 5.19 Exogenous itaconate coupled with WGP training synergise to give an enhanced training response

Graph shows that pre-treatment with itaconate prior to WGP training enhances the trained effect of cells vs just training with WGP alone. BMDMs were pre-treated with different concentrations of itaconate for 3hrs followed by training in the presence of 100μg/mL WGP stimulation for a period of 24hrs. After 24hrs cells were washed and allowed to rest according to the training protocol previously described. On day 6 cells were stimulated for 24hrs with 10μg/mL pIC. TNF (A) or IFNβ (B) production was measured after 24hrs. Cytokine levels were measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). *P<0.05; **P<0.01; (RM-One way ANOVA with Holm-Šídák's multiple comparisons test).

5.2.7 WGP induced IDT is independent of succinate accumulation

Since I showed in the previous section that itaconate accumulation was redundant for the enhanced anti-viral effects of WGP, coupled with the fact that our isotope tracing also showed a significant accumulation of succinate, I next turned my attention to how succinate accumulation might affect anti-viral responses in BMDMs. I have previously shown that inhibition of SDH, which would lead to endogenous succinate accumulation, reduced IFN and ISG responses to acute pIC challenge. Therefore, I did not expect exogenous succinate to have any enhancing effects on pIC responses. It has been shown that macrophages can respond to succinate, derived from succinic acid, leading to modulation of metabolism and immune responses²⁴⁷. I first investigated whether succinate enhanced TNF responses to acute pIC stimulation (**Fig 5.20A**). As expected, succinate had a slight but non-significant increase in TNF production after pIC stimulation. However, there was overall no significant changes in either IFN (**Fig 5.20B**) or IP-10 (**Fig 5.20C**) production after pIC stimulation in succinate pretreated BMDMs.

However, to fully rule out the role for succinate in the anti-viral trained response of WGP I stimulated BMDMs with 5mM of succinate and treated these cells the same as my training assays. On day 6 I restimulated these cells with pIC for 24hrs (**Fig 5.21**). Like the previous experiment I ran involving itaconate, succinate alone was not sufficient in driving a trained response when restimulated with pIC. WGP could enhance TNF responses to pIC, however, succinate had no significant effect (**Fig 5.21A**). Similarly, WGP could enhance IFN responses to pIC, but again succinate alone had no significant effects (**Fig 5.21B**).

Finally, I investigated whether exogenous succinate could have a synergistic training response with WGP. Like my itaconate protocol BMDMs were pretreated for 3hrs prior to WGP stimulation and after 24hrs cells were allowed to rest for 6 days. On day 6 cells were stimulated with pIC. As expected WGP training alone had a significant increase in TNF in response to pIC. However, succinate at different concentrations had no significant effect on WGP TNF training effects to pIC (**Fig 5.22A**). However, interestingly, I saw that 50 μ M succinate synergised with WGP

causing a significant increase in IFN in comparison to WGP alone in response to pIC (**Fig 5.22B**).

Taken together this data indicates that both endogenous and exogenous succinate alone is redundant in driving the enhanced antiviral responses seen in WGP treated BMDMs. However, importantly for clinical settings succinate can synergise with training agents to boost their anti-viral affects.

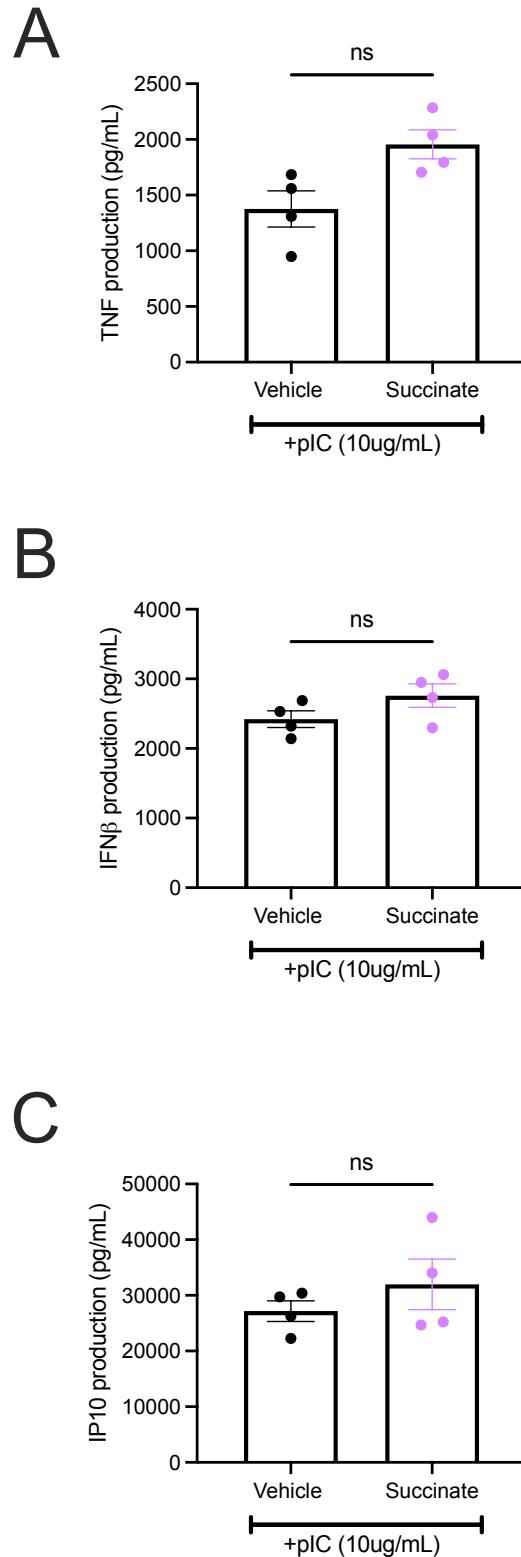
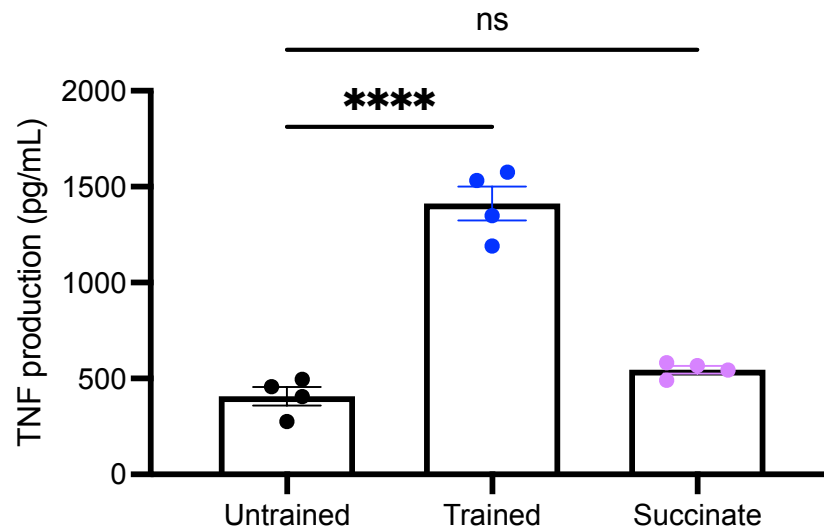


Figure 5.20 Exogenous succinate has no effect on BMDM responses to pIC stimulation

BMDMs were treated with 5mM succinate for a period of 3hrs. After 3 hours cells were stimulated for 24hrs with pIC. TNF (A), IFN β (B) and IP-10 (C) production was measured after 24hrs in pIC stimulated BMDMs. Cytokines were measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). NS: P value >0.05; (Paired student T-test).

A



B

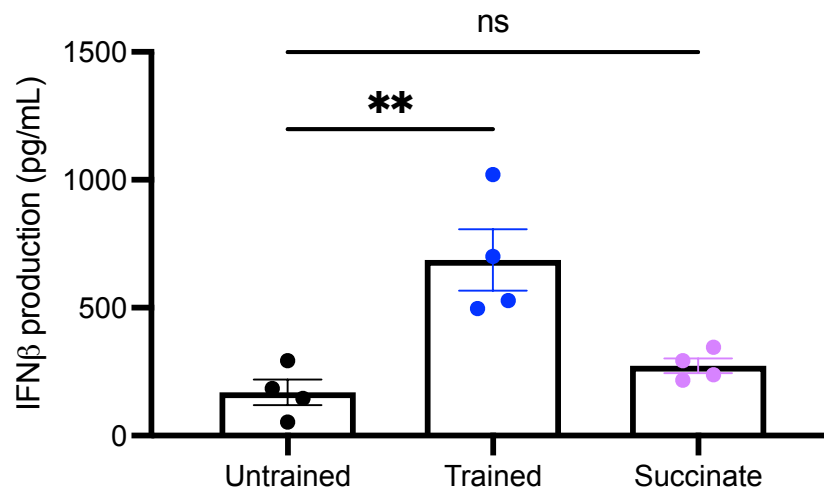


Figure 5.21 Exogenous succinate cannot induce trained immunity alone

BMDMs were treated with 5mM succinate or 100 μ g/mL WGP for a period of 24hrs. After 24hrs cells were washed and allowed to rest according to the training protocol previously described. On day 6 cells were stimulated for 24hrs with 10 μ g/mL pIC. TNF (A) or IFN β (B) production was measured after 24hrs. Cytokine levels were measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). NS: P value >0.05; **P<0.01; ****P<0.0001; (RM-One way ANOVA with Holm-Šidák's multiple comparisons test).

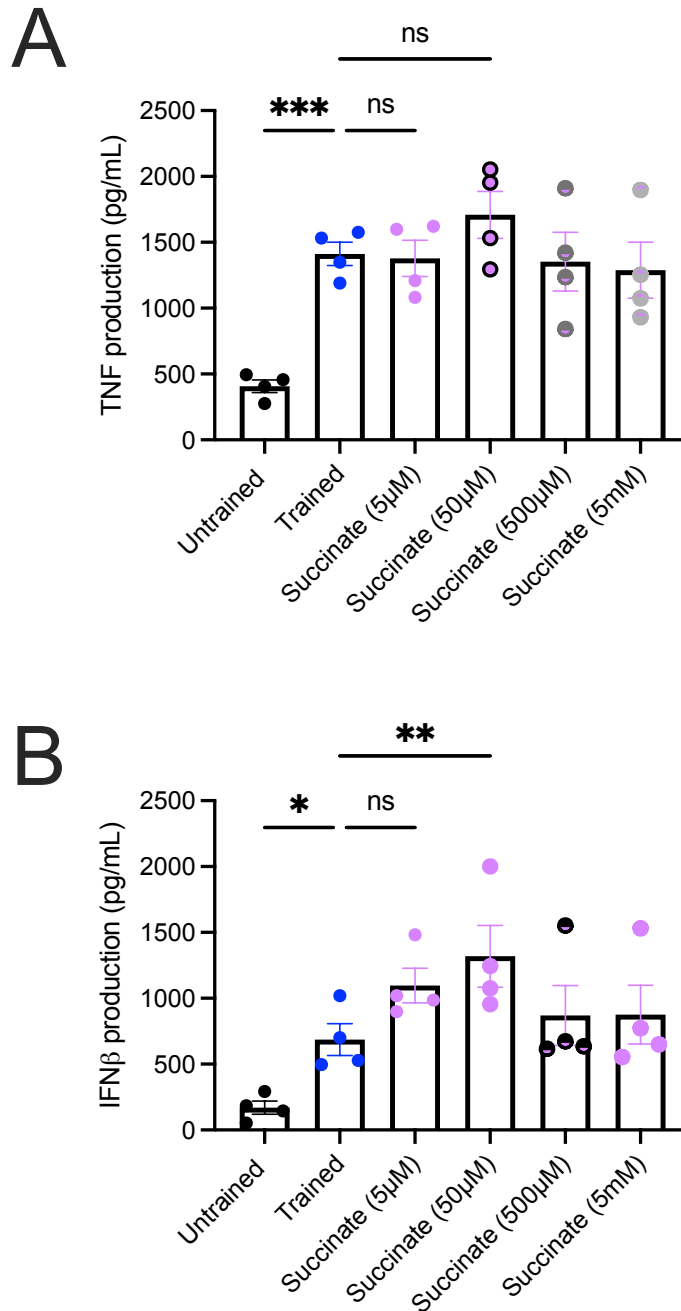


Figure 5.22 Exogenous succinate coupled with WGP training synergise to give an enhanced training response

Graph shows that pre-treatment with succinate prior to WGP training enhances the trained effect of cells vs just training with WGP alone. BMDMs were pre-treated with different concentrations of succinate for 3hrs prior to a 100μg/mL WGP stimulation for a period of 24hrs. After 24hrs cells were washed and allowed to rest according to the training protocol previously described. On day 6 cells were stimulated for 24hrs with 10μg/mL pIC. TNF (A) or IFNβ (B) production was measured after 24hrs. Cytokine levels were measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). NS: P value >0.05; *P<0.05; **P<0.01; ***P<0.001; (RM-One way ANOVA with Holm-Šidák's multiple comparisons test).

5.2.8 WGP induced IDT is replicated by fumarate accumulation

Since neither itaconate nor succinate training seemed to induce a similar phenotype to WGP anti-viral training I decided to turn my attention to the other metabolite that was accumulated in WGP treated BMDMs; fumarate. Recently, it has been shown that modulations in fumarate levels, due to repression of fumarate hydratase (FH) can impact both anti-bacterial and anti-viral responses^{248, 249} and accumulation of fumarate has been shown to be implicated in trained immunity²³⁹.

To test the hypothesis that changes in fumarate accumulation may impact anti-viral responses I first pre-treated cells with an ester of fumarate; Dimethyl Fumarate (DMF). DMF has been shown to inhibit interferon signalling and is used in the clinic to treat multiple sclerosis (MS) and psoriasis under the drug name Tecfidera^{244, 250}. First, I examined its potential as a priming agent in anti-viral responses. To do this BMDMs were primed with 10 μ M DMF for a period of 3hrs prior to stimulation with pIC (**Fig 5.23**). As expected pre-treatment with DMF inhibited TNF, IFN and IP-10 responses to pIC (**Fig 5.23A-C**).

Since DMF is widely known to inhibit STAT and NF- κ B signalling, which in turn would impact pIC responses through TLR3, I hypothesized that this might mask the direct effects of fumarate on anti-viral immunity^{251, 252}. To explore this further I used a pharmacological inhibitor of the TCA enzyme FH, FhIn1, to force cells into accumulating fumarate²⁴⁸. Cells were pre-treated for 3hrs with FhIn1 and stimulated for 4hrs after with pIC (**Fig 5.24**). Interestingly, pre-treatment with FhIn1 caused a significant increase in BMDMs ability to produce TNF production in response to pIC (**Fig 5.24A**). It was interesting to note that basal TNF levels were also slightly increased, although this comparison this did not reach statistical significance. In contrast, FH inhibition at this timepoint only induced a small non-significant increase in IFN production in response to pIC (**Fig 5.24B**, p-value = 0.59). Taken together these data indicates that accumulation of endogenous fumarate can impact macrophage responses to pIC.

Having seen that fumarate can in fact induce enhanced response to pIC when non-specific pathways, such as STAT and NF- κ B, are not affected I next wondered if

DMF induce trained immunity , since its' inhibitory effects have not been shown to be long lasting. To do this I trained BMDMs with different concentrations of DMF and WGP as a positive control (**Fig 5.25**). As was expected WGP significantly enhanced the cells TNF response to pIC. However interestingly, 10 μ M of DMF led to a small yet significant increase in TNF in response to pIC (**Fig 5.25A**). More interestingly, anything above this concentration led to a tolerance phenotype in cells. When I measured IFN production, as expected, WGP significantly enhanced the cells ability to produce IFN. Although it failed to reach statistical significance there was a trending increase in IFN with cells that had been trained with 10 μ M of DMF (**Fig 5.25B**). Interestingly, again anything above this concentration led to a tolerised phenotype in cells. Taken together this data indicates that fumarate and it's ester DMF is capable of driving anti-viral trained responses in macrophages.

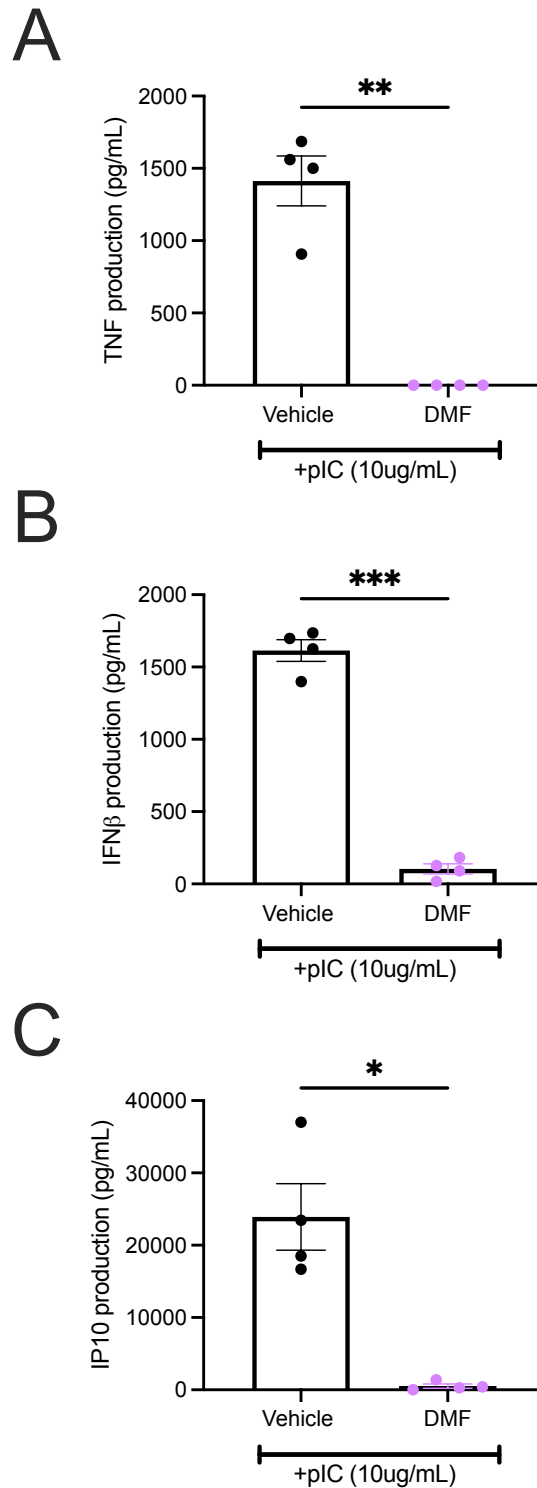


Figure 5.23 Dimethyl fumarate inhibits BMDM responses to pIC stimulation

BMDMs were treated with 10 μ M DMF for a period of 3hrs. After 3 hours cells were stimulated for 24hrs with pIC. TNF (A), IFN β (B) and IP-10 (C) production was measured after 24hrs in pIC stimulated BMDMs. Cytokines were measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). NS: P value >0.05; *P<0.05; **P<0.01; ***P<0.001; (Paired student T-test).

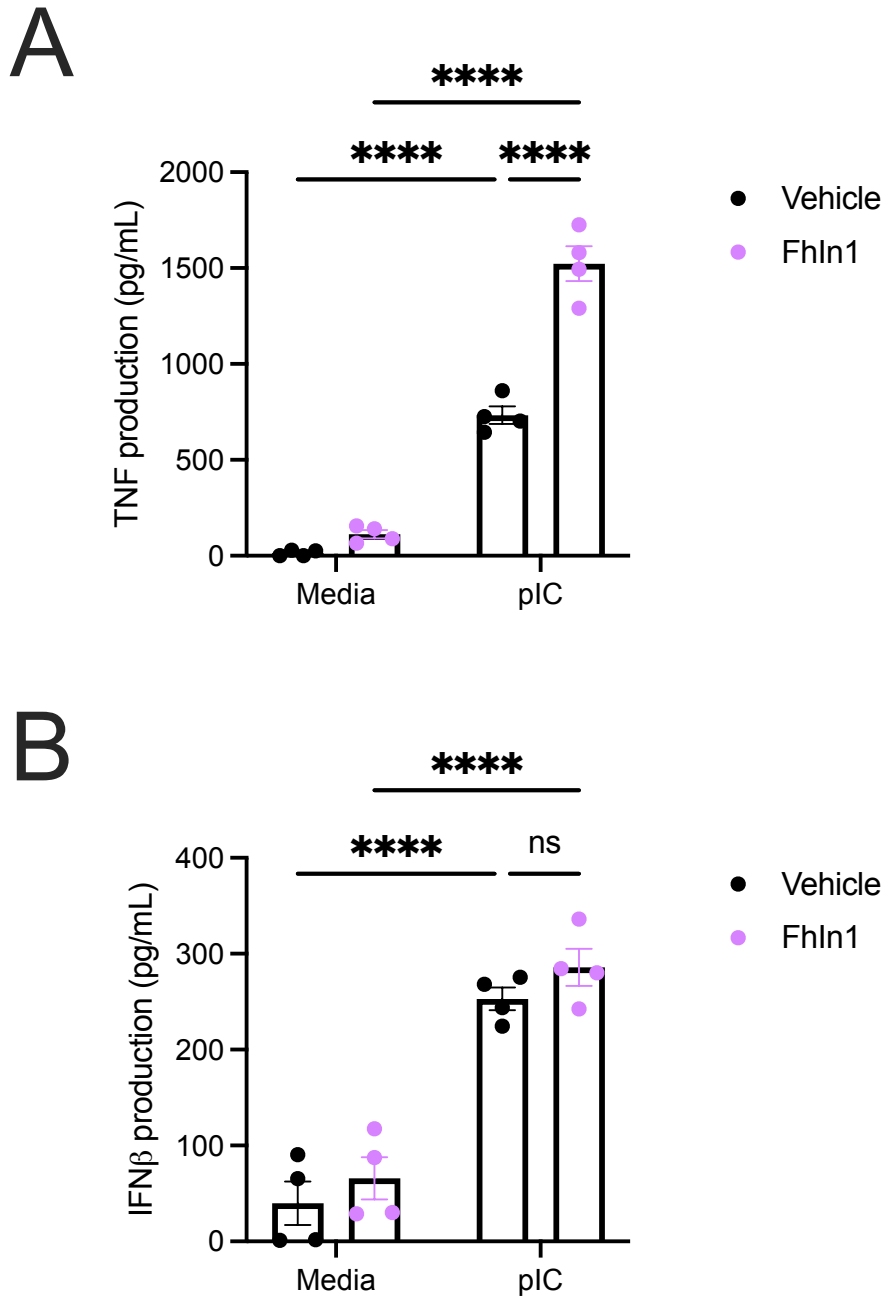


Figure 5.24 FH inhibition impacts BMDM responses to pIC

BMDMs were treated with 10 μ M FhIn1 for a period of 3hrs. After which cells were stimulated with pIC for a period of 4hrs and TNF (A) or IFN β (B) was measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). NS: P value >0.05, ****P<0.0001; (Two-Way ANOVA with Šídák's multiple comparisons test).

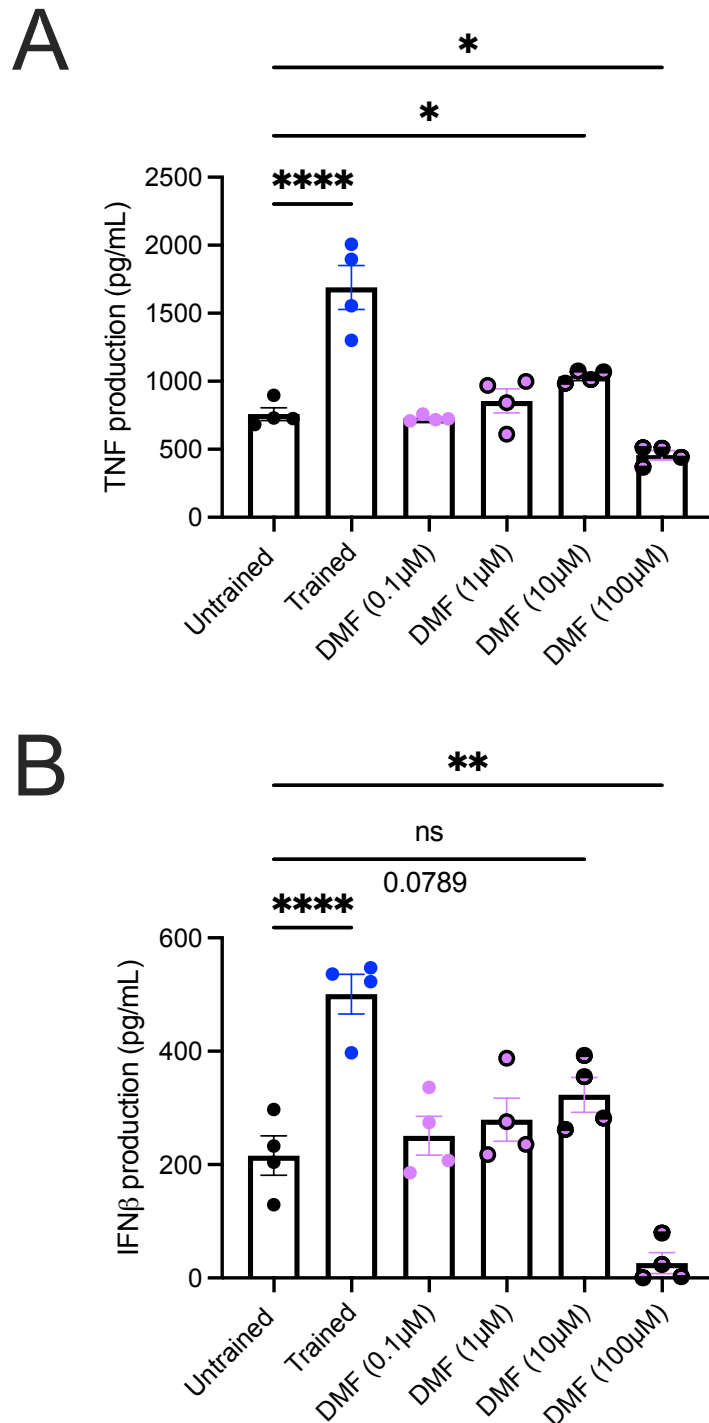


Figure 5.25 DMF can induce trained immunity or tolerance based on concentration in BMDMs

BMDMs were stimulated with different concentrations of DMF for a period of 24hrs. After 24hrs cells were washed and allowed to rest according to the training protocol previously described. On day 6 cells were stimulated for 24hrs with 10µg/mL pIC. TNF (A) or IFNβ (B) production was measured after 24hrs. Cytokine levels were measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). NS: P value >0.05; *P<0.05; **P<0.01; ***P<0.001; (RM-One way ANOVA with Holm-Šidák's multiple comparisons test).

5.2.9 WGP effects mitochondrial mass but maintains mitochondrial membrane potential

I have previously shown that WGP induces mitochondrial reprogramming with increases in oxidative metabolism and TCA cycle genes. These changes are vital for trained immunity since inhibition leads to a reduction in the training effect. However, in all these experiments *Fh* mRNA was shown to be reduced by day 3. Therefore, I wanted to investigate further how modulation of mitochondrial FH expression and activity leads to anti-viral trained immunity.

It has been shown that in the context of LPS stimulation alterations to FH via pharmacological inhibition or gene knock-out can cause enhanced mitochondrial stress and polarisation²⁴⁸. Therefore, I first wanted to explore whether WGP had similar effects, since it led to a similar reduction in the mRNA expression of FH. Since this decrease occurred at day 3, consistent with optimal changes in OCR, I decided to investigate mitochondrial membrane potential, measured by Tetramethylrhodamine methyl ester (TMRM), as well as mitochondrial mass, measured by mitotracker green (MTG) at this time-point. TMRM is cell permeable and is attracted to the mitochondria's inner membrane when there is a strong negative potential. Therefore, bright TMRM signal indicates a strong negative charge across the membrane and therefore a more active mitochondria. I also measured the ratio of membrane potential per mitochondria to test the efficiency of mitochondria in WGP treated cells (**Fig 5.26**). Here LPS was used as a positive control, since it has been well characterised that LPS can drive membrane potential without effecting the mitochondrial mass²³³. Both WGP and LPS were capable of inducing changes in the relative ratios of TMRM to MTG suggesting an increase in membrane potential per mitochondria (**Fig 5.26A**). However, examining the relative changes in TMRM alone I saw that only LPS induced a significant increase in the mitochondrial membrane potential, whereas, WGP did not (**Fig 5.26B**). In fact, further investigation showed that WGP actually reduced the staining of MTG, suggestive of reduced mitochondrial numbers or fission (**Fig 5.26C**). This did not occur in LPS treated BMDMs.

Overall this data indicates that WGP treatment does impact macrophage mitochondrial mass, but maintains the mitochondrial potential, suggesting that WGP treated macrophages have more efficient mitochondria vs untreated cells. This aligns with the hypothesis that FH reduction would increase membrane potential since there was an increase in membrane potential relative to the number or volume of mitochondria in WGP treated macrophages.

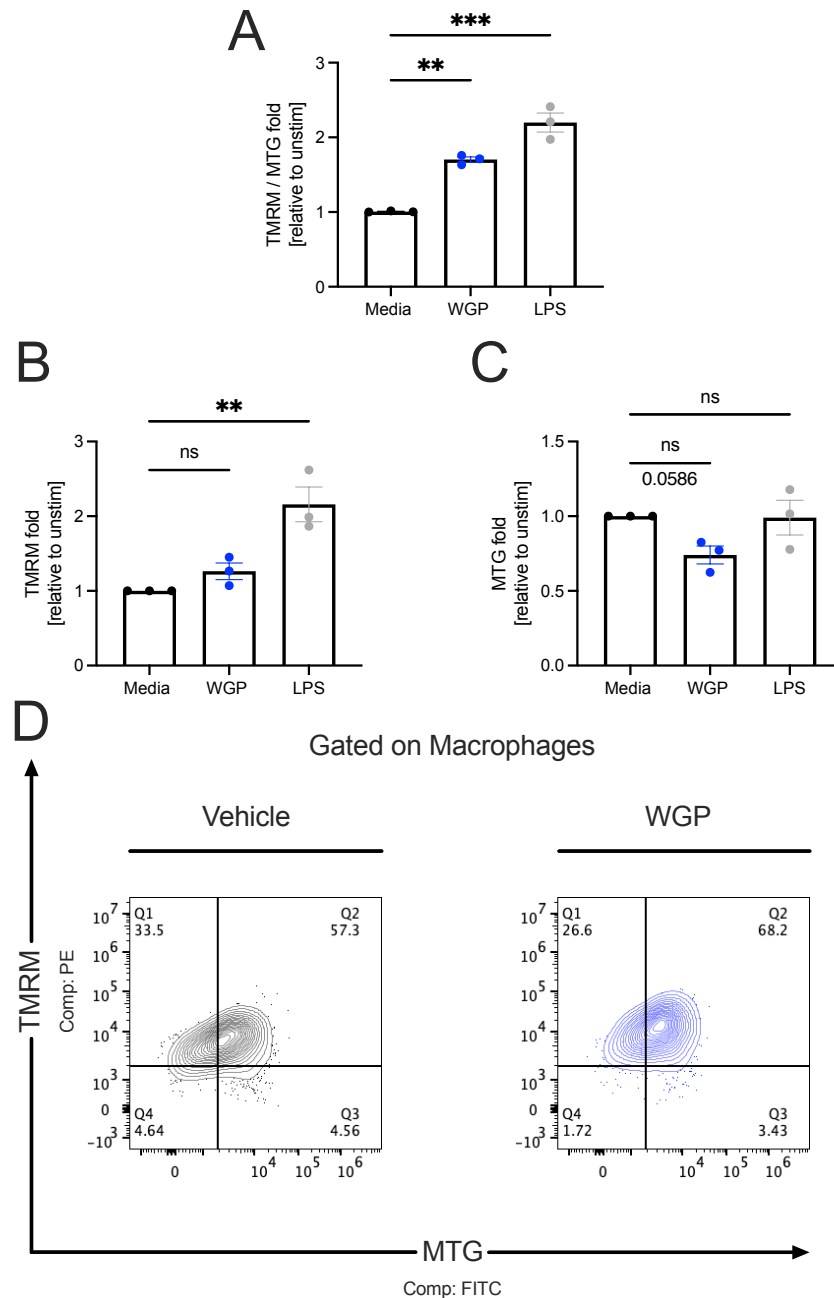


Figure 5.26 WGP induces changes in mitochondrial membrane potential relative to the volume of mitochondria in the cell

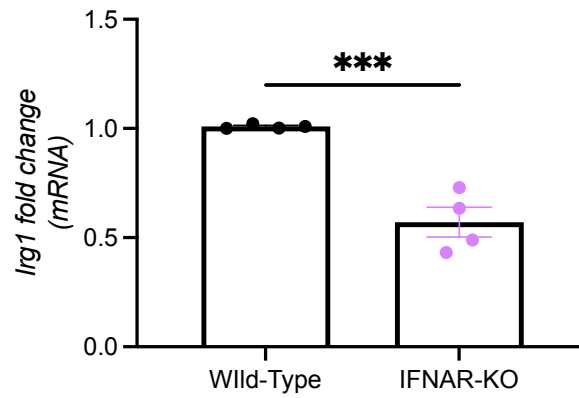
BMDMs were stimulated with 100 μ g/mL WGP or 100ng/mL of LPS for a period of 24hrs. After 24hrs cells were washed and allowed to rest for 2 days. On day 3 cells were collected on ice and stained for TMRM and MTG function according to manufactures protocol. Relative fold change of TMRM to MTG (A), fold change of TMRM relative to the MFI of the media treated control (B), fold change of MTG relative to the MFI of the media treated control (C). Flow plot showing TMRM and MTG staining of media and WGP treated BMDMs (D). Each dot is a representative of an individual experiment (Biological N = 3). NS: P value >0.05; **P<0.01; ***P<0.001; (RM-One way ANOVA with Holm-Šidák's multiple comparisons test). *Experiments performed with Dr. Hannah Prendeville (Sheedy Lab, TBSI)*

5.2.10 WGP causes alteration of fumarate hydratase and immune response gene 1 in an IFN signalling independent manner

Changes in mitochondrial polarisation coupled with changes in *Fh* and *Irg1* expression have been associated with mitochondrial RNA (mtRNA) release leading to enhanced IFN production. Since I observed that WGP induces changes in *both Irg1 and Fh* mRNA expression, I wanted to test whether these changes were dependent on IFN signalling. First, I tested whether both genes were affected by IFN itself. To do this I isolated RNA from WT and IFNAR knock out (KO) BMDMs and assessed relative basal expression of both genes in both groups (**Fig 5.27**). As has been shown by others previously^{253, 254}, expression of *Irg1* was linked to IFN signalling, with IFNAR KO BMDMs expressing significantly less *Irg1* than wild type BMDMs (**Fig 5.27A**). Next, I tested if IFN signalling had a similar role in *Fh* expression. Interestingly, I saw that in the absence of IFN signalling there was a significant increase in the relative expression of *Fh* (**Fig 5.27B**). This data suggests that IFN has an inhibitory role on *Fh*, while IFN enhances *Irg1* gene expression.

Having confirmed that basal levels of both *Irg1* and *Fh* were susceptible to changes in IFN levels I decided to investigate whether this might impact WGP-induced IDT. Overall, I have shown previously in this chapter that oxidative metabolism is not greatly impacted by loss of IFN signalling, however, I wanted to confirm that this was true for these genes as well since both are implicated in further IFN production^{243, 248}. mRNA was isolated from WGP treated BMDMs, either WT or KO, after 6 days Training/treatment and expression of the two genes was measured by qPCR (**Fig 5.28**). WGP was capable of driving enhancement of *Irg1* gene expression in both WT and KO BMDMs, showing that this induction was independent of IFN signalling (**Fig 5.28A**). Similarly, WGP stimulation caused a repression in *Fh* gene expression in both WT and KO BMDMs (**Fig 5.28B**). Taken together this data indicates that while baseline IFN signalling can regulate *Irg1* and *Fh* expression, in the context of WGP stimulation IFN signalling is not required for the reprogramming of metabolic genes.

A



B

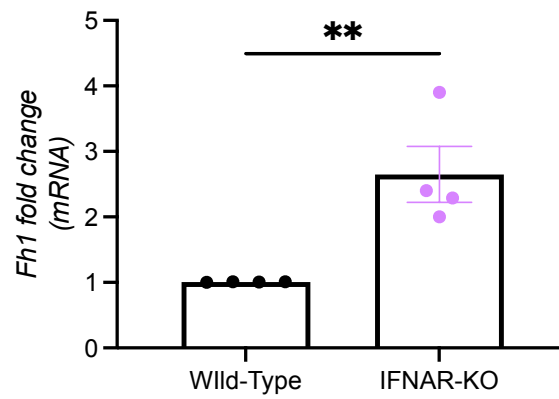


Figure 5.27 IFN signalling impacts core TCA cycle enzymatic gene expression

RNA isolated from unstimulated BMDMs from wild type or IFNAR knock out mice. Relative expression of *Irg1* (A) or *Fh1* (B) mRNA transcripts. Each dot is a representative of an individual experiment (Biological N = 4). **P<0.01; ***P<0.001; (Unpaired T-test).

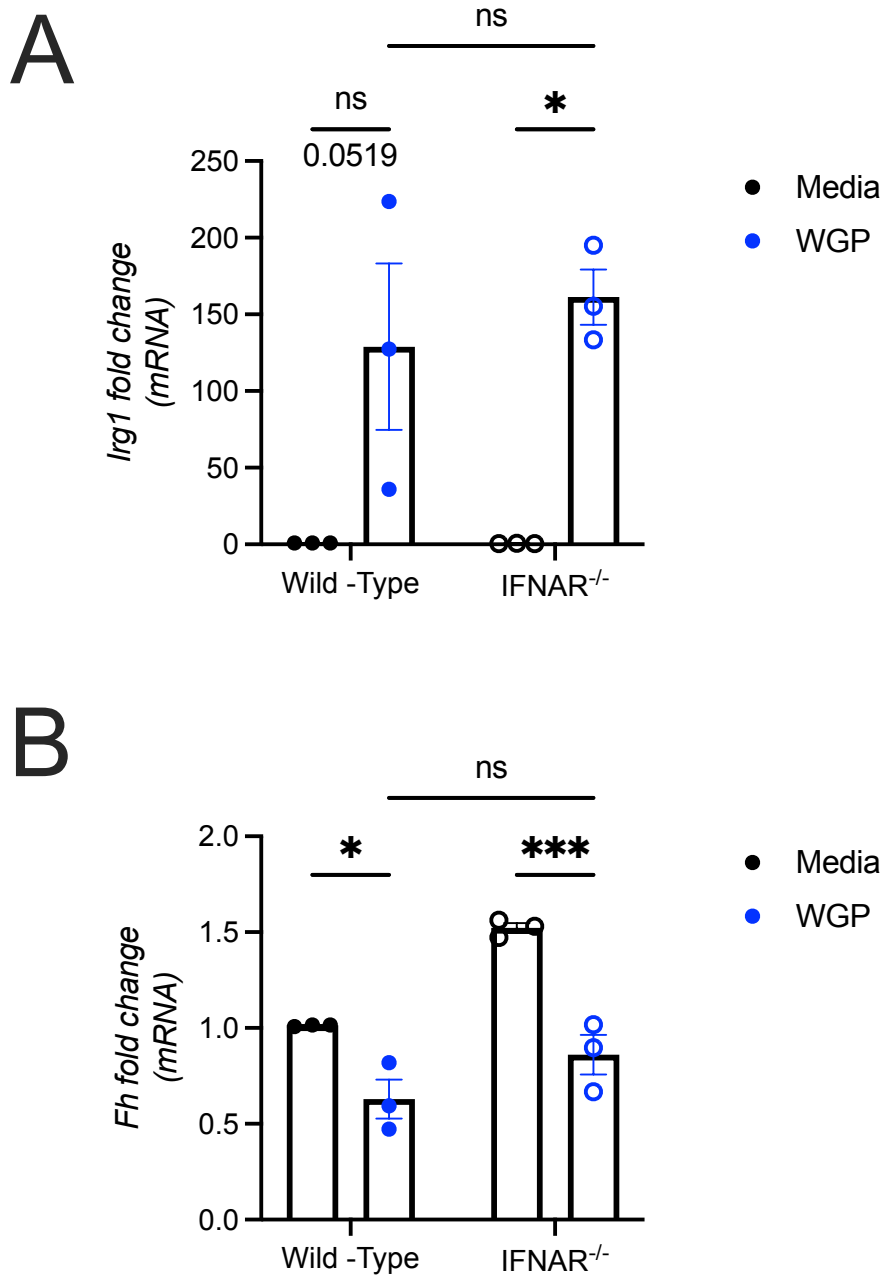


Figure 5.28 Changes in TCA enzyme genes are independent of IFN signalling

Wild type and IFNAR KO BMDMs were treated with 100µg/mL WGP for 24hrs. After 24hrs, cells were washed and allowed to rest for 6 days. On day 6, RNA was isolated and qPCR performed. Relative expression of *Irg1* (A) and relative expression of *Fh* (B). Each dot is a representative of an individual experiment (Biological N = 3). NS: P value >0.05, *P<0.05; ***P<0.010; (Two-Way ANOVA with Šídák's multiple comparisons test).

5.2.10 WGP stimulation leads to mtRNA release independent of IFN signalling driving anti-viral interferon dependent training

Since both itaconate and fumarate immunometabolic rewiring have been associated with mitochondrial nucleic acid release^{243, 248}, I hypothesized that this could be the cause of the enhanced anti-viral trained response. To test this, I fractionated the cytosol from the nuclear fraction of the cells using digitonin buffer extraction as shown in the material and methods section (**Fig 2.1**). Since mitochondrial nucleic acid should be unique to the nuclear fraction under steady state conditions, I measured the levels of mitochondrial (mt) D-loop expressed RNA within the cytosolic fraction via qPCR (**Fig 5.29**). Here, I used LPS pretreated with oligomycin for 3hrs as a positive control as it has been well characterised that enhancing mitochondrial stress causes mtRNA release. Oligomycin + LPS caused a significant increase in the amount of mtRNA being released into the cytosol as shown (**Fig 5.29A**). Interestingly, three days post WGP there was also a significant increase in mtRNA being released as shown by the significant increase in D-loop (**Fig 5.29A**). Taken together this data indicates that WGP induces the ability of BMDMs to release mtRNA from the mitochondria a novel finding that might explain anti-viral responses.

Having seen that WGP could induce the release of mt nucleic acid I next wanted to investigate whether this might occur in an IFN dependent manner. To do this I treated wild type and IFNAR KO BMDMs with WGP as previously described and isolated fractions on day 3. As I have shown WGP stimulation caused an increase in mtRNA release with increased expression of D-loop in the cytosolic fraction (**Fig 5.29B**). Interestingly, there was still an increase in D-loop expression found in the cytosol of WGP treated IFNAR KO BMDMs, highlighting that mtRNA release was independent of IFN signalling. This lines up with what I observed with both my overall metabolic responses as well as the changes in *Fh* and *IRG1* gene expression. Therefore, I theorise that the release of mtRNA is driven via these immunometabolic changes which subsequently drives the IFN-dependent anti-viral trained responses seen.

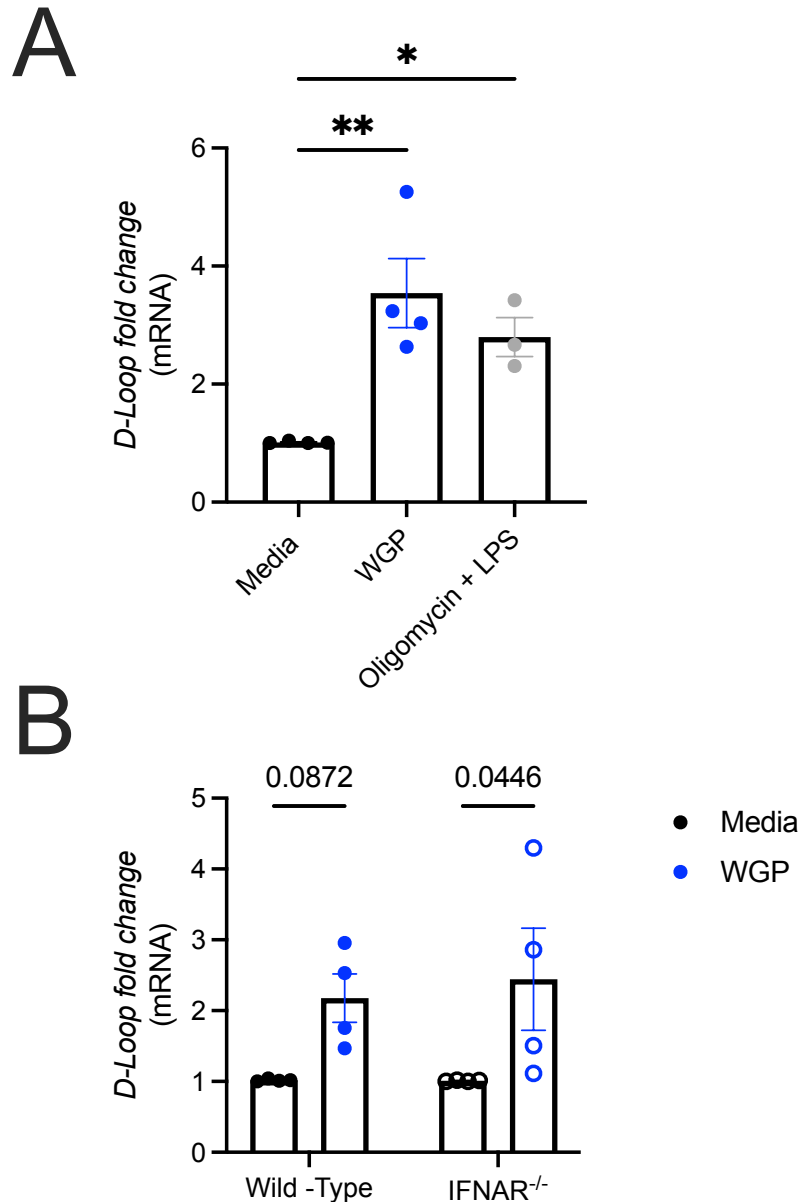


Figure 5.29 BMDMs release mtRNA three days after WGP stimulation

BMDMs were stimulated for 24hrs with 100µg/mL WGP. After 24hrs cells were washed and allowed to rest for 2 days. After 72hrs, cells were detached from plates and cytosolic fraction was separated from nucleic fraction via digitonin extraction. After extraction RNA was isolated from media or WGP treated BMDMs and qPCR was performed to confirm presence of D-loop. (A) mtRNA D-loop expression in cytosol of media treated or WGP treated BMDMs and (B) mtRNA D-loop expression in cytosol of media treated or WGP treated wild type vs IFNAR Knock out BMDMs. Each dot is a representative of an individual experiment (Biological N = 4, +control N = 3). NS: P value >0.05, *P<0.05; ([A] Paired T-test or [B] Two-Way ANOVA with Šidák's multiple comparisons test) *mtRNA release experiments were performed in collaboration with Yukun Min and Dr. Mihai Sularea (O'Neill Lab and O'Farrelly Lab, TBSI).*

5.2.11 WGP stimulation leads to increased antiviral signalling genes 3 days post stimulation

From the RNA seq data set I have previously discussed (Chapter 4) I showed that by day 6 there were increased anti-viral signalling through changes in receptor and signalling gene expression. Therefore, having seen these changes in mitochondrial RNA release at day 3 I wanted to confirm that these changes also corresponded with ISG upregulation and receptor gene expression. To test this, I isolated RNA from day 3 BMDMs that had been stimulated with WGP. I chose this day since I had seen the greatest increase in metabolic activity and highest mtRNA release. The first set of genes I measured were involved in interferon signalling (**Fig 5.30**). There was overall no significant increase in *Irf3* gene expression (**Fig 5.30A**). However, when I explored signalling genes further down the IFN signalling cascade I started to identify genes of interest. Firstly, I saw that the expression of *Irf7* was significantly enhanced 3 days post WGP stimulation (**Fig 5.30B**). I also saw that the ISG / regulator of interferon signalling gene *Ifi204* was also significantly enhanced (**Fig 5.30C**). Taken together this data indicates that downstream IFN signalling genes are affected by WGP stimulation potentially through the recognition of mt nucleic acid in the cytosol.

Having seen that downstream interferon genes were altered 3 days post WGP stimulation I next decided to investigate whether nucleic acid sensing genes were also increased 3 days post WGP stimulation. This would be in line with mt nucleic acid influencing macrophage IFN signalling responses. I turned my attention to three receptor genes of interest; *Tmem173* the gene that encodes for STING1 important in the recognition of cytosolic double stranded (ds) DNA, *Ifih1* which encodes for MDA5 which recognises cytosolic dsRNA and *Ddx58* the gene that encodes for RIG-I which recognises both dsRNA and 5'-triphosphate RNA. WGP stimulation had no overall effect on the expression of *Tmem173* (**Fig 5.31A**). However, interestingly 3 days post WGP stimulation there was a significant increase in the expression of both *Ifih1* and *Ddx58* (**Fig 5.31B-C**). Taken together this suggests that WGP treated macrophages enhance their expression of antiviral receptors and signalling genes potentially through the recognition of mtRNA in the cytosol.

Since I had seen these changes with WGP-treatment, I wanted to investigate if these changes occurred independently of IFN signalling. To do this I again trained BMDMs generated from either wild-type or IFNAR KO mice and collected RNA on day 3. Since I had seen the greatest consistency in the increase in *Ddx58* I decided to analyse the expression of this gene (**Fig 5.32**). As expected WGP significantly enhanced the expression of this gene three days post stimulation. Interestingly, although there was a slight increase in the KO this effect was not significant. I also saw that loss of interferon signalling impacted the expression of *Ddx58* which is well characterised since *Ddx58* is itself an ISG^{255,256}. Taken together this data indicates that WGP stimulation leads to metabolic changes that cause the release of mtRNA into the cytosol. This release can then be recognised by cytosolic receptors with trigger IFN signalling leading to primed ISG responses.

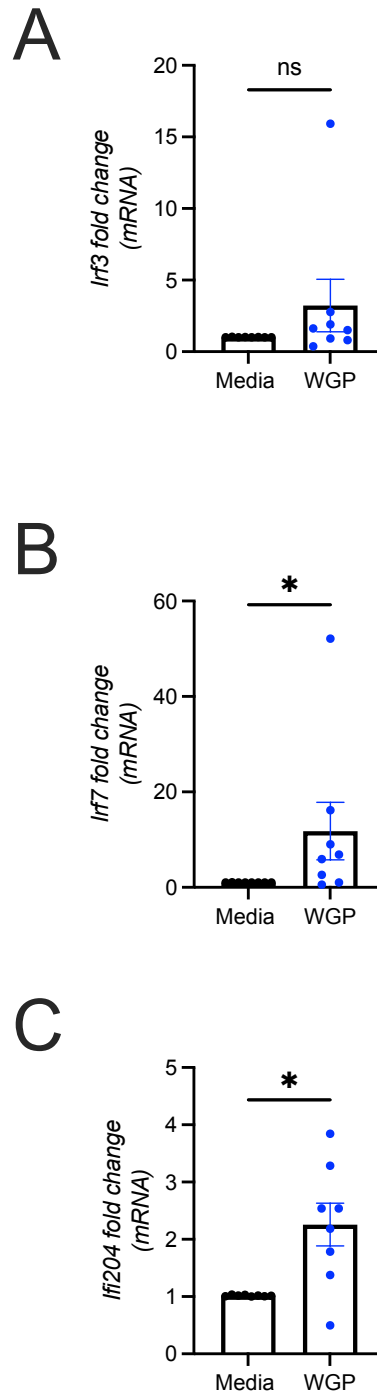


Figure 5.30 WGP drives changes in key anti-viral signalling genes 3 days post stimulation

BMDMs were stimulated for 24hrs with 100 μ g/mL WGP. After 24hrs cells were washed and allowed to rest for 2 days. After 72hrs RNA was isolated from media or WGP treated BMDMs and qPCR was performed for (A) *Irf3*, (B) *Irf7* and (C) *Ifi204*. Each dot is a representative of an individual experiment (Biological N = 8). Normality tests were performed for each gene of interest (A) Paired Wilcoxon test, (B) paired Wilcoxon test (C) paired T-test. NS: P value >0.05, *P<0.05.

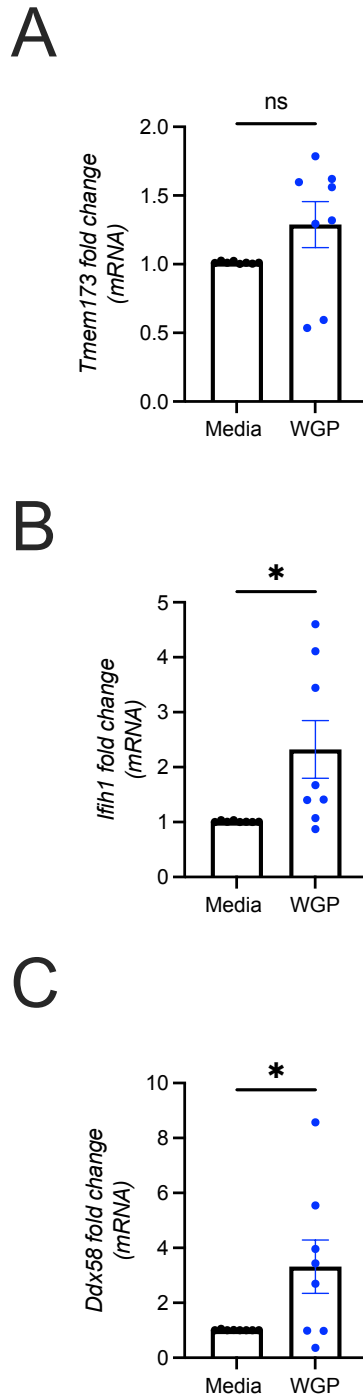


Figure 5.31 WGP drives changes in key anti-viral receptor genes 3 days post stimulation

BMDMs were stimulated for 24hrs with 100 μ g/mL WGP. After 24hrs cells were washed and allowed to rest for 2 days. After 72hrs RNA was isolated from media or WGP treated BMDMs and qPCR was performed for (A) *Tmem173*, (B) *Ifih1* and (C) *Ddx58*. Each dot is a representative of an individual experiment (Biological N = 8). Normality tests were performed for each gene of interest (A) Paired T-test, (B) paired Wilcoxon test (C) paired Wilcoxon test. NS: P value >0.05, *P<0.05.

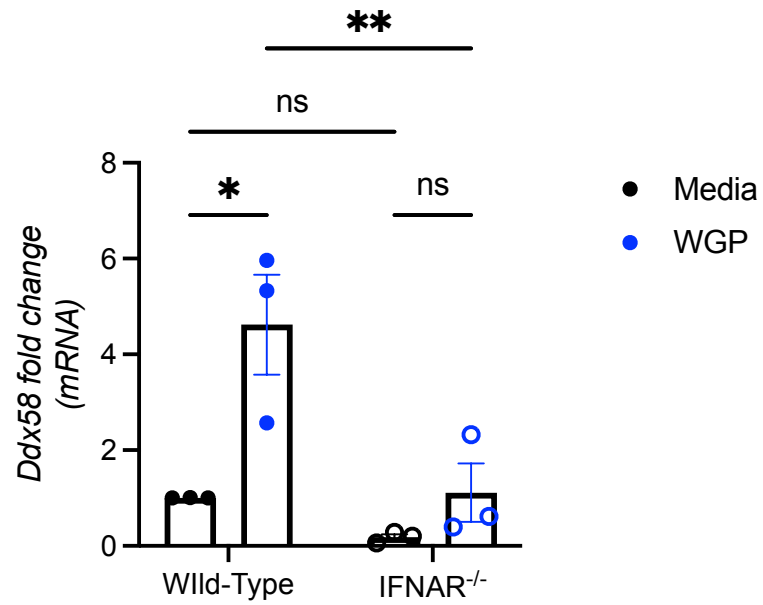


Figure 5.32 Increase in anti-viral receptor expression is dependent on IFN signalling

BMDMs were stimulated for 24hrs with 100 μ g/mL WGP. After 24hrs cells were washed and allowed to rest for 2 days. After 72hrs, RNA was isolated from media or WGP treated BMDMs and qPCR was performed. Each dot is a representative of an individual experiment (Biological N = 3). NS: P value >0.05, *P<0.05, **P<0.005; (Two-Way ANOVA with Šídák's multiple comparisons test)

5.2.12 mtRNA release is important for WGP induced interferon dependent trained immunity

Having seen that WGP treated cells release mtRNA coupled with enhanced IFN signalling and RNA sensing in the cytosol, I hypothesized that by blocking mitochondrial RNA synthesis I could reduce or block the IFN dependent training effect I have described over the past two sections. To do this I used the well described inhibitor of RNA polymerase, MT11²⁵⁶. I decided to trial different timepoints to see which would give the most effective results. First, I wanted to see if inhibition of RNA polymerase just prior to the restimulation would impact WGP mediated IFN-dependent training. 3hrs prior to pIC stimulation cells were treated with IMT1 followed by an 8hr stimulation with pIC (**Fig 5.33A**). In both the vehicle as well as the inhibitor group, WGP treatment significantly enhanced the cells' ability to produce IFN β . There was no significant difference between the two groups. This suggested that the release of mtRNA did not induce a priming effect but instead may be a long-term memory affect induced at an earlier timepoint. However interestingly, I did notice that untrained cells that had been given the inhibitor had a lower ability to induce IFN post pIC challenge, ($p=0.195$), potentially hinting at the idea that mitochondrial nucleic release is required for a normal anti-viral immune response.

Since, I had seen no differences between vehicle and IMT1 trained groups, I hypothesized that since mtRNA release occurred at 3-days post WGP treatment, I might have to inhibit its production at this earlier timepoint. Therefore, I added IMT1 inhibitor to BMDMs three days after WGP stimulation, to match the increase in mtRNA release. The inhibitor was maintained for the remainder of the training protocol and was removed prior to pIC stimulation, to prevent any effects stemming directly from the inhibitor. Again, although both vehicle and IMT1 treated trained cells had a significant increase in IFN response to pIC relative to their untrained cells, I noted a non- significant reduction in the cells ability to enhance IFN in cells treated with IMT1 (**Fig 5.33B**, $p=0.0524$). This data indicates that mtRNA release is partially important for inducing the enhanced anti-viral effects indicative of WGP IDT. Notably, there was a lot of variation across this experiment which suggests inhibitor treatments and conditions may require further optimization.

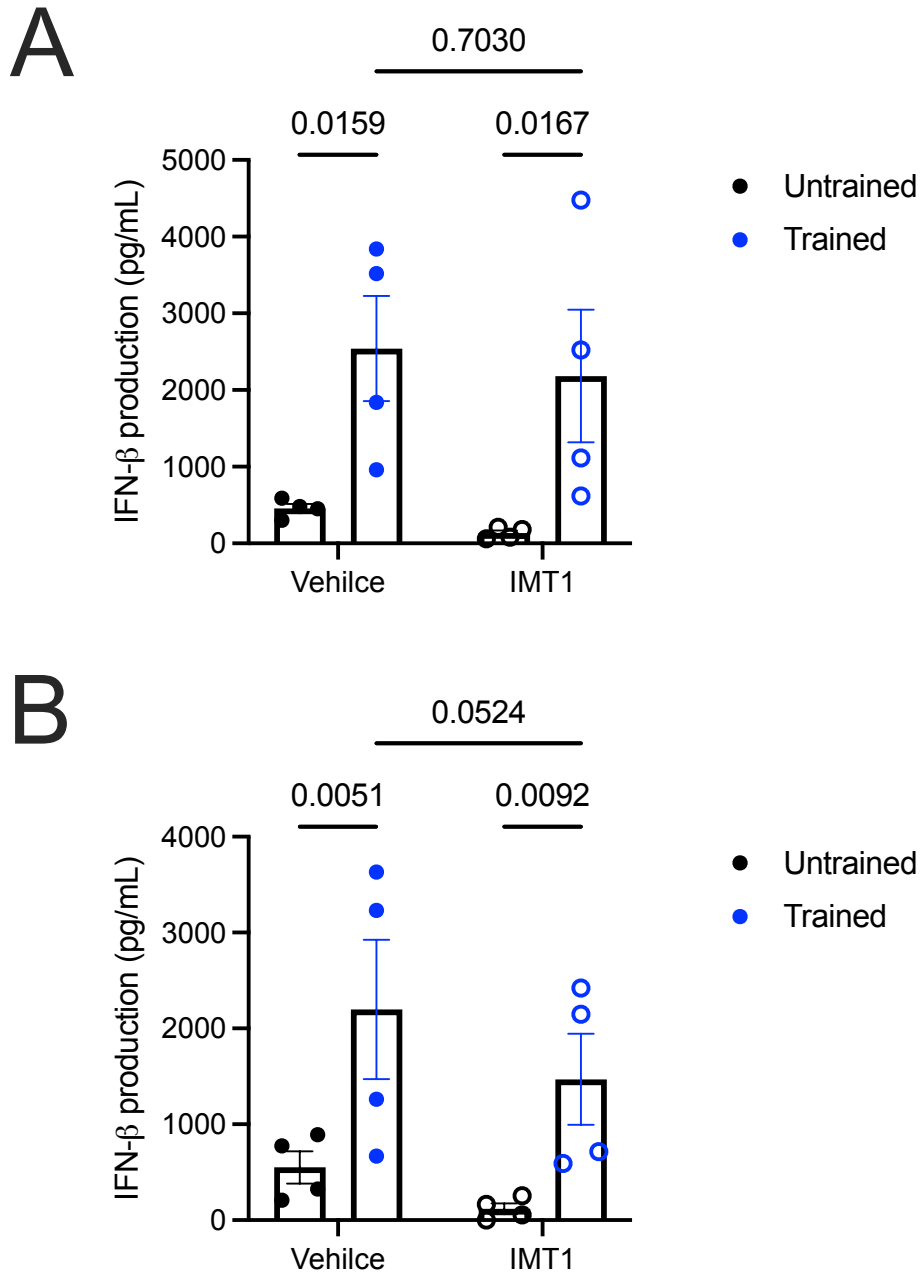


Figure 5.33 Increase in anti-viral trained responses are dependent on mtRNA release

BMDMs were stimulated for 24hrs with 100 μ g/mL WGP. After 24hrs cells were washed and allowed to rest for 2 days. After 72hrs, cells were treated with either media (A) or 10 μ M of the mitochondrial RNA polymerase inhibitor IMT1 (B). Cells were rested until day 6 where cells were either treated with IMT1 3hrs prior to stimulation (A) or were stimulated with 10 μ g/mL pIC for 8hrs (B). In both cases cells were stimulated for 8hrs with 10 μ g/mL pIC. Each dot is a representative of an individual experiment (Biological N = 4). Exact P-values shown; (Two-Way ANOVA with Šidák's multiple comparisons test)

5.3 Discussion

Following on from the work described in Chapter 4 I wanted to further breakdown and tease apart the immunological mechanism via which a fungal ligand can confer IFN induced trained immunity. In the field of fungal immunology the importance of type I interferons in anti-fungal immunity has been recently described¹⁶⁸. However, this knowledge is crucially lacking in many of the trained immunity papers utilising β -glucan in the literature. Additionally, the ability of fungal ligands to drive non-canonical IFN production is really only defined to dendritic cell populations, and thus it was interesting to explore this initial response in macrophage models^{168, 169}.

Importantly, the training field also has a gap in the literature describing how signalling from the initial priming aspect of β -glucan challenge can trigger metabolic changes that influences the cells ability to undergo long term functional reprogramming. In this regard, I explored two possible hypotheses; either changes in type I interferon effects macrophage metabolism to drive trained immunity or WGP induced metabolism impacts anti-viral responses including type I interferon. Both were viable options with literature showing that type I interferon was capable of reprogramming dendritic cell metabolic responses for enhanced functional activity as well as other groups highlighting the importance of metabolic reprogramming in enhancing type I interferon responses in macrophages^{164, 243, 248}. Therefore overall the work from this chapter expanded on known literature from other fields of immunobiology and added it to the context of trained immunity.

I firstly wanted to investigate the role of interferon signalling in β -glucan induced trained immunity. From the previous chapter I showed that enhanced anti-viral functions, labelled as interferon-dependent training (IDT), were dependent on interferon signalling. However, I did not expand on how a fungal ligand such as β -glucan could induce such a potent anti-viral response. As mentioned, to understand the crux of this signalling I turned to fungal immunology where it has been well characterised that fungal ligands, such as β -glucan derived from zymosan, can drive potent IFN responses in-vitro and in-vivo^{168, 169}. I expanded on this knowledge

showing that early exposure to purified β -glucan, WGP, was enough to drive a weak, yet significant, interferon and ISG response in macrophages in-vitro. These results were shown to be dependent on type I interferon signalling showing that β -glucan stimulation drives a feedback loop on the type I interferon response. These early responses were linked with changes in expression of IRFs, such as IRF1 and IRF5. These two IRFs were of interest as they have been shown to both regulate the IFN response to fungal ligands, but also to regulate themselves¹⁶⁹. Similar to what Gringhuis, S.I. *et al*, showed in dendritic cells recognition of fungal β -glucan by macrophages drove an increase in the expression of both *Irf1* and *Irf5* after 2hrs of stimulation. Similar to their results I also showed that IRF1 was maintained 6 to 8hrs post stimulation whereas IRF5 returned to baseline. The authors showed that IRF5 was vital in driving an IFN response to fungal ligands whereas IRF1 inhibited this response via blocking the binding of IRF5 and IRF7 to the *IFNB* promoter. Therefore, it is interesting to postulate whether this tight regulation contributed to my difficulty in accurately detecting IFN at both expression and protein levels. This was a key limitation encountered and impacting my approaches in this chapter. To infer Type I IFN production I had to rely on functional readouts like ISG expression induced by WGP treatment and examine these in the context of IFNAR-deficiency to elucidate their dependence of Type I IFN production. This was further complicated by the fact that for in-vitro mouse experiments there is no commercially available assay for measuring low levels of type I IFN accurately. Further, much of the previous work on fungal ligands that can induce IFN was performed in more professional IFN producing cells, such as (p)dendritic cells. It would be interesting to test whether in these cell types WGP has a greater ability in driving IFN in a similar metabolic/mtRNA dependent mechanism which we could use to show proof of concept of this model. In conclusion for this section I confirmed that WGP drives an early, yet weak interferon response that enhances the expression of some IRFs and ISGs.

Having confirmed that WGP does in fact drive an acute IFN response my next question was whether this was the causative agent of metabolic reprogramming. Wu, D. *et al* have shown that in dendritic cells IFN alters oxidative phosphorylation which enables for further immune function such as inflammatory cytokine and

heightened IFN production¹⁶⁴. Therefore, I hypothesized that the initial interferon production by WGP could be the facilitator in driving the metabolic shifts that I observed. However, IFNAR KO cells treated with WGP maintained their ability to enhance glycolysis and OxPhos vs their untreated controls. Some aspects of metabolism were dampened in the absence of IFN signalling in WGP treated cells, however, this was probably explained due to the fact that loss of IFN signalling had a major effect on cells basal respiratory rate. This has implications for anti-IFN therapies due to the fact that basal interferon signalling seems to play a role in maintaining a cells 'normal' cellular metabolic state. Therefore, it would be interesting to postulate whether anti-IFN therapies may act through modulating cellular metabolism or whether they have direct effects against IFN responses themselves. In fact it has been shown that the common MS therapeutic, Tecfidera, which acts on exacerbated IFN responses can modulate glycolytic responses showing similar responses to what I have shown with IFNAR KO mice²⁵⁷. Therefore, although my hypothesis was proven incorrect, early interferon signalling does not lead to enhanced metabolic responses, I have added to the current literature by showing that type I IFN itself is an important regulator of baseline metabolism and thus plays a much more complex role than just anti-viral signalling. In conclusion baseline type I IFN acts as a metabolic signalling molecule, potentially maintaining immune cells in a primed state to prevent infections.

Since I saw that WGP induced IFN did not impact cellular metabolism, I next turned my attention to whether cellular metabolism would impact IFN responses. Many groups have published on this in the context of bacterial immunity^{243, 248} and viral immunity²⁴⁹ but in the context of trained immunity the role metabolites have directly on responses is poorly defined. I sought to investigate whether metabolites themselves can enhance anti-viral immunity as well as investigating their potential to act as training agents. The trained immunity literature is full of data on various metabolites that accumulate during the training phase^{120, 239, 258}. Since this has not been described for WGP I wanted to investigate these changes further using Carbon-13 labelled glucose isotope tracing. Glucose uptake was effected by WGP treatment with WGP treated macrophages enhancing glucose uptake, however, interestingly metabolic pathways and labelling of glycolytic pathways were not significantly changed by WGP. This was interesting since I saw increased lactate in

the media as well as enhanced ECAR on Seahorse. However, these findings may be explained from increased flux through glycolysis, without blocking or accumulating upstream metabolites. This was also coupled with the fact that I did not see any significant changes in the isotopologues of both glycolytic and TCA cycle metabolites potentially pointing towards another fuel source in macrophage training. This would explain why simply inhibiting glycolysis in our system had no impact on trained immunity, as highlighted in previous chapters. In conclusion I hypothesise that WGP trained macrophages become metabolically 'flexible' utilising any fuel source available to them. However, what is evident here is that maintained mitochondrial metabolism is vital in this aspect, that is, trained cells may use any fuel source available to them as long as they can feed TCA cycle and metabolic mechanisms.

On that note I turned my intention to the metabolites that were accumulated within the TCA cycle; itaconate, succinate and fumarate. All three have been shown to be uniquely important in regulating inflammatory signals in macrophages^{233, 243, 248, 253}. Importantly they have also been shown to be important in viral immunity^{249, 259, 260}. Therefore, I wanted to explore the possibility of two hypothesis; either metabolite accumulation leads to direct responses (priming) or these metabolites can induce training. In terms of priming, itaconate was the only metabolite that increased TNF and IFN responses to pIC challenge, with its derivative 4-OI inhibiting these responses. Interestingly, one of itaconate's main intracellular functions, inhibition of SDH²⁴⁶, seemed to negatively impact responses to pIC with SDH inhibition by AA5 reducing both IFN and ISG changes. However, when ACOD1 was inhibited by citraconate IFN responses were also inhibited. This suggests that in viral immunity itaconate may play a role that does not involve SDH inhibition but may act on enhancing interferon responses in a yet undefined manner. However, itaconate alone was not capable of driving a training effect, although interestingly, itaconate synergised with WGP training enhancing anti-viral responses. This result was fascinating as it suggested that trained immunity was not fixed but could be modulated by pairing with metabolites. This coupled with functional IDT in IRG1 KO cells suggested that itaconate alone was not the sole contributor to the training response. Similarly, succinate alone could neither prime nor train macrophages, but similar to itaconate could be harnessed as a synergistic enhancer of training. The

most interesting results I discovered were with the metabolite fumarate. Priming with the fumarate ester DMF inhibited IFN responses and I have shown in previous sections that DMF inhibited IDT as well. Yet inhibition of FH which induces intracellular accumulation of fumarate enhanced TNF responses to pIC and even more interestingly DMF was able to induce mild trained immunity over vehicle control, significantly boosting TNF responses as well as boosting IFN responses. I hypothesize that DMF acts based on how it is metabolised by the cell. Studies have shown that DMF is metabolised to MMF in-vitro²⁶¹, which may indicate that the longer cells are allowed to rest the greater the metabolic conversion and thus changes in immunological effects. Greater understanding of how DMF is metabolised over time in cells would be needed to better test this hypothesis however.

I was also able to show that WGP induced similar effects to what occurs during LPS mediated fumarate accumulation²⁴⁸. There was an increase in mitochondrial membrane potential, however, this increase was per mitochondria since mitochondrial volume was reduced by WGP (Reduced MTG). There was also a reduction in FH gene expression, which may explain fumarate accumulation. All of this was coupled with release of mitochondrial RNA, which like the metabolic changes observed, was independent of interferon signalling. This RNA release was important in enhancing the trained responses to pIC, which may occur through mtRNA recognition in the cytosol, inducing an IFN response and thus priming IFN signalling genes and anti-viral receptors. Here I hypothesise that WGP causes metabolic reprogramming of macrophages which can cause hyper polarisation of the mitochondria. This coupled with changes in TCA cycle metabolites can trigger the release of mt nucleic acid (measured here as mtRNA) into the cytosol, which occurs in a type I IFN independent manner. Cytosolic mtRNA can then be recognised by cytosolic receptors which leads to type I IFN signalling and ISG priming of macrophages.

The major limitations to my results thus far in showing IDT is a deeper metabolic profiling of cells over different timepoints to pin point the aspartate-arginosuccinate shunt which is a major facilitator of anti-viral responses linked with fumarate accumulation²⁴⁹ and needing to tease apart priming from training aspects. I have

shown that IDT is definitely dependent on epigenetic modifications since inhibition with MTA and CPH inhibits these effects, however, I am aware that ATAC sequencing to better understand what anti-viral genes may be susceptible to IDT would greatly improve the significance of my findings. It has been shown that certain genes are susceptible to IFN epigenetic modifications, termed 'memory' ISGs, and it would be interesting to test whether these are similar to epigenetically primed WGP genes¹⁸⁴. Similarly, I also understand that I need to investigate the role enhanced gene expression of receptors and IFN signalling has on responses. I plan on investigating protein expression of these receptors to rule out any primed phenotype in IDT. However, taken together the data indicates a working hypothesis that WGP induces a form of anti-viral protection known as interferon-dependent trained immunity (IDT) as shown in **Fig 5.34**.

Therefore, I purpose that metabolic alteration, specifically in *Fh* downregulation, has two major impacts on macrophage trained immunity. Firstly, reduction in *Fh* and increased expression of *Ass1*, (measured in the RNA-seq in Chapter 4), suggests changes in a pathway known as the argininosuccinate shunt. The literature has never shown how fumarate can exist the mitochondria to confer epigenetic modifications. I hypothesise that the argininosuccinate shunt may provide this link. Here, oxaloacetate can be shuttled from the TCA to be further metabolised into cytosolic fumarate. This cytosolic fumarate may accumulate due to reduced cytosolic *Fh* expression which may then be used for the epigenetic modifications known to occur in TI¹²¹. At the same time, the reduced expression of mitochondrial *Fh* may also cause hyperpolarisation of the mitochondrial membrane which can impact release of mt nucleic acid²⁴⁸. This can then prime macrophages for enhanced anti-viral responses as discussed previously. Therefore, WGP induces a dual hit model conferring both classical TI as well as non-canonical IDT. I shall use this mechanism to explain my results over the following chapter in an in-vivo setting.

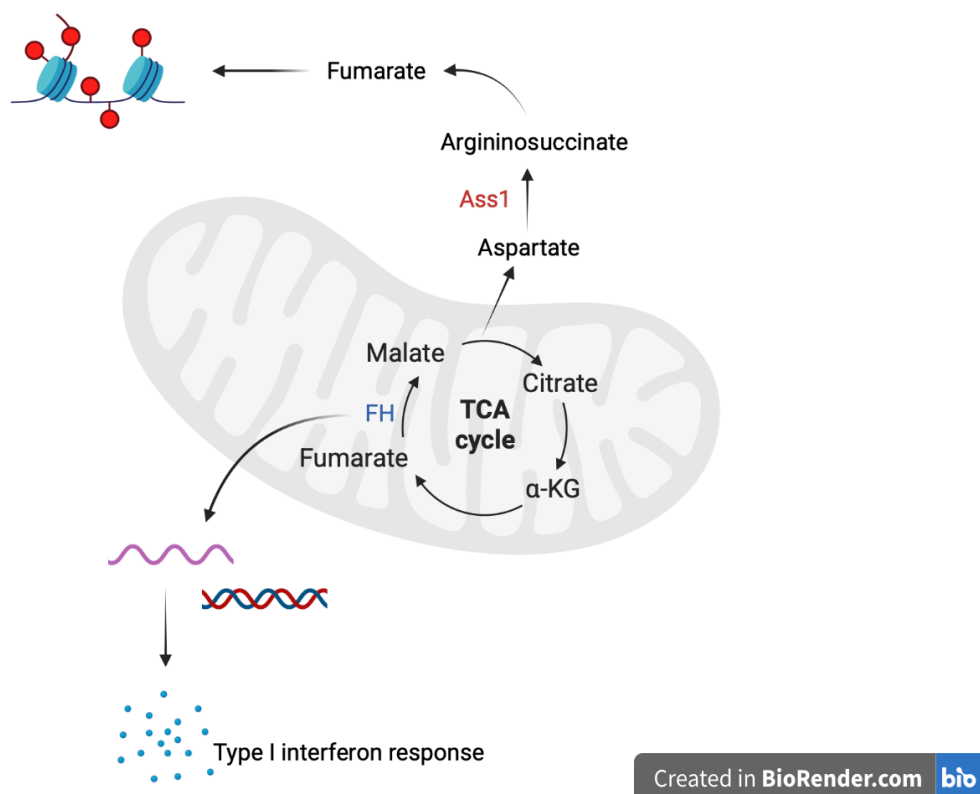


Figure 5.34 Schematic showing the proposed mechanism of action for interferon-dependent training

Three days after WGP stimulation macrophages undergo metabolic reprogramming of their TCA cycle. Importantly there is a reduction in the expression of *Fh*. At the same time there is an increase in genes involved in the aspartate-argininosuccinate shunt, such as *Ass1*. The reduction in *Fh* induces mitochondrial stress facilitating the release of mtRNA into the cytosol driving IFN signalling and inducing an enhanced anti-viral state within macrophages. Outside of the TCA increased expression of *Ass1* facilitates the breakdown of aspartate to argininosuccinate which can be utilised by the cell to facilitate cytosolic fumarate important for epigenetic modifications as seen in classical trained immunity.

Chapter 6. Dietary feeding of WGP β -glucan drives IFN dependent anti-viral signalling

6. 1 Introduction

Vaccination or parenteral delivery strategies have long been employed in the training field to induce trained immunity^{89, 101}. Most of these studies have either focused on changes in peripheral immune cells (peripheral training) or on changes on bone marrow stem and progenitor cells (central training)^{89, 90}. However, recently it is emerging that oral delivery of training agents, such as β -glucan, can also cause similar responses^{99, 197, 198}.

Moreso, a growing body of evidence has pointed to the role for type I IFN in inducing trained immunity. Firstly, groups have shown that IP delivery of β -glucan can lead to enhanced neutrophil responses to combat tumours as well as viral infection dependent on IFN signalling^{35, 262}. It has also been shown that viral infections can trigger non-specific immunity in the lungs against tumours, bacterial infection and non-specific viral infection^{92, 93, 95}. However, no exact mechanism has linked the broad-spectrum responses observed. Since they are triggered by viral infection it would be interesting to hypothesise that type I IFN links these responses.

Taking all this together it is important to also identify current gaps in the literature that I wanted to explore in this chapter. Firstly, it is poorly understood what causes increased bone marrow progenitor changes, termed haematopoiesis, which is often associated with a bias towards monocyte progenitors (myelopoiesis) or granulocyte progenitors (granulopoiesis). Similarly, there is also a gap in the literature in whether resident immune cells are “trained” or whether they have been replaced by bone marrow derived immune cells that have been trained centrally.

Therefore, I hypothesise that WGP feeding will lead to enhanced central and peripheral cellular changes that are mediated by metabolic and epigenetic changes. Since I saw that interaction with WGP causes macrophages to increase anti-viral signalling I also hypothesise that these changes will occur in a type I IFN dependent manner. Finally, I hope to show that mature cells have enhanced responses to viral ligand or viral infection both ex-vivo and in-vivo.

Experiments in chapter 6 were performed alongside Dr. Anna Ledwith, as stated in figure legends

6.2 Results

6.2.1 Oral delivery of WGP triggers uptake by gut myeloid cells

So far I have shown that the yeast β -glucan WGP was capable of driving anti-viral trained immunity and interferon signalling in an in-vitro setting. However, since WGP is sold commercially as an oral supplement (Wellmune®) I wanted to test whether these effects could be translated into an in-vivo model. Two in-vivo models were performed over the course of my PhD (**Fig 6.01A-B**). Since WGP was orally delivered to the mice I first wanted to explore whether gut immune cells could recognise WGP. To do this, I labelled WGP with a fluorescent label, 5-(4,6-Dichlorotriazinyl) Amino fluorescein (5-DTAF). Mice were either injected intraperitoneally (IP) or orally gavaged (OG) with this labelled WGP and culled 3 days after. Various organs were collected, immune cells isolated and phagocytes were analysed based on the mean fluorescent intensity (MFI) of DTAF. Since many of the previous experiments focused on macrophage WGP uptake I focused on these cells in the gut. There was an increase in DTAF signal in cells I defined as CD45⁺CD11b⁺Ly6C⁺F4-80⁺ pancreatic macrophages 3 days after IP injection (**Fig 6.02A**). This was consistent with what has previously been published¹⁰¹. However, I was very interested in the ability of gut macrophages to take up WGP. These cells were also defined as CD45⁺CD11b⁺Ly6C⁺F4-80⁺ cells, however, I appreciate I should include CD64, MHC-II and exclusion markers such as Ly6G for excluding neutrophils. There was a significant increase in DTAF signal in macrophages that were found within the colon lamina propria (LP) (**Fig 6.02B**). There were no changes in DTAF labelling in colon intra epithelial layer (IEL) macrophages nor in small intestine IEL or LP macrophages (**Fig 6.02C**). Overall this data indicates that WGP is recognised by macrophages in the colon of mice that had been orally gavaged WGP.

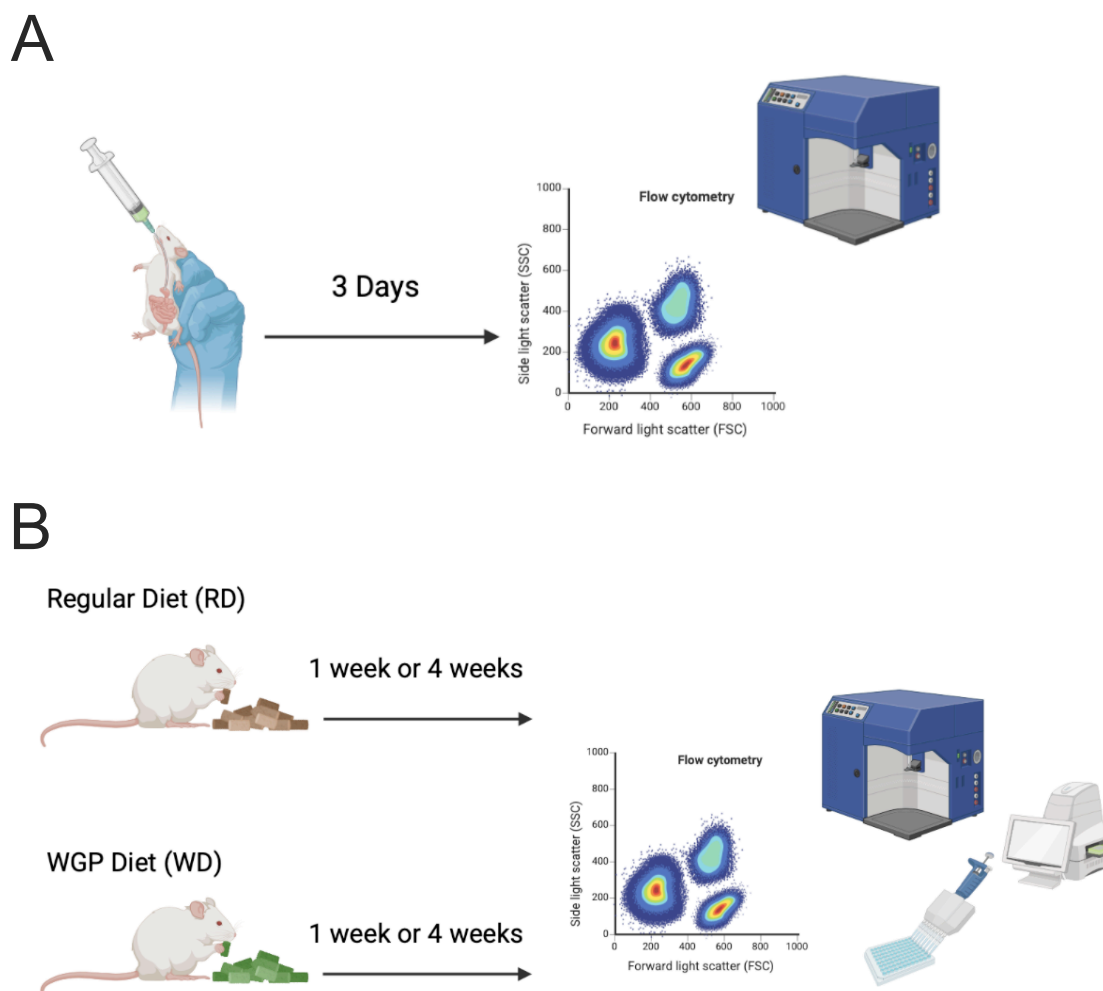
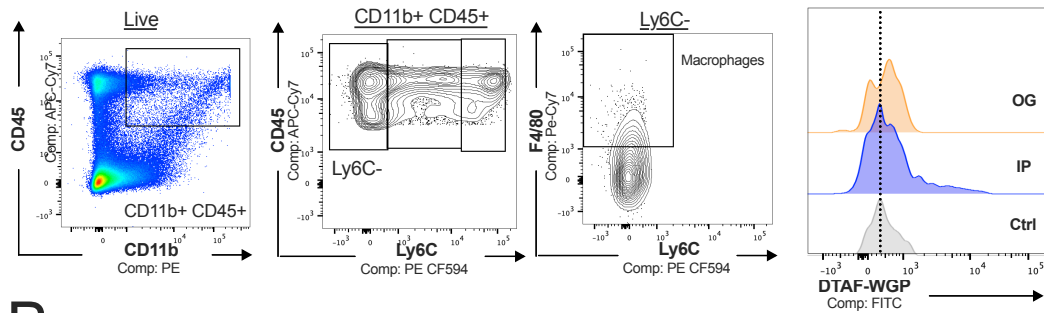


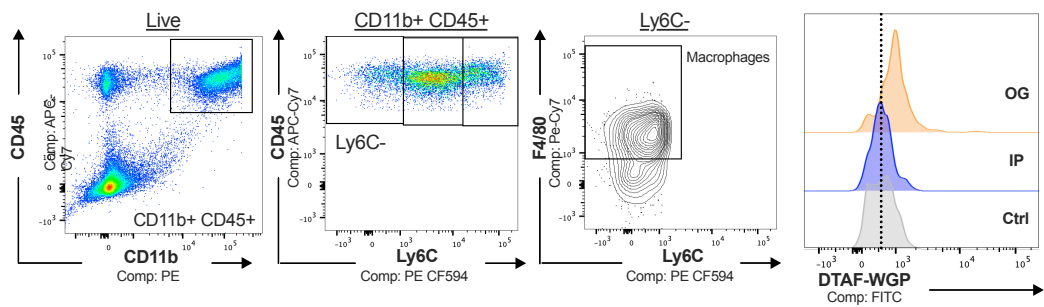
Figure 6.1 Models used for oral delivery of WGP into mice

(A) Mice were orally gavaged DTAF labelled WGP and were culled three days later. Organs were processed and generated into a single-cell suspension. Cells were stained for flow cytometry analysis and ran on the Cytec Aurora. (B) For most experiments mice were fed a WGP supplemented diet (0.05% WGP per chow W/W). Mice were either fed the diet for 1 week or 4 weeks depending on the assay and were culled using CO₂ asphyxiation. Organs were processed as previously described and cells were either stained for flow cytometry or cultured for ex-vivo experiments.

A (Pancreas)



B (Colon lamina propria)



C

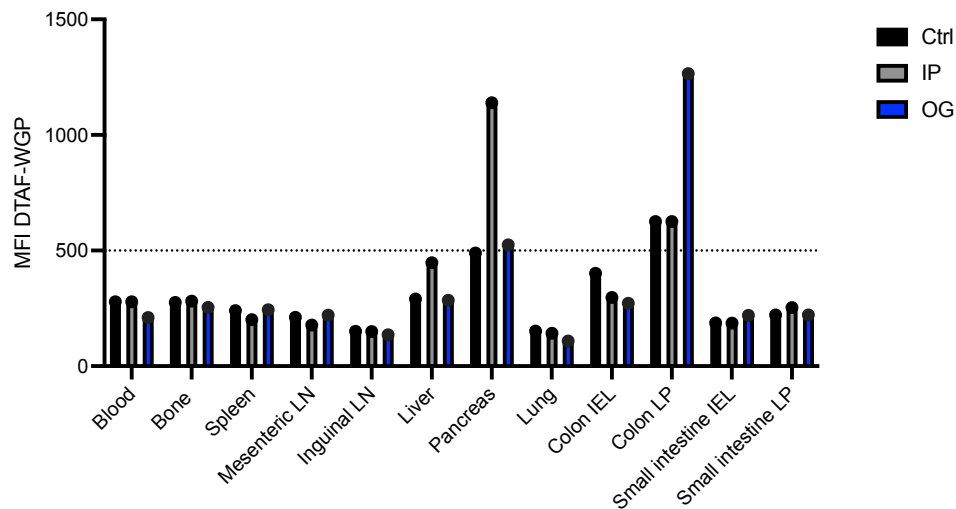


Figure 6.2 DTAF-labelled WGP is taken up by colon lamina propria macrophages

Mice were orally gavaged DTAF labelled WGP and were culled three days later. Organs were processed and generated into a single-cell suspension. Cells were stained for flow cytometry analysis and ran on the Cytec Aurora. (A) Gating strategy showing uptake of DTAF labelled WGP by pancreatic macrophages after IP injection, but not after oral gavage. (B) Gating strategy showing uptake of DTAF labelled WGP by colon lamina propria macrophages after oral gavage but not after IP injection. (C) MFI of DTAF labelled WGP in different organs taken from mice administered WGP. *Experiment performed alongside Dr. Anna Ledwith.*

6.2.2 1-Week WGP feeding induces weak interferon signal in blood serum and bone marrow aspirate

It has been previously shown that in IP injection strategies β -glucan can induce trained immunity via IFN signalling in the bone marrow²⁶². Since I saw that WGP was recognised by gut macrophages and monocytes I hypothesized that this would trigger IFN signalling, via the mechanism I described in results chapters 2 and 3. In order to test this hypothesis the levels of IFN and inflammatory cytokine in both the blood serum as well as in the bone marrow aspirate were measured (**Fig 6.03**). I also attempted to measure changes in cytokine levels in gut immune cell populations, but to no avail. I therefore, first decided to investigate whether 1-week WGP feeding enhanced IFN levels in blood serum (**Fig 6.03A**). I was able to detect a weak IFN signature in both regular diet (RD) fed mice as well as in WGP diet (WD) fed mice. There was no significant difference between the two groups, although WD mice did seem to have slightly elevated levels of IFN in the serum. Next, I wanted to see if inflammatory cytokine levels linked to IFN signalling were increased. This was to make sure that WD did not just cause increased inflammation in mice, which might impact results further in my studies. To do this I measured the concentration of IP-10 in the blood serum (**Fig 6.03B**). Overall, I found that IP-10 levels were not reliably detectable in blood serum in either diets, thus ruling out increased IFN linked inflammation at this timepoint.

Having seen that there was a weak IFN signal in blood serum I next turned my attention to the bone marrow aspirate. Again, IFN β levels were similar in both RD and WD mice, yet there was a slight but non-significant increase in IFN β in the WD bone marrow aspirate (**Fig 6.03C**). Unlike in the blood serum, IP-10 was more reliably detectable in the bone marrow aspirate in both diets (**Fig 6.03D**). There was no overall significant difference between the two diets although again WD mice had less overall IP-10 in the bone marrow aspirate. Overall this data indicates that there is a weak, but importantly not statistically significant, change in IFN levels in both blood and bone marrow 1 week post WGP feeding. It is important to note, as with chapter 4, that the R&D ELISA used to detect murine IFN β , although useful for detecting IFN in stimulated cells, has a much lower sensitivity and lower accuracy

for detecting cytokines in the femtogram/mL range and thus is not ideal for detecting lower amounts of IFN induced by fungal ligands. In order to get around this I attempted to train BM with differing concentrations of rIFN β from regular diet fed mice (**Fig 6.03E**). These cells were then differentiated into macrophages and stimulated with pIC. I saw that at low concentrations of IFN training BMDMs had heightened TNF response to pIC, however, at higher concentrations this effect was lost and instead the cells had lower responses. This data suggests that low concentrations of IFN can induce trained immunity in BM cells which provides further evidence for my IDT model.

In collaboration with Dr. Darragh Duff's group, I have also been able to utilise SIMOA assays to measure these changes in the whole blood and supernatant of human cells stimulated with WGP, which show increases in both IFN α , IFN β and IFN ω (Data not shown here). Therefore, I hypothesize that it is also possible that lower amounts of IFN (tonic IFN) promote training responses, while higher concentrations lead to inflammatory overload and tolerance.

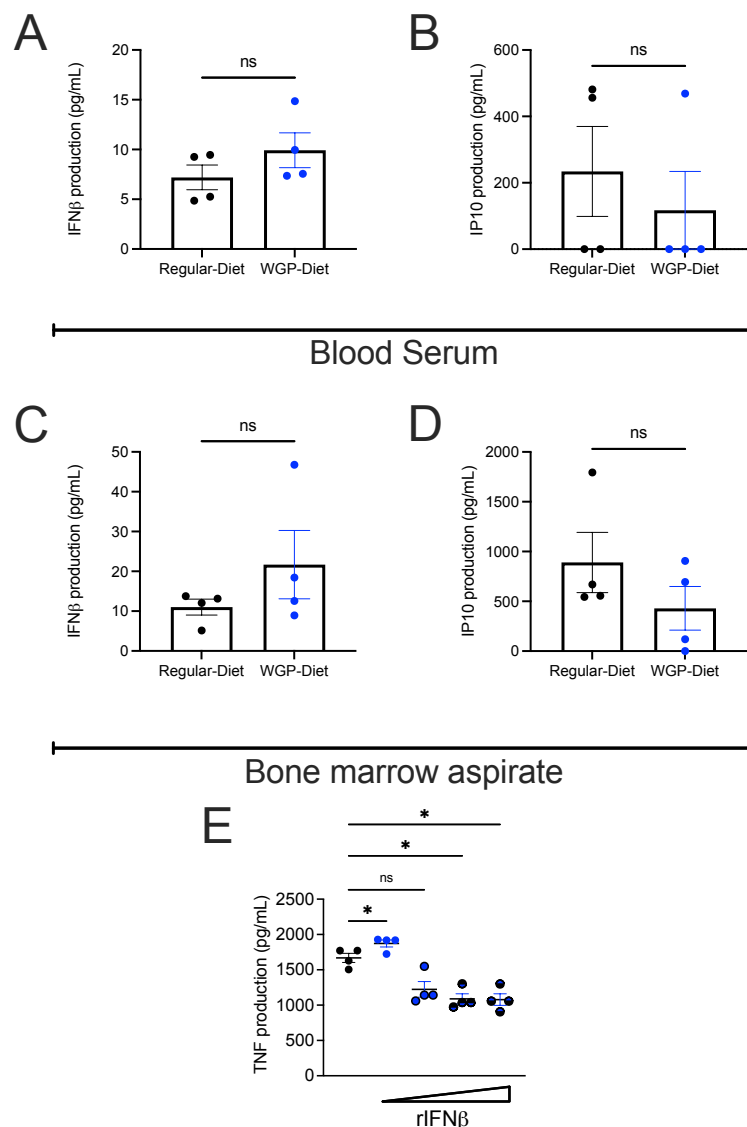


Figure 6.3 1-week dietary feeding induces a weak IFN signature in blood and BM aspirate, which is sufficient to induce a trained effect in-vitro

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section (A-D). Blood was drawn through the right ventricle of the heart using EDTA treated needle and collection tubes. Bone marrow aspirate was collected by flushing 500 μ L PBS through the tibias and femurs of each mouse. All supernatant was treated with phosphatase and protease inhibitors to prevent loss of cytokine during freezing. Protein concentration of IFN β (A) and IP-10 (B) in blood serum samples and IFN β (C) and IP-10 (D) in bone marrow aspirate as measured by ELISA. (E) Bone marrow was taken from regular diet fed mice and treated with four different concentrations of recombinant IFN β (1pg/mL to 1,000pg/mL). Cells were washed after 24hrs and allowed to differentiate into macrophages over 6 days. On day 6 cells were stimulated with pIC for 4hrs and levels of TNF were measured by ELISA. Each dot is a representative of an individual experiment (Biological N = 4). NS = P value >0.05 (Unpaired T-test with Welch's correction).

6.2.3 1-Week WGP feeding induces changes in IFN production by CD169+ macrophages, but not pDCs in the bone marrow

Other groups have shown that IP delivery of β -glucan causes an enhancement of IFN signalling in the bone marrow through plasmacytoid dendritic cells (pDCs)¹¹⁹. Therefore, I wanted to test whether the weak interferon signal I had detected in the bone marrow was being induced by mature bone marrow cells, such as either pDCs or CD169+ macrophages which have been shown to induce interferon in response to β -glucan²⁶².

I first decided to investigate pDCs. I used a similar gating strategy to Kalafati *et al*, who described pDCs as CD45+Lin-CD11b-CD11c+B220+BST2+. I first examined if 1-week WGP feeding induced changes in the frequency or cell number of pDCs, all which could play a role in changing IFN levels in the bone marrow. I did not see any significant changes in total pDC cell numbers in the bone marrow (**Fig 6.04A**). Similarly, I also didn't measure any significant increase in the relative percentage frequency of pDCs within the bone marrow (**Fig 6.04B**). Taken together this data indicates that 1-week WGP feeding does not alter pDC populations within the bone-marrow.

Having seen that 1-week WGP feeding has no impact on pDC populations, I next examined if there were functional changes in their ability to produce type I IFN. To do this I stained bone marrow for intracellular IFN α production using a FITC conjugated antibody. I measured both the percentage positive pDCs within the bone marrow as well as the mean fluorescent intensity (MFI) of pDCs. I did not see any significant differences between IFN α percentage positive pDCs in either diets (**Fig 6.05A**). Similarly, there was no significant difference between MFIs of regular diet or WGP diet fed mice (**Fig 6.05B**). This was also shown by the histogram plot with no real change between regular diet and WGP diet (**Fig 6.05C**). Taken together this data indicates that 1-week WGP feeding had no impact on pDC populations or IFN production within the bone marrow.

Next, I investigated changes in cell number and frequency of CD169+ macrophages. These macrophages have been identified to be both sensitive to IFN

signalling as well as being drivers of IFN^{263, 264}. Similar to the pDC results there were no significant differences in either cell number (**Fig 6.06A**) or relative frequency of these cells in the bone marrow (**Fig 6.06B**). Therefore, WGP feeding does not induce alterations in these cell types.

Having observed that 1-week WGP feeding has no effect on either cell number nor frequency of CD169+ macrophages, I examined whether the percentage positivity for cells expressing IFN α altered as well as the MFI of these positive cells. 1-week of WGP feeding caused a significance increase in the percentage of CD169+ macrophages that were positive for IFN α (**Fig 6.07A**). However, the MFI of these cells were not significantly different to the regular diet (**Fig 6.07B**). This suggest that while each individual cell is producing the same amount of IFN α , overall, there are more CD169+ macrophages producing IFN α within the bone marrow compartment in mice fed β -glucan WGPs.

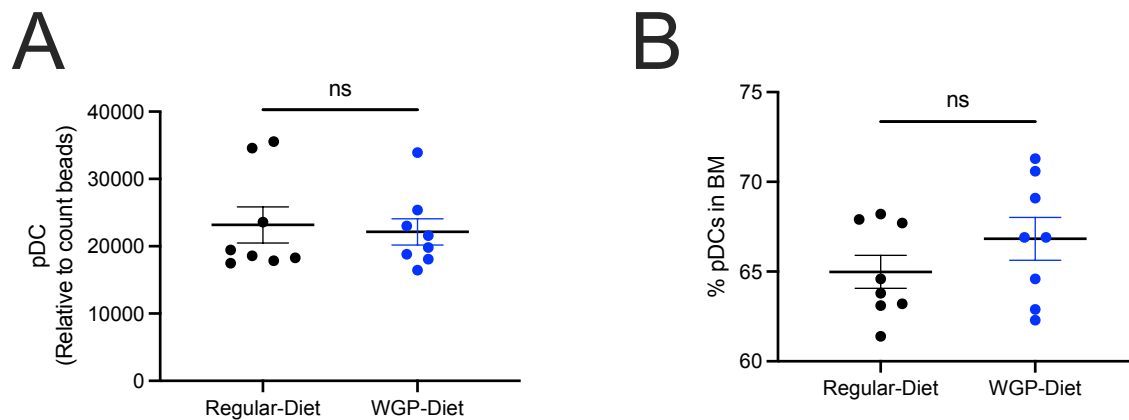


Figure 6.4 Flow analysis of pDC cell number and relative frequency within the bone marrow in regular diet and WGP diet fed animals

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and stained for flow analysis. pDC populations were gated on, and count beads were used to normalise cell number. (A) Graph shows cell number of pDCs (normalised to count beads) and (B) relative frequency of pDCs within the bone marrow. (Biological N = 14). NS = P value >0.05 (Unpaired Mann-Whitney T-test post normalisation testing).

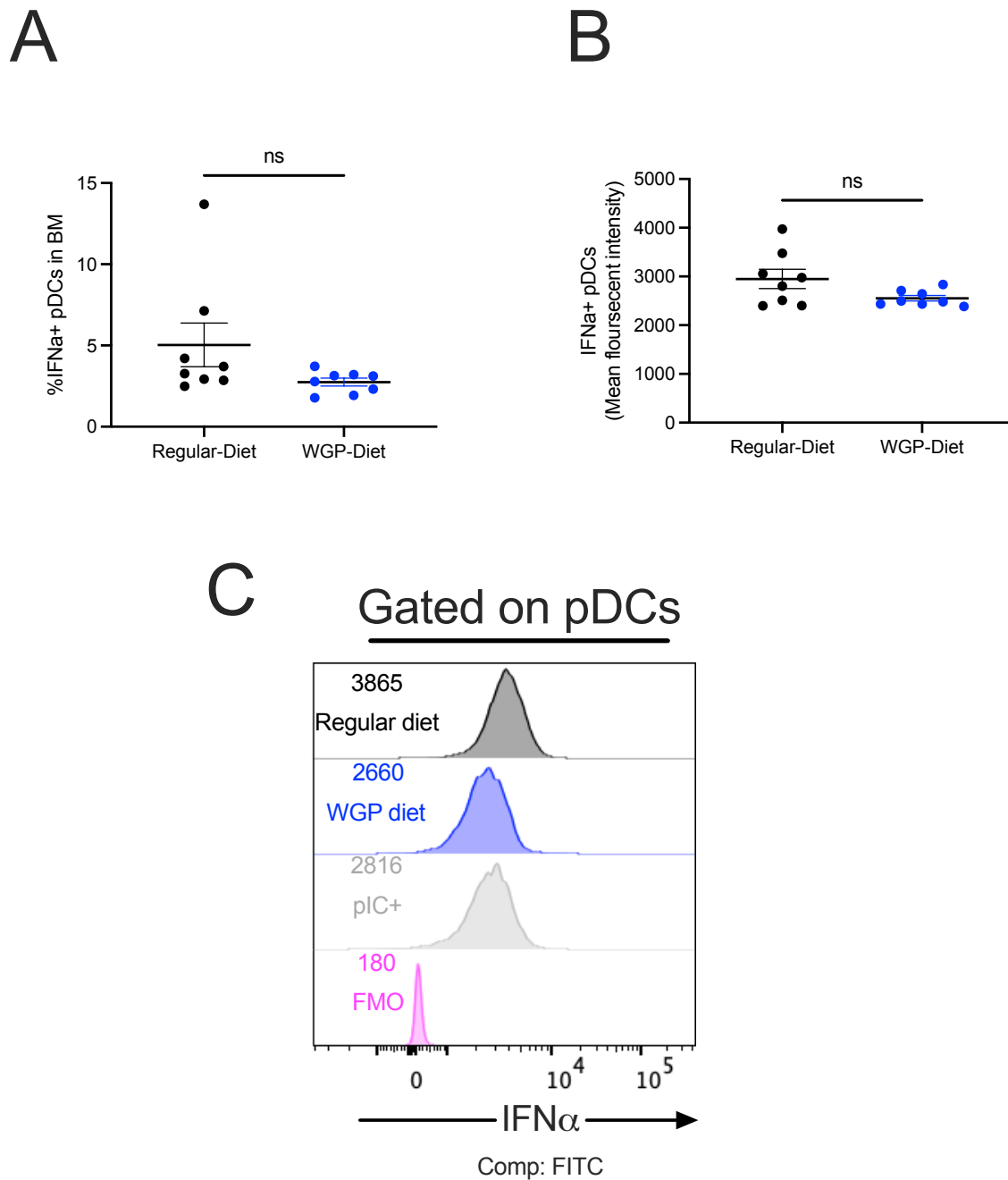


Figure 6.5 Flow analysis of IFN α producing pDC cells within the bone marrow in regular diet and WGP diet fed animals

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and stimulated with golgi block for a period of 4hrs to prevent cytokine release. (A) Graph shows relative frequency of IFN α producing pDCs and (B) mean fluorescent intensity (MFI) of IFN α staining. Representative histogram of regular diet (black), WGP diet (Blue), cells stimulated with pIC as a positive control (grey) and fluorescence minus one control (pink). (Biological N = 8). NS = P value >0.05 (Unpaired Mann-Whitney T-test post normalisation testing).

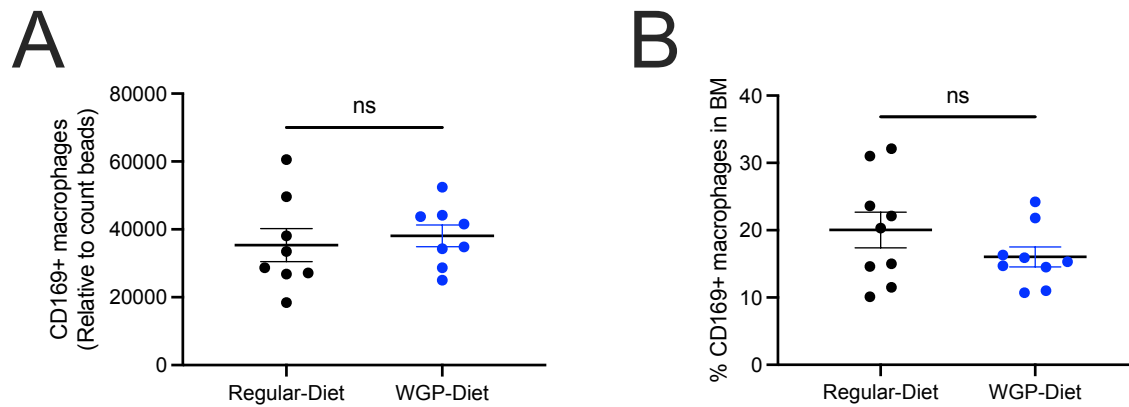


Figure 6.6 Flow analysis of CD169+ macrophage cell number and relative frequency within the bone marrow in regular diet and WGP diet fed animals

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and stained for flow analysis. CD169+ macrophage populations were gated on, and count beads were used to normalise cell number. (A) Graph shows cell number of CD169+ macrophages (normalised to count beads) and (B) relative frequency of CD169+ macrophages within the bone marrow. (Biological N = 8). NS = P value >0.05 (Unpaired Welch's T-test post normalisation testing).

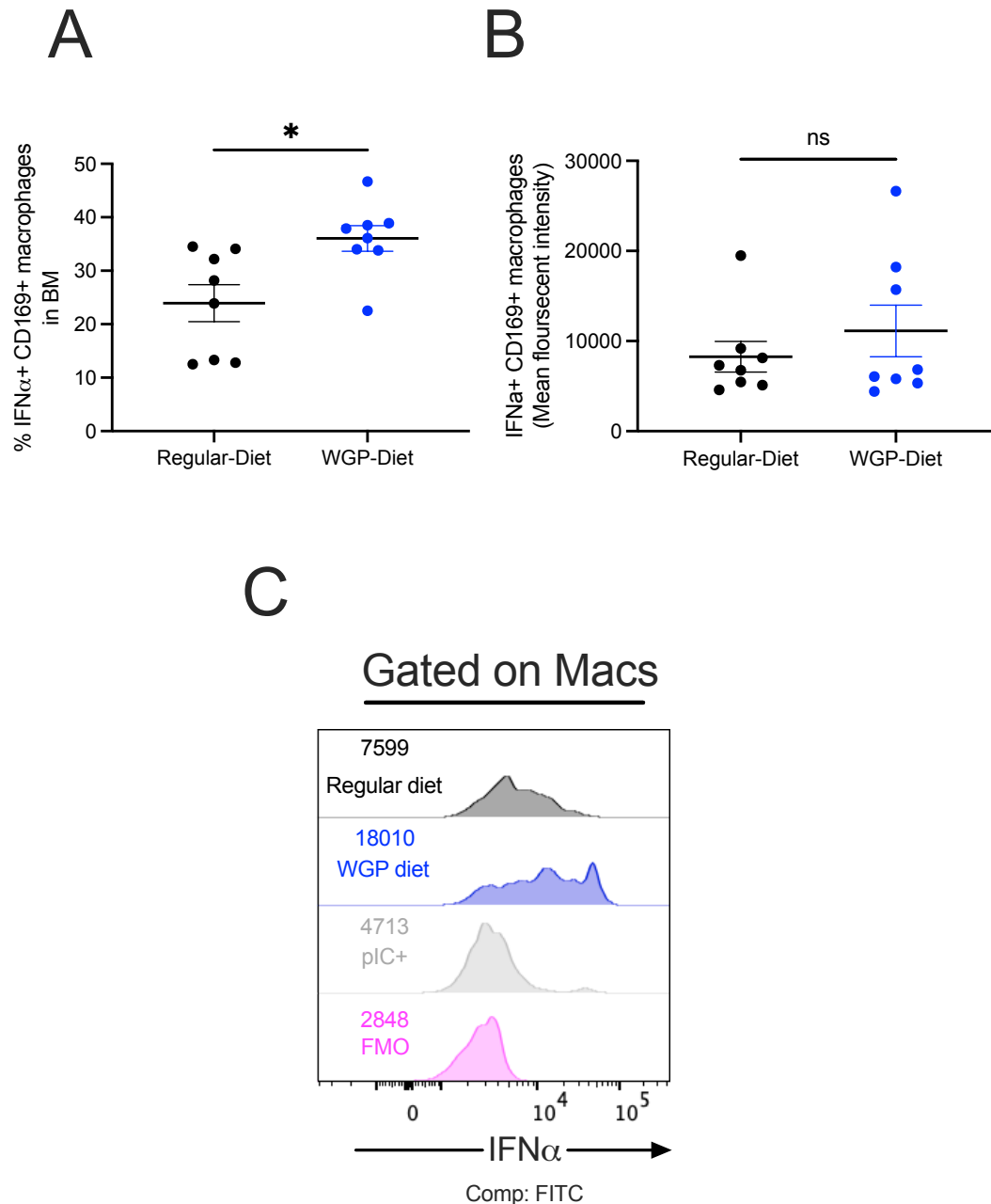


Figure 6.7 Flow analysis of IFN α producing CD169+ macrophages within the bone marrow in regular diet and WGP diet fed animals

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and stimulated with golgi block for a period of 4hrs to prevent cytokine release. (A) Graph shows relative frequency of IFN α producing CD169+ macrophages and (B) mean fluorescent intensity (MFI) of IFN α staining. (C) Representative histogram of regular diet (black), WGP diet (Blue), cells stimulated with pIC as a positive control (grey) and fluorescence minus one control (pink). (Biological N = 8). NS = P value >0.05, *P-value <0.05; ((A) Unpaired T-test with Welch's correction and (B) Unpaired Mann-Whitney T-test post normalisation testing).

6.2.4 1-Week WGP feeding induces changes in the relative proportion of bone marrow progenitor cell subtypes

It has been shown that increases in bone marrow interferon signalling causes changes in bone marrow progenitors with expansion of specific haematopoietic stem and progenitor cells (HSPCs)^{119, 262}. Therefore, I also decided to investigate changes in proportions of progenitor cells in the bone marrow. The first subset of cells I measured were the total HSCs, defined by gating as lineage-Sca-1+cKit+ (LKS+). Feeding mice WGP diet for 1 week led to a significant increase in the relative percentage of these cells within the bone marrow (**Fig 6.08A**). Since I saw these increases, I wanted to understand what cells might be affecting this increase. Within the HSPC populations are long-term (LT) HSCs, short-term (ST) HSCs and multi-potent progenitor cells (MPPs). Interestingly, there was a significant reduction in the frequency of LT-HSCs (**Fig 6.08B**) with a significant increase in both ST-HSCs (**Fig 6.08C**) and MPP (**Fig 6.08D**) populations within the total bone marrow. Taken together this data indicates that 1-week WGP feeding can induce a phenotypical switch in the bone marrow compartment, with more pluripotent stem-cells committing to differentiation.

Next, I decided to investigate the more lineage biased cell populations within the bone marrow. The first group I looked at were the MPP3 and MPP4 cell populations, which represent myeloid and lymphoid biased cells respectively. Interestingly, 1-week WGP feeding also caused a significant increase in the proportion of MPP3 (**Fig 6.09A**) and no significant differences in the MPP4 population (**Fig 6.09B**) within the bone marrow compartment. I also measured the frequency of more committed cell progenitors such as common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). Again, surprisingly 1-week of WGP feeding was enough to cause a relative proportional expansion of both CLPs (**Fig 6.09C**) and CMPs (**Fig 6.09D**) within the total bone marrow. Taken together this data indicates that 1-week WGP feeding is capable of inducing shifts in committed progenitor cells with no bias for either myeloid or lymphoid cell types.

Having seen that 1-week WGP feeding can induce changes in the relative proportions of HSPCs I next wanted to investigate the impact type I IFN has on

these changes. It has been shown that a member of the Ly6 family, Sca-1, is sensitive to type I interferon signalling and its up-regulation causes expansion of various HSPCs within the bone marrow^{265, 266}. Therefore, to test this I investigated the MFI of Sca-1 in the different HSPCs in my two diets. Firstly, I saw that Sca-1 expression did not significantly change between diets within the total HSPCs or LKS+ cells (**Fig 6.10A**). However, there was a bigger, though non-significant change when I looked at the expression of Sca-1 in LT-HSCs (**Fig 6.10B**). Interestingly, this was not the case for the ST-HSC population with no significant differences between the two diets (**Fig 6.10C**). Finally, I did see that Sca-1 expression was significantly increased in the MPP population in mice that had been fed WGP for 1-week (**Fig 6.10D**). Taken together this data indicates that WGP feeding enhances Sca-1 expression in specific HSPC subsets. Indicative of type 1 interferon signalling.

Next, I decided to investigate the more lymphoid or myeloid committed progenitors. Interestingly even though the overall MFI of the MPP group was significantly increased there was no significant changes in the MFI of Sca-1 between diets in both MPP3 or MPP4 progenitors (**Fig 6.11A-B**). However, a very interesting results was that in committed progenitors such as CMPs and CLPs there were significant, and trending increases in the MFI of Sca-1 after 1-week of WGP feeding in both cell types (**Fig 6.11C-D**). Taken together this data indicates that 1-week WGP feeding is significant enough to enhance the expansion of the ISG Sca-1 in myeloid and lymphoid committed progenitor cells.

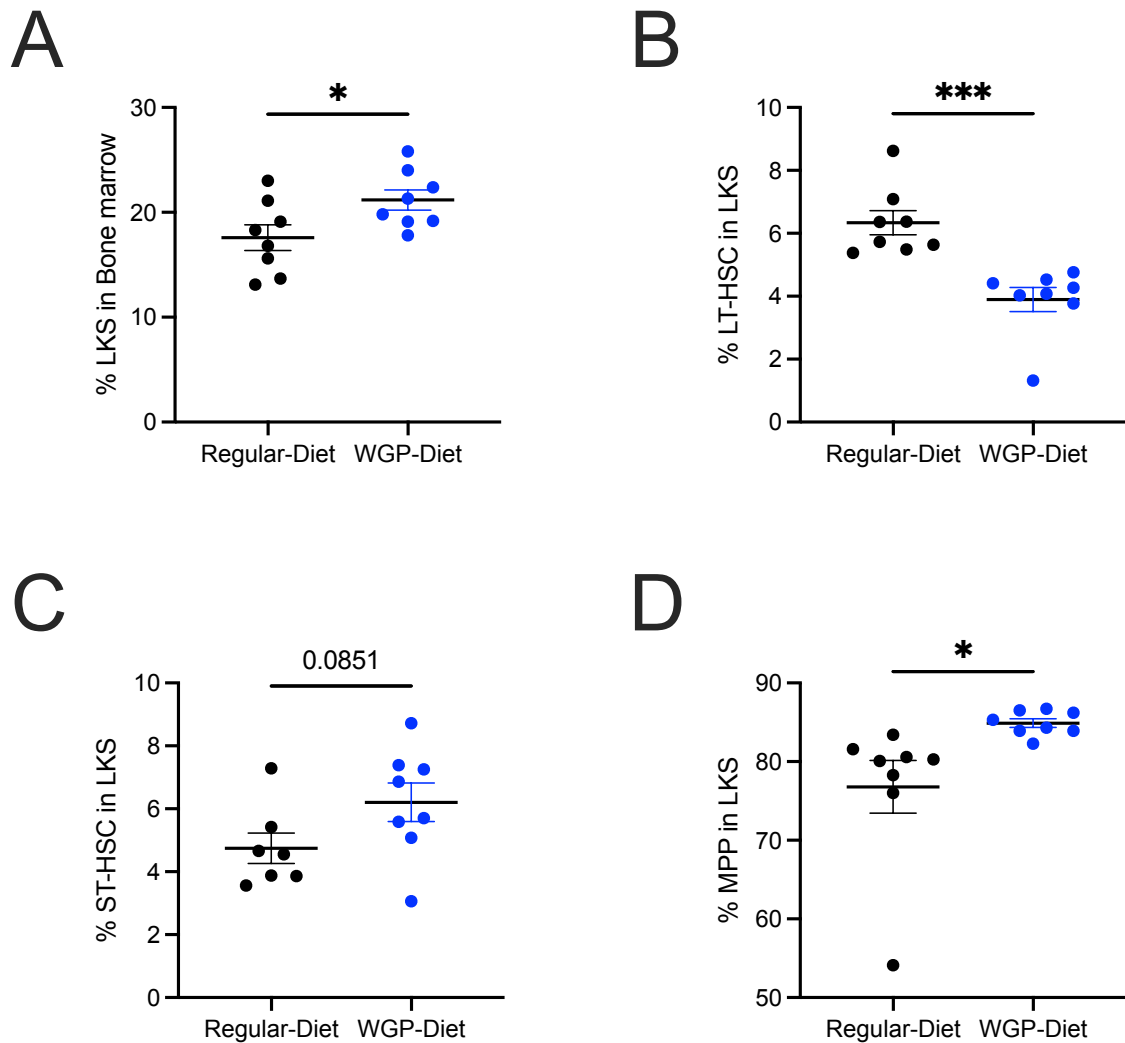


Figure 6.8 Flow analysis showing relative proportions of bone marrow stem cells (HSCs) 1-week post WGP feeding

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and processed and stained for flow cytometry analysis. (A) Graph shows relative proportions of Lineage-cKIT+Sca-1+ (LKS) cells, (B) long-term haematopoietic stem cells (LT-HSC), (C) short term haematopoietic stem cells (ST-HSC) and (D) multi-potent progenitor cells (MPPs) within the bone marrow. (Biological N = 8). *P-value < 0.05, **P-value < 0.005, ***P-value < 0.001; (Unpaired T-test with Welch's correction (A, C & D) and Unpaired T-test with Mann-Whitney correction (B) post normalisation testing). *Experiment performed with Dr. Anna Ledwith.*

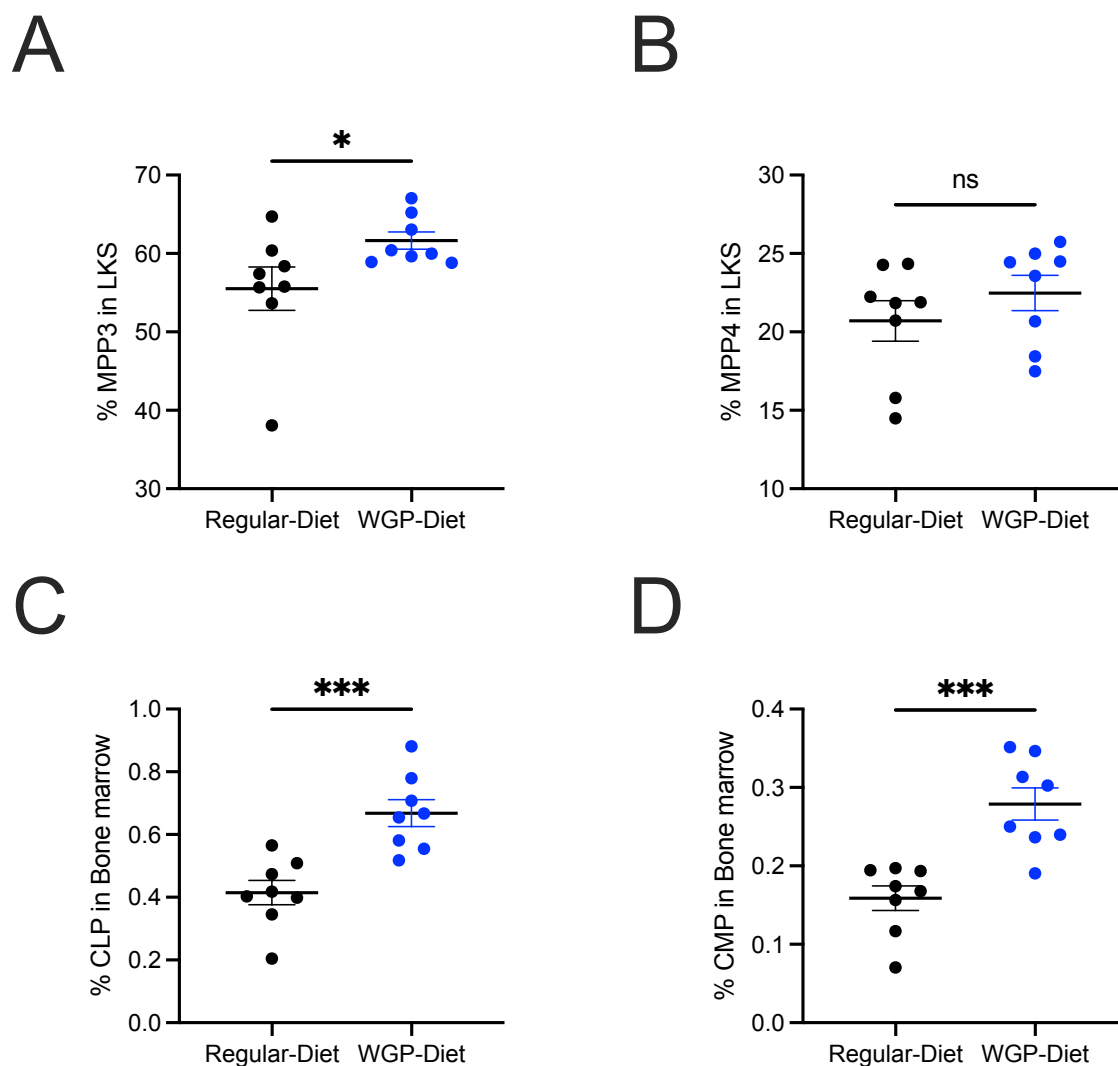


Figure 6.9 Flow analysis showing relative proportions of bone marrow progenitor cells (HPCs) 1-week post WGP feeding

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and processed and stained for flow cytometry analysis. (A) Graph shows relative proportions of MPP3, (B) MPP4, (C) common lymphoid progenitor cells (CLPs) and (D) common myeloid progenitor cells (CMPs) within the bone marrow. (Biological N = 8). *P-value <0.05, ***P-value <0.001; (Unpaired T-test with Mann-Whitney test (A) or Welch's test (B-D) post normalisation testing). *Experiment performed with Dr. Anna Ledwith.*

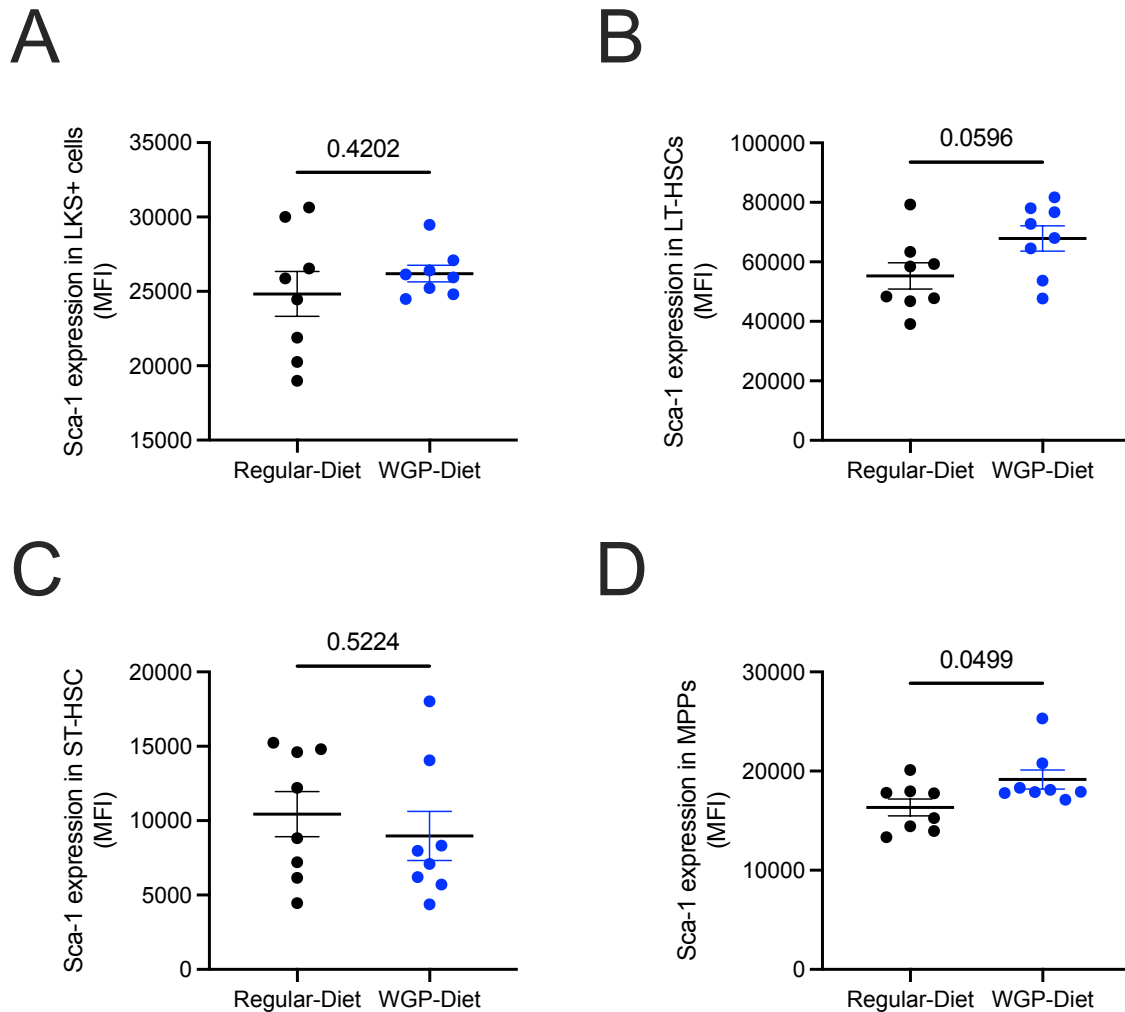


Figure 6.10 Flow analysis showing changes in Sca-1 expression (MFI) in HSC populations 1-week post WGP feeding

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and processed and stained for flow cytometry analysis. (A) Graph shows mean fluorescent intensity (MFI) of Sca-1 in Lineage-cKIT+Sca-1+ (LKS) cells, (B) long-term haematopoietic stem cells (LT-HSC), (C) short term haematopoietic stem cells (ST-HSC) and (D) multi-potent progenitor cells (MPPs) within the bone marrow. (Biological N = 8). Exact P-value shown; (Unpaired T-test with Welch's correction (A, B & D) and Unpaired T-test with Mann-Whitney correction (C) post normalisation testing).

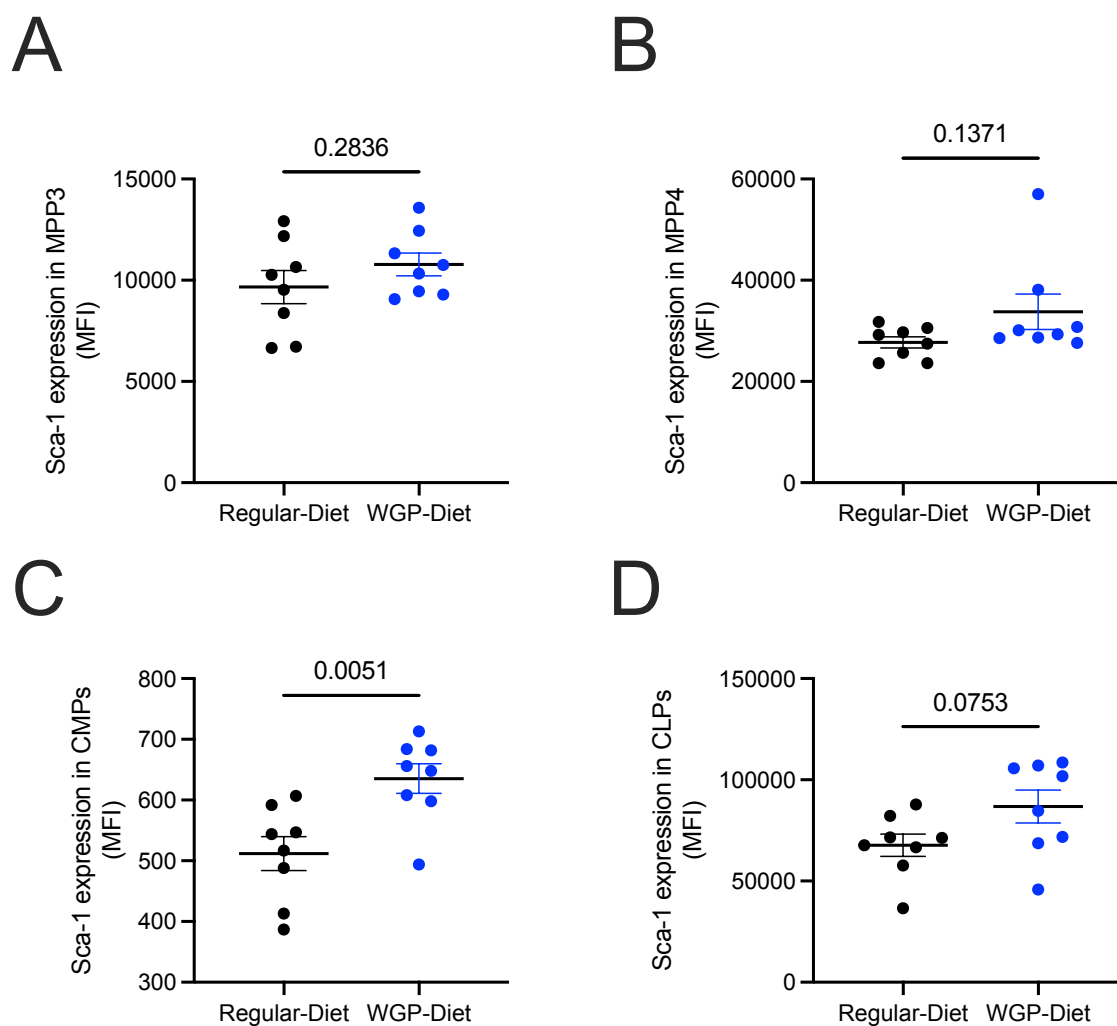


Figure 6.11 Flow analysis showing changes in Sca-1 expression (MFI) in bone marrow progenitor cells (HPCs) 1-week post WGP feeding

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and processed and stained for flow cytometry analysis. (A) Graph shows mean fluorescent intensity (MFI) of Sca-1 in MPP3, (B) MPP4, (C) common lymphoid progenitor cells (CLPs) and (D) common myeloid progenitor cells (CMPs) within the bone marrow. (Biological N = 8). Exact P-value shown; (Unpaired T-test with Welch's correction post normalisation testing).

6.2.5 1-Week WGP feeding induces changes in the metabolic phenotype of specific HSPC subsets

Since I had seen changes in the expansion of bone marrow stem and progenitor cells I wanted to investigate if these cells had an altered metabolic phenotype. It has been suggested that systemic changes in IFN levels can induce heightened HSPC metabolic effects post IP injection of β -glucan¹¹⁹. Since these authors saw heightened glycolytic reprogramming of these HSPCs, particularly LT-HSCs, I first decided to analyse the expression of GLUT1 via flow cytometry¹¹⁹. Here, I investigated changes in the MFI of glut-1 in the LKS+ HSC compartment (Total LKS+ cells, LT-HSC, ST-HSC and MPPs). Surprisingly, there was a significant reduction in the MFI of glut-1 in the LKS+ compartment of cells taken from animals that had been fed a WGP diet for 1-week (**Fig 6.12A**). This led me to investigate the individual components of the LKS+ cells. Interestingly, there was also a reduction in the MFI of glut-1 in LT-HSC population (**Fig 6.12B**). In contrast, there were no significant differences observed in the MFI of glut-1 in the ST-HSC population (**Fig 6.12C**). However, again like the LT-HSC population the MPPs also had a significant reduction in the MFI of glut-1 (**Fig 6.12D**). To make sure that these effects weren't due to cell size differences I normalized the MFI of glut-1 to the MFI of FSC-H for each cell type (Data not shown) and saw that this had no effects on the results I have shown. Taken together this data indicates that 1-week WGP feeding leads to altered expression of glut-1 independent of cellular size.

To further investigate these changes, I saw I next decided to analyse the expression of glut-1 within the MPP populations, MPP3 and MPP4, as well as within the CLP and CMP populations. As was seen with the total MPPs, there were significant reduction in the MFI of glut-1 in both the MPP3 and MPP4 populations, suggesting that not one population was the cause of this reductive shift (**Fig 6.13A-B**). I next investigated the more committed progenitor populations; both CMPs and CLPs (**Fig 6.13C-D**). I saw that similar to the stem cells both these populations had reduced MFI of glut-1. This suggested that changes in glut-1 expression were not just restricted to early progenitors but affected the entire HSPC niche.

To further analyse these changes in glut-1 expression I decided to undertake a deeper analysis of the metabolic phenotype of the bone marrow compartment. To do this I utilised a modern flow-based assay, known as single cell energetic metabolism by profiling translation inhibition (SCENITH), and taking advantage of click-chemistry to perform the flow analysis. The protocol is shown in **Fig 6.14**, however, briefly bone-marrow cells were enriched for HSPCs using cKIT⁺ selection and after cells were plated in 96-well plates and incubated +/- oligomycin or 2DG for 30 mins. After this timepoint cells were incubated with puromycin, which is taken up by cells and incorporated into ribosomes or newly synthesised proteins which has been shown to be relative to their energetic complexity²⁶⁷. Cells were then stained for flow and fixed using PFA. After 24hrs, an Alexa flour-azide was then chemically “clicked” to the puromycin, and samples were measured via flow.

I first analysed the HSC populations, including total LKS⁺ cells, LT-HSCs and ST-HSCs. In a separate plate no puromycin was added to cells to control for quiescence and act as a click FMO. Within the LKS⁺ population there was a significant difference between the no puromycin and the puromycin only groups for both regular diet and WGP diet fed mice (**Fig 6.15A**). Overall, there was no significant differences between the regular diet and WGP diet groups, although WGP fed animals seemed to have slightly less MFI of puromycin. The most interesting differences however was that there was a significant reduction in puromycin MFI with 2DG inhibition for both regular diet and WGP diet versus their relative controls. However, LKS⁺ cells were more sensitive to glycolytic inhibition since they were significantly lower than the regular diet group. Oligomycin did not seem to have any effect on puromycin MFI in either group. This suggests that LKS⁺ cellular metabolism is dependent on glycolysis over oxphos and that LKS⁺ cells from WGP fed mice are more dependent on glucose metabolism than regular diet fed mice.

Next, I investigated these changes at a deeper cellular level. First, I tested whether these changes occurred at the LT-HSC level (**Fig 6.15B**). Interestingly, there were no significant differences between puromycin incubated and no puromycin cells for both regular diet and WGP diet suggestive of a metabolically quiescent phenotype. Inhibition with 2DG as well as oligomycin overall had no significant effect on these cells. Next, I analysed the ST-HSC population (**Fig 6.15C**). Again, as seen with the

LT-HSC population there were no significant differences between diets or between the puromycin and no puromycin groups again suggesting metabolic quiescence. However, there was a significant reduction in puromycin MFI between the no inhibitor and 2DG inhibited ST-HSCs from WGP fed mice, suggesting that WGP feeding led to a more glucose reliant ST-HSC.

Since I had seen that the LT-HSC and ST-HSC groups were relatively metabolically quiescent I next decided to investigate more progenitor committed populations. I firstly investigated the total MPP populations (**Fig 6.16A**). Unlike the previous populations there were significant differences between the puromycin incubated and no puromycin cells, showing that these cells had a level of metabolic activity. There was a non-significant reduction in the metabolic profile of MPPs from WGP fed mice as shown by reduced puromycin MFI. Both groups had significant reductions in metabolic activity when inhibited with 2DG, but oligomycin had no effect on the MFI of puromycin. Interestingly, MPPs from WGP fed mice had reduced puromycin MFI relative to MPPs from regular diet fed mice. Therefore, these cells were more sensitive to 2DG inhibition suggesting that they are more dependent on glucose metabolism. To further quantify this, I also analysed the committed MPPs. Firstly, MPP3s incubated with puromycin had a significant increase in their MFI relative to no puromycin incubation in both diets (**Fig 6.16B**). 2-DG inhibition also significantly reduced puromycin MFI in both diets showing that these cells are sensitive to glycolytic inhibition. However, unlike in the total MPPs and LKS⁺ cells, there were no significant differences between either diets, suggesting the MPP3 cells from both diets are equally sensitive to glycolytic inhibition. Finally, I also analysed the MPP4 population (**Fig 6.16C**). Like what I saw with the previous MPP populations both groups were metabolically active with increased puromycin MFI relative to the no puromycin control. Again, there was a non-significant reduction between the MPP4s from the regular diet and WGP diet fed mice in the no inhibitor group. However, interestingly only MPP4s from WGP fed mice were sensitive to 2DG inhibition with a significant reduction relative to the no inhibitor control. Further these cells were also more sensitive to glycolytic inhibition since they also had a significant reduction relative to the MPP4 cells treated with 2DG from regular diet fed mice. Overall, this data suggests that within the LKS⁺ population the MPPs are the major metabolically active cells and that

WGP MPP4s within the MPP population have a higher dependence on glucose metabolism versus MPP4s from regular diet fed mice.

Having seen metabolic changes in the biased MPP populations I decided to investigate metabolic changes in committed progenitors such as CLPs and CMPs. Firstly, I saw no significant differences between the MFIs of the no puromycin and the puromycin no inhibitor control in either diet within the CLPs (**Fig 6.17A**). This again may suggest a metabolically quiescent cellular type. 2DG also had no significant effect on CLPs from WGP fed mice. However, 2DG inhibition significantly reduced puromycin MFI in CLPs from regular diet fed mice. This was reflected also in a significant difference between the 2DG treated CLP regular diet and WGP diet fed mice. Oligomycin had no significant changes in either group. I next investigated the CMPs (**Fig 6.17B**). CMPs from both regular diet and WGP diet fed mice had significant differences in the puromycin incubated and no puromycin incubated groups once again suggesting a metabolically active cell type. 2DG also caused a significant reduction in puromycin MFI in both diets. However, 2DG caused significantly greater inhibition of puromycin MFI once again suggesting that CMPs from WGP fed mice were more metabolically dependent on glucose than CMPs from regular diet fed mice. Oligomycin had no significant effects on both diets.

Taken together this data indicates that although WGP feeding induces changes in the MFI expression of glucose transporter such as glut-1, overall HSPCs from WGP fed mice have no enhanced metabolic activity but have been metabolically altered to rely more heavily on glucose metabolism suggesting a metabolic shift induced by WGP feeding, which is consistent across multiple populations of cells in the bone marrow.

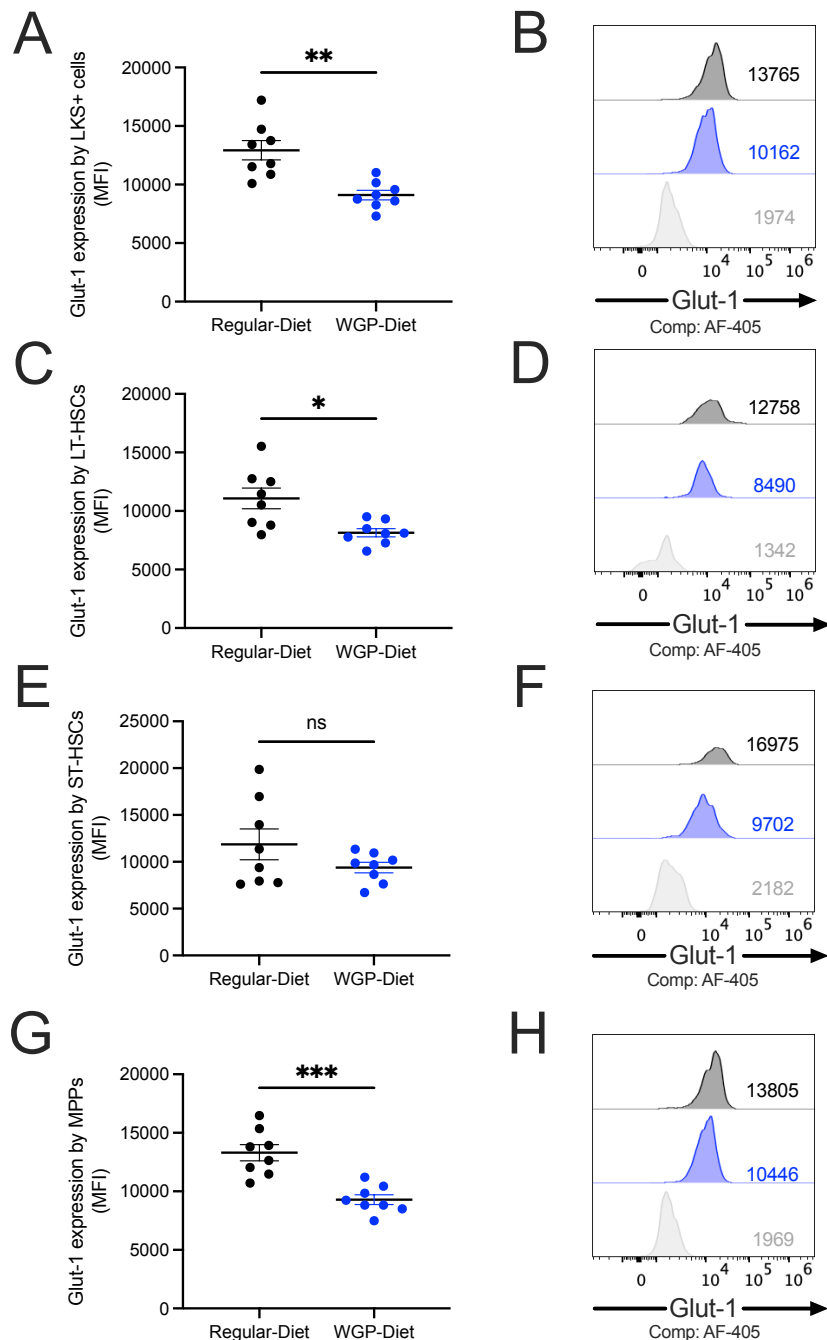


Figure 6.12 Flow analysis showing changes in Glut-1 expression (MFI) in bone marrow HSCs 1-week post WGP feeding

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and processed and stained for flow cytometry analysis. (A) Graph shows mean fluorescent intensity (MFI) of Glut-1 in Lineage-cKit+Sca-1+ (LKS) cells, (B) long-term haematopoietic stem cells (LT-HSC), (C) short term haematopoietic stem cells (ST-HSC) and (D) multi-potent progenitor cells (MPPs) within the bone marrow. (Biological N = 8). *P-value <0.05, **P-value <0.005 or ***P-value <0.001; (Unpaired T-test with Welch's correction post normalisation testing). *Experiment performed with Dr. Anna Ledwith.*

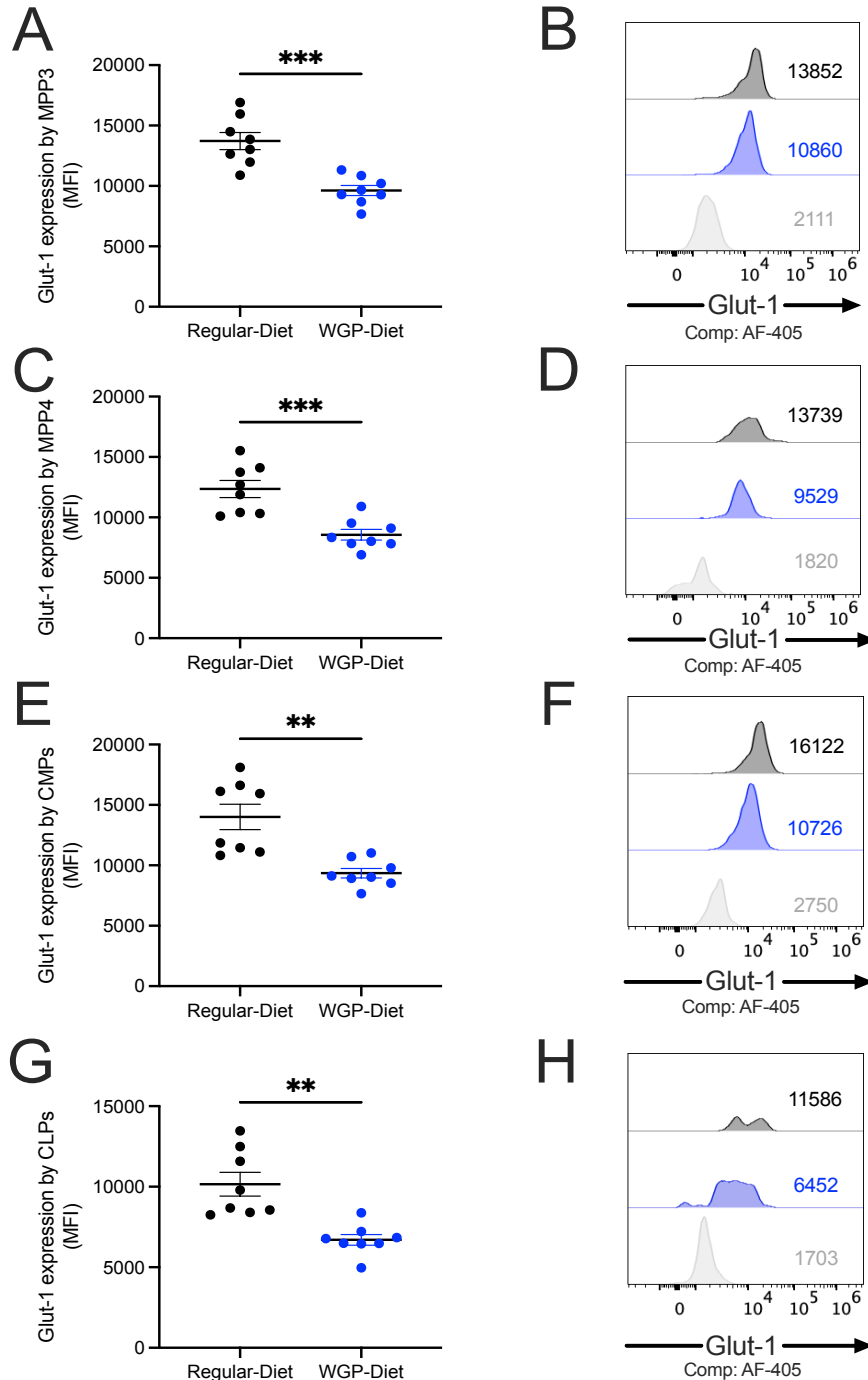


Figure 6.13 Flow analysis showing changes in Glut-1 expression (MFI) in bone marrow progenitor cells (HPCs) 1-week post WGP feeding

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and processed and stained for flow cytometry analysis. Graph shows mean fluorescent intensity (MFI) of Sca-1 in MPP3 (A), MPP4 (B), common lymphoid progenitor cells (CLPs) (C) and common myeloid progenitor cells (CMPs) (D) within the bone marrow. (Biological N = 8). **P-value < 0.005 or ***P-value < 0.001; (Unpaired T-test with Welch's correction post normalisation testing). *Experiment performed with Dr. Anna Ledwith.*

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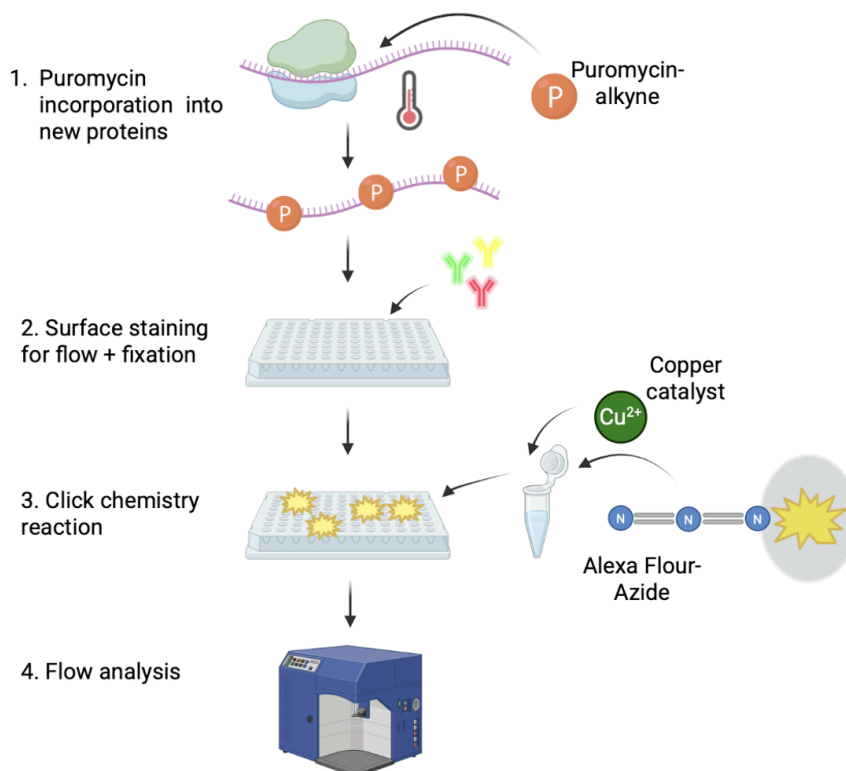


Figure 6.14 Graphical description of puromycin incorporation and click-chemistry reaction

Puromycin has been shown to be incorporated into protein synthesis which is tightly related to metabolic turnover of cells²⁶⁷. Once puromycin has been incorporated into cells, these were stained with flow antibodies as normal and then fixed using PFA. Prior to flow cytometry analysis, click-chemistry was performed where an Alexa flour-azide was attached to the puromycin in the cells using a copper catalyst. Once cells had been clicked standard flow analysis was performed on the Cytex Aurora.

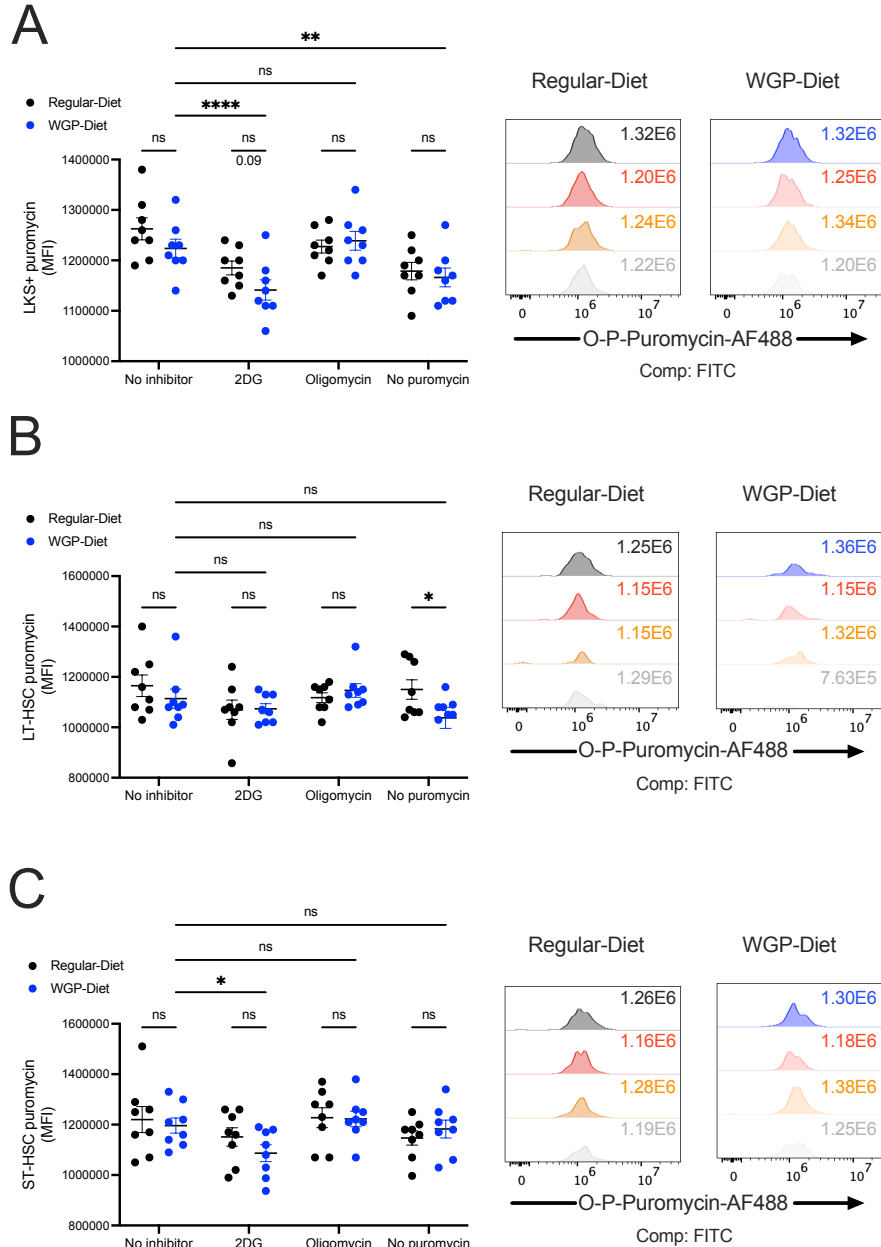


Figure 6.15 Click-chemistry metabolic flow analysis of HSCs 1-week post regular or WGP supplement diets

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted, incubated for 30 mins with $1\mu\text{M}$ oligomycin or 100mM 2DG, followed by another 30 min incubation with puromycin. After which, cells were processed and stained for flow cytometry analysis. (A) Graph shows mean fluorescent intensity (MFI) of puromycin uptake in LKS+ cells, (B) LT-HSCs or (C) ST-HSCs as well as the relative histograms. No puromycin was used as a negative control to account for certain HSCs may exhibit a metabolically quiescent state. (Biological N = 8). No significance (NS) = P-value > 0.05, *P-value < 0.05, **P-value < 0.005 or ***P-value < 0.001; (Paired 2-Way ANOVA testing with Šídák's multiple comparison test, following normalisation testing). *Experiment performed with Dr. Anna Ledwith.*

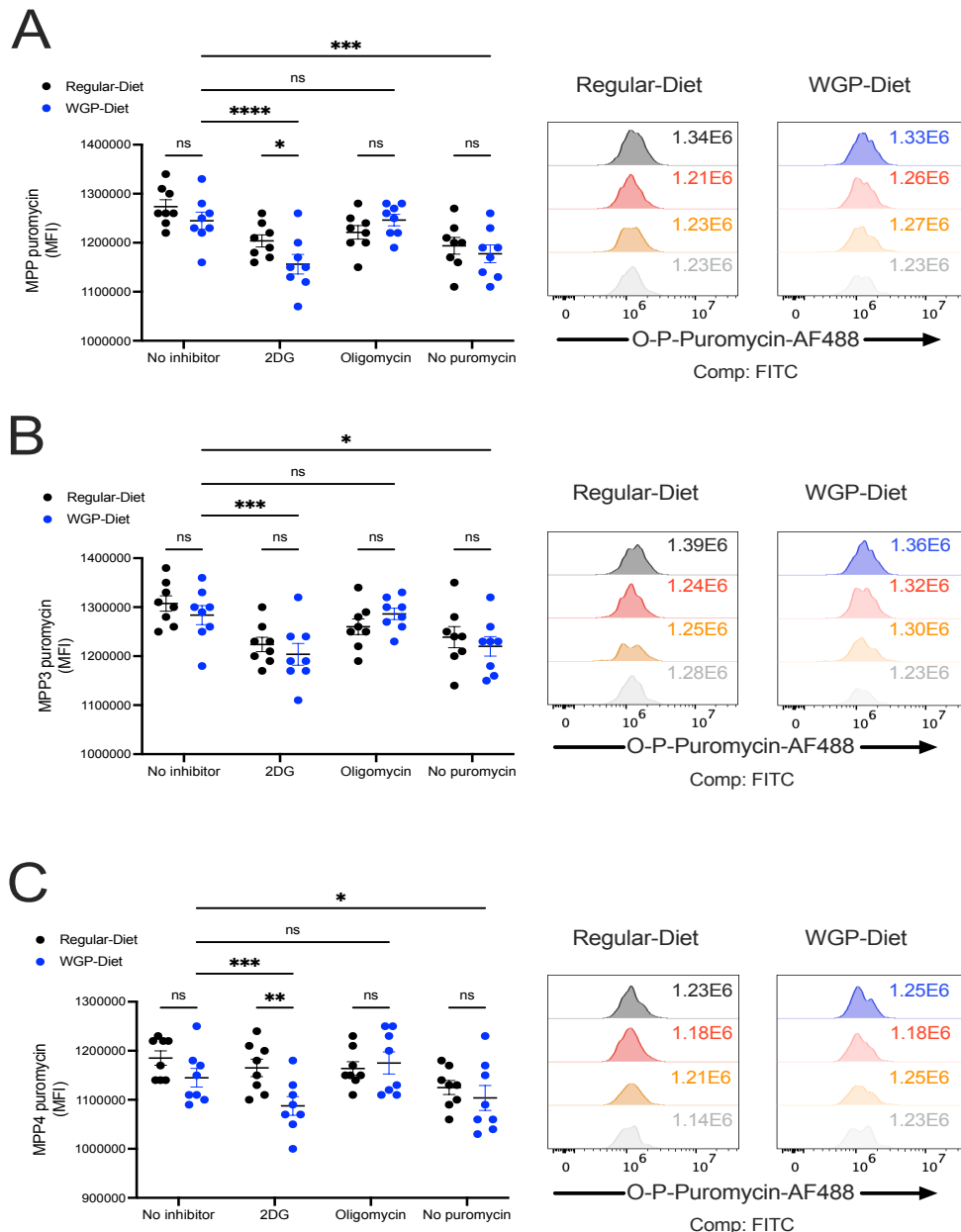


Figure 6.16 Click-chemistry metabolic flow analysis of MPPs 1-week post regular or WGP supplement diets

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted, incubated for 30 mins with 1 μ M oligomycin or 100mM 2DG, followed by another 30 min incubation with puromycin. After which, cells were processed and stained for flow cytometry analysis. (A) Graph shows mean fluorescent intensity (MFI) of puromycin uptake in total MPPs, (B) MPP3s or (C) MPP4s as well as the relative histograms. No puromycin was used as a negative control to account for certain HSCs may exhibit a metabolically quiescent state. (Biological N = 8). No significance (NS) = P-value > 0.05, *P-value < 0.05, **P-value < 0.005, ***P-value < 0.001 or ****P-value < 0.0001; (Paired 2-Way ANOVA testing with Šídák's multiple comparison test, following normalisation testing). *Experiment performed with Dr. Anna Ledwith.*

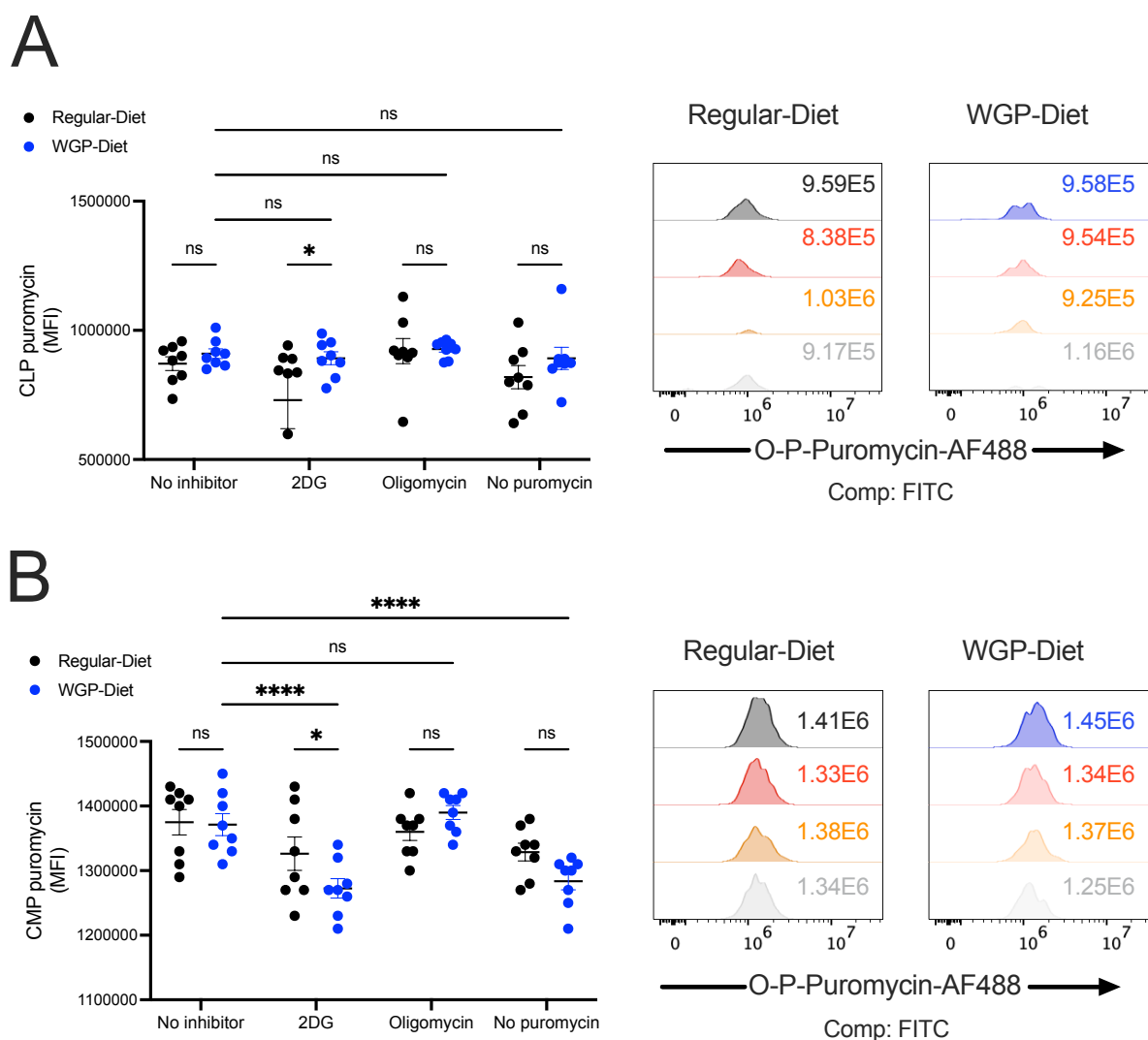


Figure 6.17 Click-chemistry metabolic flow analysis of CLPs and CMPs 1-week post regular or WGP supplement diets

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 1-week. After the 1 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted, incubated for 30 mins with 1 μ M oligomycin or 100mM 2DG, followed by another 30 min incubation with puromycin. After which, cells were processed and stained for flow cytometry analysis. (A) Graph shows mean fluorescent intensity (MFI) of puromycin uptake in CLPs or (B) CMPs. No puromycin was used as a negative control to account for certain HSCs may exhibit a metabolically quiescent state. (Biological N = 8). No significance (NS) = P-value > 0.05, *P-value < 0.05 or ****P-value < 0.0001; (Paired 2-Way ANOVA testing with Šídák's multiple comparison test, following normalisation testing). *Experiment performed with Dr. Anna Ledwith.*

6.2.6 Bone marrow IFN expression is further enhanced after 4-weeks of WGP feeding

Since I had seen that IFN responses, bone marrow changes and metabolic changes occurred as early as 1-week post feeding I wanted to investigate if this effect would persist and would it be enhanced. WGP is sold as an oral supplement for human consumption often in packs for 4-weeks of consumption. Therefore, I decided to test whether 4-weeks of feeding led to enhanced responses. To test this, I again measured the level of IFN and inflammatory cytokines within the bone marrow aspirate (**Fig 6.18**). 4-weeks of WGP feeding lead to significant increase of IFN β within the bone marrow aspirate (**Fig 6.18A**). This showed that IFN signalling persisted with 4-weeks of feeding. I also measured the levels of the inflammatory ISG IP-10 (**Fig 6.18B**). There was no significant increase or trend in this cytokine after 4-weeks of WGP feeding. Taken together this data indicates that 4-weeks of WGP feeding leads to enhanced IFN levels within the bone marrow without impacting inflammatory cytokine expression such as IP-10.

IFN- β within Bone marrow aspirate (pg/mL)

Regular-Diet WGP-Diet

0.0556

Diet Group	IFN- β (pg/mL)
Regular-Diet	0
Regular-Diet	0
Regular-Diet	10
Regular-Diet	11
Regular-Diet	12
Regular-Diet	13
WGP-Diet	11
WGP-Diet	13
WGP-Diet	14
WGP-Diet	15
WGP-Diet	23

IP-10 within Bone marrow aspirate (pg/mL)

Diet Group	Mean (pg/mL)	Individual Data Points (pg/mL)
Regular-Diet	~410	~350, ~400, ~410, ~420, ~430, ~450
WGP-Diet	~420	~310, ~320, ~330, ~340, ~350, ~360, ~370, ~380, ~390, ~400, ~410, ~420, ~430, ~440, ~450, ~460, ~470, ~480, ~490, ~500, ~510, ~520, ~530, ~540, ~550, ~560, ~570, ~580, ~590, ~600, ~610, ~620, ~630, ~640, ~650, ~660, ~670, ~680, ~690, ~700, ~710, ~720, ~730, ~740, ~750, ~760, ~770, ~780, ~790, ~800, ~810, ~820, ~830, ~840, ~850, ~860, ~870, ~880, ~890, ~900, ~910, ~920, ~930, ~940, ~950, ~960, ~970, ~980, ~990, ~1000

0.3223

Regular-Diet WGP-Diet

Mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-week. After the 4 week feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted, and aspirate was collected as previously described. ELISA was performed on bone marrow aspirate to investigate levels of (A) IFN β or (B) IP-10. (Biological N = 5). Exact P-value shown on graph; (Mann-Whitney T test (A) and unpaired T test (B), post normalisation testing).

6.2.7 4-weeks WGP feeding leads to expansion of HSPCs in a type I interferon dependent manner

Since I had seen sustained IFN signalling after 4 weeks of WGP feeding I wanted to investigate the impact this might have on bone marrow haematopoiesis. It has been shown previously that chronic exposure to type I interferon leads to bone marrow exhaustion and loss of haematopoiesis²⁶⁸. Therefore, I predicted that 4 weeks WGP feeding would lead to altered bone marrow haematopoiesis in a type I interferon dependent manner. In order to test this hypothesis wild type and IFNAR knock out (IFNAR^{-/-}) mice were fed WGP for a period of 4 weeks and their bone marrow populations were analysed via flow cytometry. First, I investigated whether 4 weeks WGP feeding had an impact on the LKS⁺ populations within the bone marrow (**Fig 6.19**). Similar to what I saw after 1 week of WGP feeding there was a significant increase in the relative frequency of LKS⁺ cells within the bone marrow after 4 weeks of WGP feeding (**Fig 6.19B**). Interestingly, IFNAR^{-/-} mice that were fed WGP for 4 weeks failed to significantly increase the LKS⁺ populations within the bone marrow (**Fig 6.19C**). Taken together this data indicated that the increase in the LKS⁺ populations within the bone marrow were dependent on type I interferon signalling.

Next, I decided to investigate what populations within the LKS⁺ cells were driving these changes. LT-HSCs (CD48-CD150⁺), ST-HSCs (CD48⁺CD150⁺) and MPP (CD48⁺CD150⁻) cells were investigated according to the gating strategy (**Fig 6.20A**). The relative frequency of LT-HSCs was significantly reduced by 4 weeks of WGP feeding in wild type animals (**Fig 6.20B**). Interestingly, this did not occur in the IFNAR^{-/-} animals fed WGP (**Fig 6.20C**). Similarly, the relative frequency of ST-HSCs was significantly reduced in wild type animals fed WGP for 4 weeks, which did not occur in IFNAR^{-/-} animals (**Fig 6.20D-E**). This was similar to results previously published showing that chronic interferon signalling represses stem cell expansion in the bone marrow as well as in preliminary data from our lab^{268 99}. However, in contrast to this reduction wild type animals that were fed WGP had a significant expansion of MPP cells within the LKS population (**Fig 6.20F**). Again, this did not occur within the IFNAR^{-/-} animals fed WGP for 4 weeks (**Fig 6.20G**). Taken together

this data indicates that 4 weeks of WGP feeding induces changes in haematopoiesis that occurs in a type I interferon manner.

Having seen that there was a significant expansion of MPP cells within the LKS, I investigated whether this was due to expansion of myeloid and lymphoid bias cells, MPP3 and MPP4. After 4 weeks of WGP feeding there was a significant increase in the relative frequency of MPP3 biased cells within the LKS population (**Fig 6.21B**). In IFNAR^{-/-} animals this did not occur (**Fig 6.21C**). Next, I wanted to investigate whether MPP4 populations were altered. After 4 weeks of WGP feeding there was a significant increase in the relative frequency of MPP4 cells in wild type animals (**Fig 6.21D**). In contrast as I have shown throughout IFNAR^{-/-} animals fed WGP lacked the ability to enhance the MPP4 populations (**Fig 6.21E**). Again, this data highlights the importance of type I interferon in induce changes within the bone marrow during WGP feeding.

Finally, I wanted to investigate changes in more matured progenitor cells such as CMPs, CLPs and common granulocyte-myeloid progenitors (GMPs). GMPs were gated on LIN⁻ cells that were CD127⁻ but CD16/32⁺CD34⁺ and CMPs were gated on LIN⁻ cells that were CD127⁻ but CD16/32⁻CD34⁺ as shown in **Fig 6.22A**. Unlike after 1 week of WGP feeding there were no significant changes in CMPs after 4 weeks feeding in either wild type or IFNAR^{-/-} animals (**Fig 6.22B-C**). Similarly, 4 weeks WGP feeding had no impact on GMP populations within the bone marrow of wild type and IFNAR^{-/-} animals (**Fig 6.22D-E**). I also investigated changes in CLPs which were gated on LIN⁻ cells that were CD127⁺cKIT⁺Sca-1⁺ (**Fig 6.23A**). Similar to the results I observed with CMPs and GMPs, the relative frequency of CLPs was not expanded by WGP feeding in neither wild type nor IFNAR^{-/-} animals (**Fig 6.23B-C**). Taken together this data indicates that 4 weeks of WGP feeding induces changes in the bone marrow niche significantly different to only 1 week of feeding and that these changes occur in a type I interferon dependent manner.

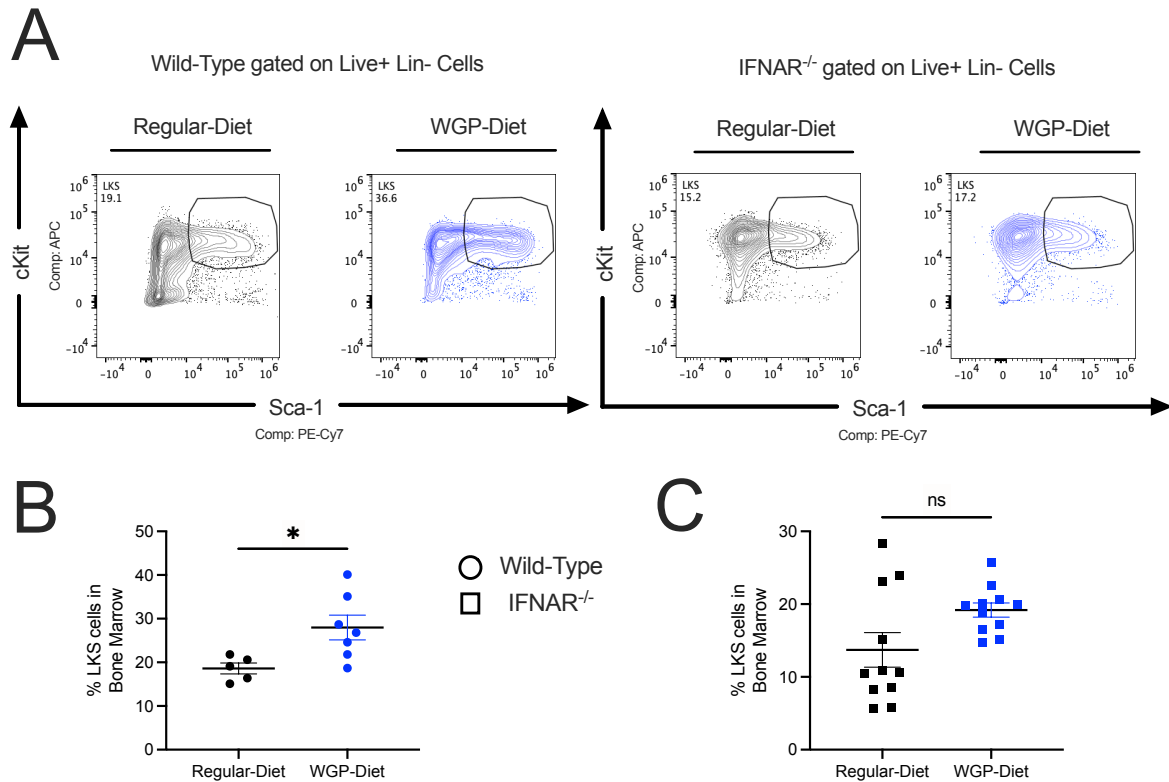


Figure 6.18 4-Weeks WGP feeding leads to significant enrichment of LKS⁺ stem cells in the bone marrow dependent on type I interferon signalling

Wild-type and IFNAR^{-/-} mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. After the 4 weeks feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and processed and stained for flow cytometry analysis. (A) Graph shows gating strategy for LKS⁺ cells gated on live LIN⁻ cell populations, (B) percentage of LKS⁺ cells in the bone marrow of wild-type animals fed regular diet or WGP diet or (C) percentage of LKS⁺ cells in the bone marrow of IFNAR^{-/-} animals fed regular diet or WGP diet. ([B] Biological N = 5 or 7, [C] N = 11). NS = not significant P-value >0.05 or *P-value <0.05; ([B] Unpaired T-test with Welch's correction post normalisation testing or [C] Mann-Whitney test post normalisation testing). *Experiment performed with Dr. Anna Ledwith.*

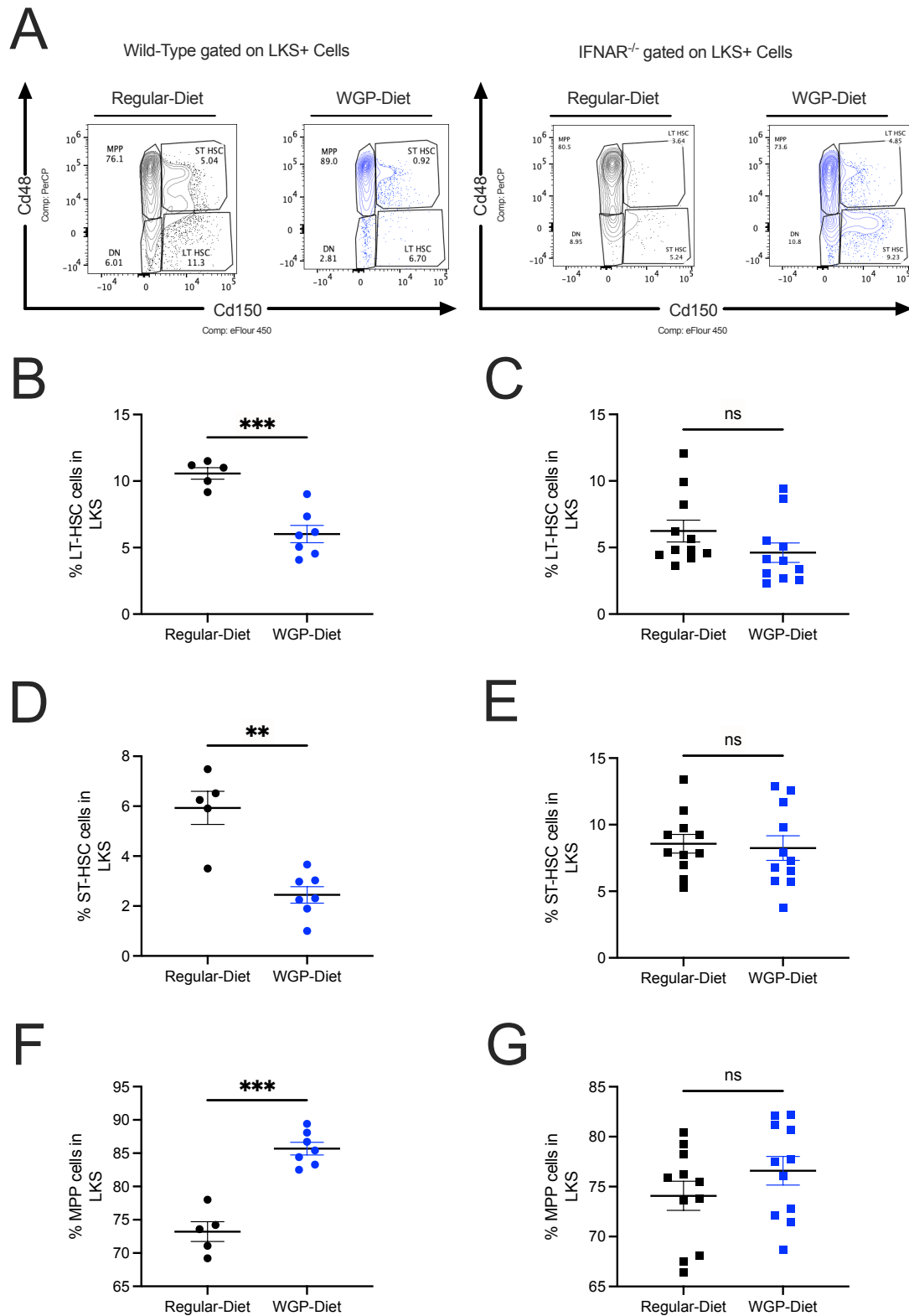


Figure 6.19 4-Weeks WGP feeding leads to significant relative population changes in HSCs in the bone marrow dependent on type I interferon signalling

Wild-type and IFNAR^{-/-} mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. After the 4 weeks feeding period mice were culled according to protocol outlines

in the material and methods section. Bone marrow was extracted and processed and stained for flow cytometry analysis. (A) Graph shows gating strategy for HSCs gated on live LIN⁻ LKS⁺ cell populations, (B) percentage of LT-HSCs in the LKS of wild-type animals fed regular diet or WGP diet, (C) percentage of LT-HSCs in the LKS of IFNAR^{-/-} animals fed regular diet or WGP diet, (D) percentage of ST-HSCs in the LKS of wild-type animals fed regular diet or WGP diet, (E) percentage of ST-HSCs in the LKS of IFNAR^{-/-} animals fed regular diet or WGP diet, (F) percentage of MPPs in the LKS of wild-type animals fed regular diet or WGP diet or (G) percentage of MPPs in the LKS of IFNAR^{-/-} animals fed regular diet or WGP diet. ([B] Biological N = 5 or 7, [C] N = 11). NS = not significant P-value >0.05, **P-value <0.005 or ***P-value <0.001; ([B, D-G] Unpaired T-test with Welch's correction post normalisation testing or [C] Mann-Whitney test post normalisation testing). *Experiment performed with Dr. Anna Ledwith.*

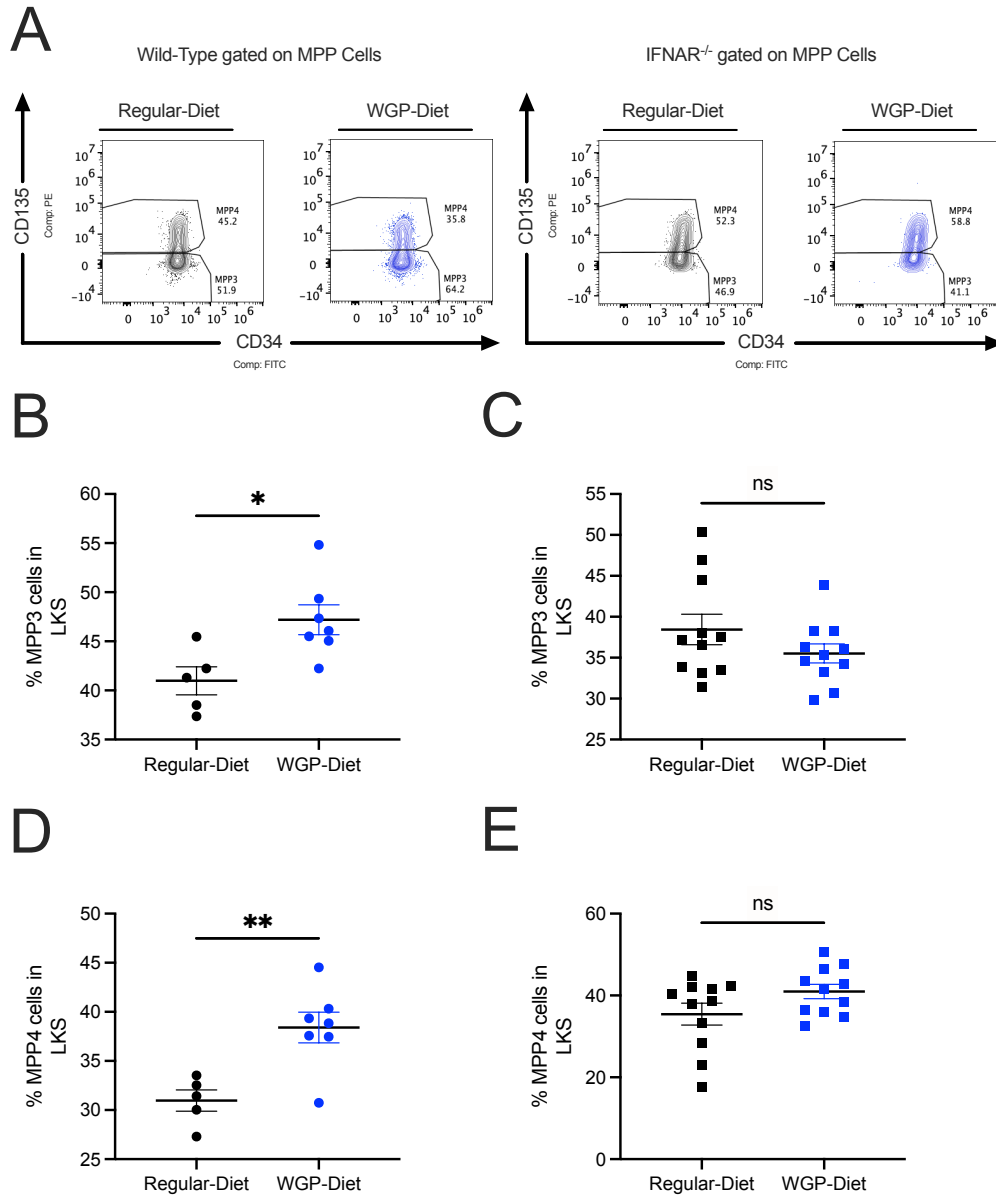


Figure 6.20 4-Weeks WGP feeding leads to significant changes in MPP populations in the bone marrow dependent on type I interferon signalling

Wild-type and IFNAR^{-/-} mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. After the 4 weeks feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and processed and stained for flow cytometry analysis. (A) Graph shows gating strategy for MPP populations gated on live LIN⁻ LKS⁺ MPP⁺ populations, (B) percentage of MPP3 in the LKS of wild-type animals fed regular diet or WGP diet, (C) percentage of MPP3 in the LKS of IFNAR^{-/-} animals fed regular diet or WGP diet, (D) percentage of MPP4 in the LKS of wild-type animals fed regular diet or WGP diet, (E) percentage of MPP4 in the LKS of IFNAR^{-/-} animals fed regular diet or WGP diet. ([B & D] Biological N = 5 or 7, [C & E] N = 11). NS = not significant P-value >0.05, *P-value <0.05 or **P-value <0.005; (Unpaired T-test with Welch's correction post normalisation testing). *Experiment performed with Dr. Anna Ledwith.*

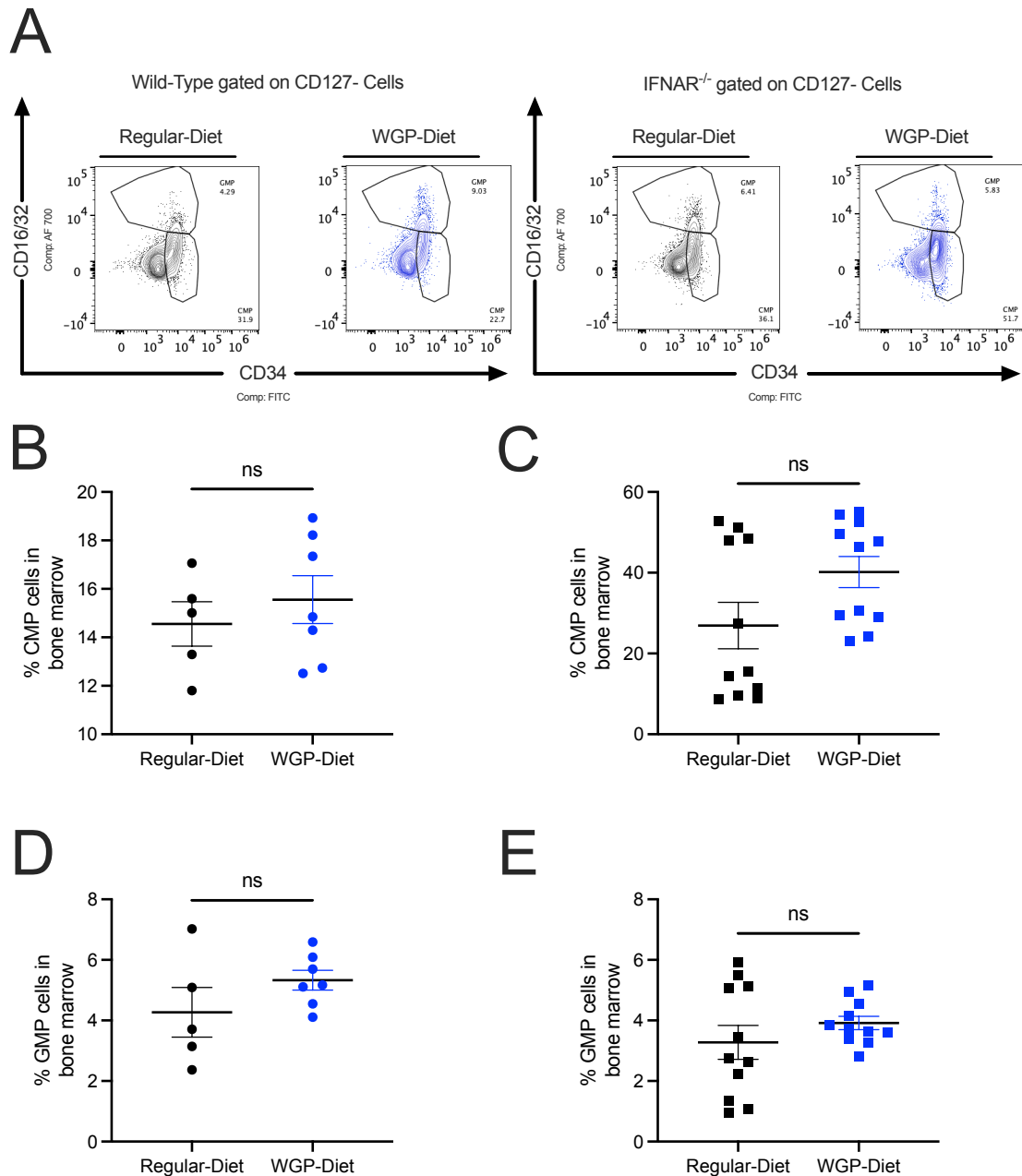


Figure 6.21 4-Weeks WGP feeding has no significant effect on the relative CMP or GMP populations in the bone marrow

Wild-type and IFNAR^{-/-} mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. After the 4 weeks feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and processed and stained for flow cytometry analysis. (A) Graph shows gating strategy for CMP and GMP populations gated on live LIN⁻ CD127⁻ populations, (B) percentage of CMPs in the bone marrow of wild-type animals fed regular diet or WGP diet, (C) percentage of CMPs in the bone marrow of IFNAR^{-/-} animals fed regular diet or WGP diet, (D) percentage of GMPs in the bone marrow of wild-type animals fed regular diet or WGP diet, (E) percentage of GMPs in the bone marrow of IFNAR^{-/-} animals fed regular diet or WGP diet. ([B & D] Biological N = 5 or 7, [C & E] N = 11). NS = not significant; (Unpaired T-test with Welch's correction post normalisation testing). *Experiment performed with Dr. Anna Ledwith.*

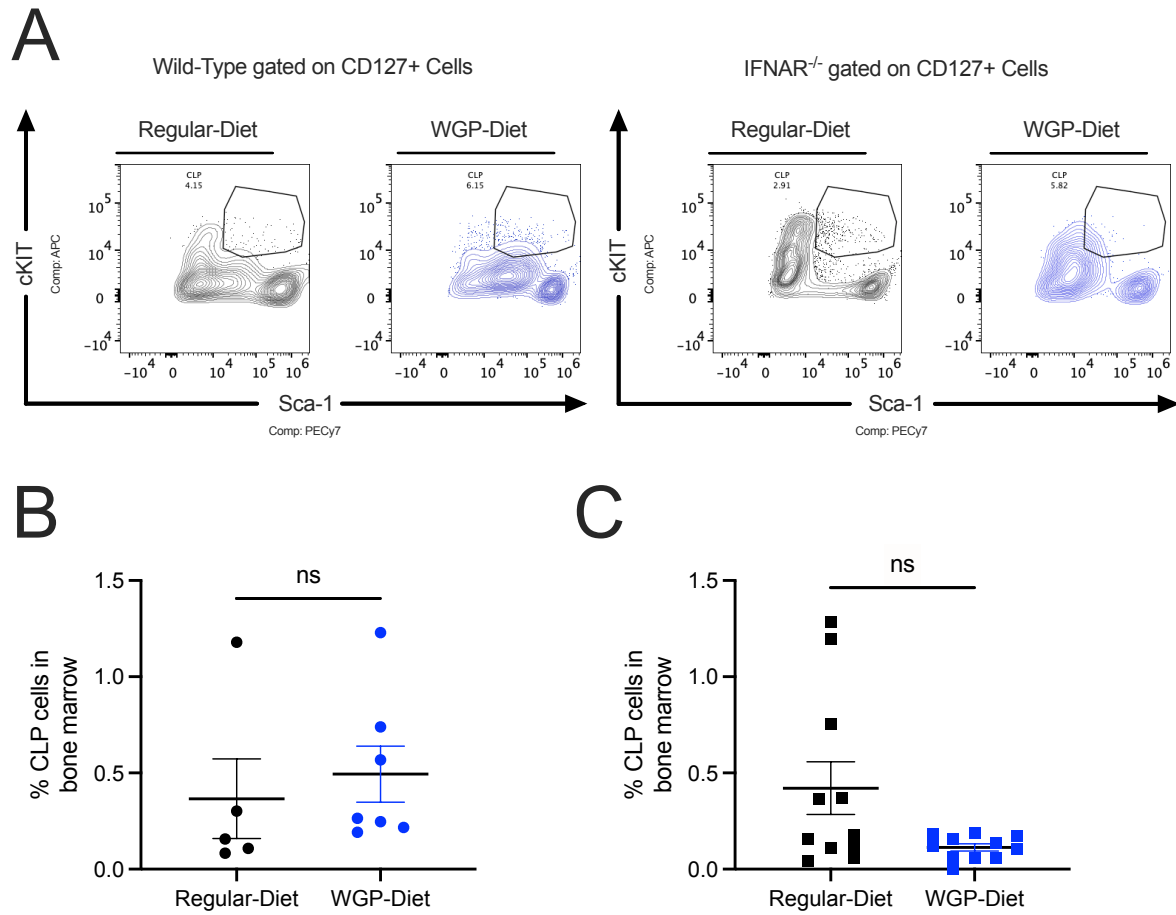


Figure 6.22 4-weeks WGP feeding has no impact on CLP populations within the bone marrow

Wild-type and IFNAR^{-/-} mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. After the 4 weeks feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and processed and stained for flow cytometry analysis. (A) Graph shows gating strategy for CLP populations gated on live LIN⁻ CD127⁺ populations, (B) percentage of CLPs in the bone marrow of wild-type animals fed regular diet or WGP diet, (C) percentage of CLPs in the bone marrow of IFNAR^{-/-} animals fed regular diet or WGP diet. ([B] Biological N = 5 or 7, [C] N = 11). NS = not significant; (Mann-Whitney test post normalisation testing). *Experiment performed with Dr. Anna Ledwith.*

6.2.8 4-weeks WGP feeding leads to epigenetic and enhanced antiviral responses in the mature progeny of bone marrow stem cells in a type I interferon dependent manner

Having seen that 4-weeks of WGP feeding led to changes in the bone marrow compartment in a type I interferon dependent manner, I wanted to test whether mature myeloid progeny would hold characteristics of trained immunity. I hypothesized that this would occur in a type I interferon dependent manner. To test this, bone marrow was taken from both wild-type and IFNAR^{-/-} animals fed both diets and were matured into BMDMs over a 7-day period, as previously described. Once matured cells were counted and either stained for flow cytometry or were plated for live viral infection with herpes simplex virus (HSV).

I first wanted to investigate whether BMDMs derived from bone marrow of WGP fed animals had a different epigenetic signature to those fed a regular diet (**Fig 6.24**). To do this I used an antibody against H3K27ac, a classical histone modification observed in trained immunity²⁵⁸. I first observed that in all cases H3K27ac expression was significantly higher than the FMO control as shown on the respective histograms (**Fig 6.24A**). Next, I also showed that in the wild types, BMDMs derived from animals fed a WGP rich diet had significantly elevated levels of H3K27ac compared with animals fed a regular diet. This suggests that WGP feeding for 4 weeks enhances epigenetic modifications that are carried through to the mature progeny, even after the training stimulus has been removed. However, in contrast there was no significant statistical differences between both diets in the IFNAR^{-/-}, suggesting that epigenetic modifications in the mature cells were induced in a type I interferon dependent manner.

Since I had seen that these mature cells had an epigenetic signature following 4 weeks of WGP feeding I next investigated whether these cells also showed functionally enhanced anti-viral potential. As mentioned, cells were infected with HSV at an MOI of 5 virus per cell for 24hrs and supernatant was tested for changes in cytokine production, mainly TNF and IFN β . I first saw that in the absence of IFNAR signalling BMDMs could not significantly increase their ability to induce TNF (**Fig 6.24B**). However, in the wild-type animals even though they could respond to

the viral infection WGP feeding had no significant effects on TNF induction. However, when I measured IFN β levels in the supernatant I saw that BMDMs derived from WGP fed mice had significantly elevated IFN β levels vs the BMDMs that were derived from mice fed a regular diet (**Fig 6.24C**). Importantly, this enhanced IFN response was not possible in the IFNAR^{-/-} animals highlighting that in the absence of IFN signalling macrophages differentiated from IFNAR deficient animals were still not able to effectively enhance their IFN response to viral infection. Taken together this data shows that changes in bone marrow progenitors during 4 weeks of WGP is sufficient to induce long lasting epigenetic and anti-viral cytokine responses in mature macrophage populations. This also highlights that WGP can not enhance anti-viral responses without effective IFN signalling.

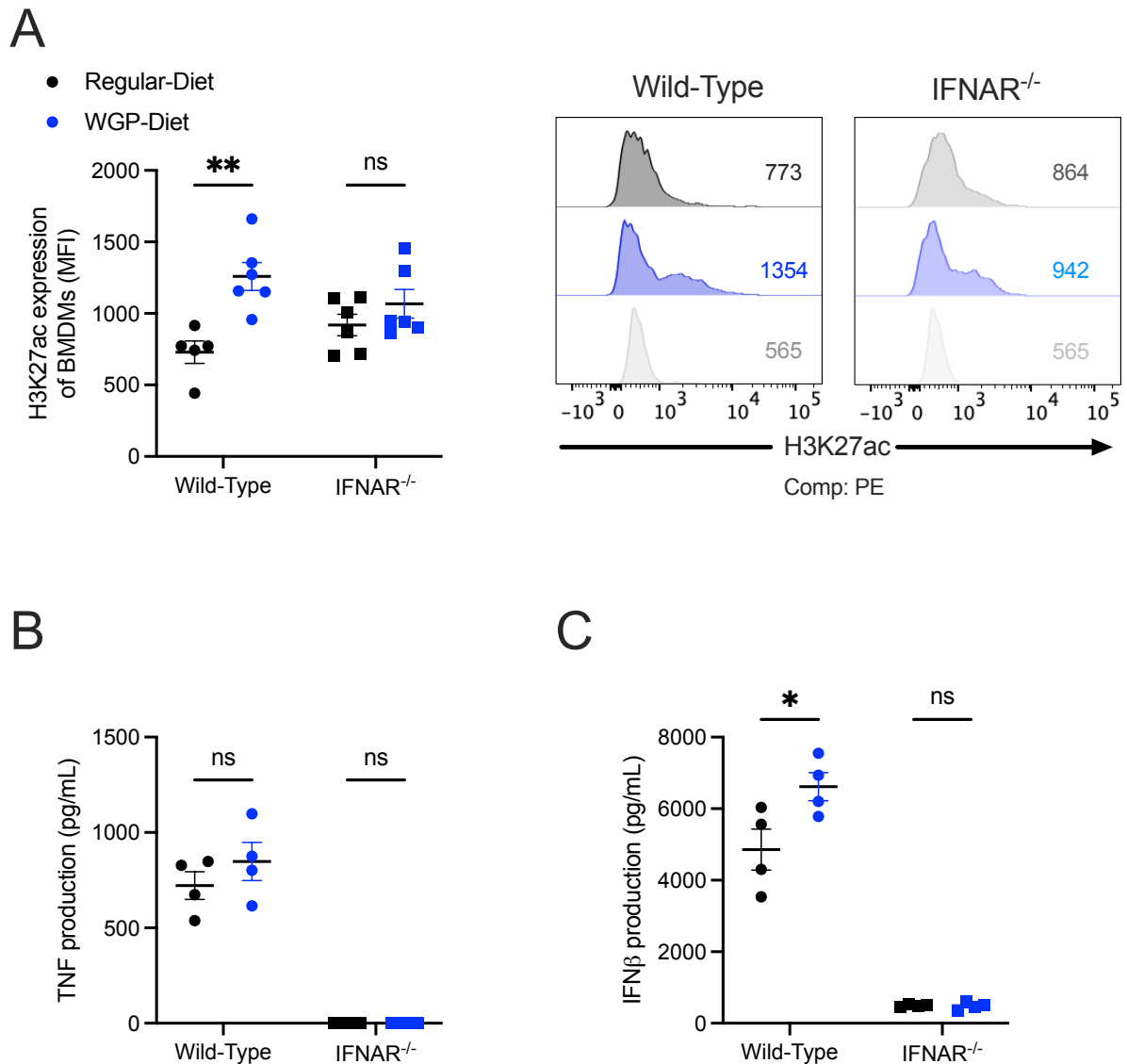


Figure 6.23 4-weeks WGP feeding leads to long lasting epigenetic and anti-viral properties after bone marrow macrophage differentiation

Wild-type and IFNAR^{-/-} mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. After the 4 weeks feeding period mice were culled according to protocol outlines in the material and methods section. Bone marrow was extracted and allowed to differentiate into bone marrow derived macrophages (BMDMs). BMDMs were then stained for intranuclear histone staining for flow cytometry analysis as well as histograms showing (A) MFI values of control (black), WGP (blue) or FMO (light grey) or were infected for 24hrs with multiplicity of infection (MOI) 5 of herpes simplex virus (HSV). (A) Graph shows MFI of H3K27ac in wild type and IFNAR^{-/-} mice fed regular diet or WGP supplemented diet, (B) TNF or (C) IFNβ in the supernatant of infected cells as measured by ELISA. (Biological N = 5 or 6). NS = not significant, *P-value <0.05 or **P-value <0.005; (Two-way ANOVA with Šídák multiple comparison test post normalisation testing).

6.2.9 4-weeks WGP feeding has no impact on the relative proportion of circulating leukocytes but leads to enhanced epigenetic modification dependent on type I IFN signalling

Having seen changes in bone marrow progenitors and the impact 4 weeks of feeding has on matured and differentiated bone marrow macrophages I wanted to investigate if there were changes in circulating leukocytes. To do this blood was drawn from mice after 4 weeks of feeding and processed for flow cytometry as previously described. It has been shown that training inducers, such as BCG and β -glucan, can cause changes in the expansion or function of leukocytes such as T cells, neutrophils and monocytes in circulation^{35, 90, 269}. Therefore, I first wanted to see whether there were changes in the composition or epigenetic signature of circulating T cells, neutrophils and monocytes. T cells were gated on live cells that were CD3⁺ but Ly6g⁻ and neutrophils were gated on live cells that were Ly6g⁺ and CD3⁻ (**Fig 6.25A**). 4-weeks feeding with WGP had no significant impact on the proportion of T cells within the circulation of mice in both wild-type or IFNAR^{-/-} animals (**Fig 6.25B-C**). Similarly, 4-weeks of WGP feeding did not impact the relative proportion of circulating neutrophils in wild type or IFNAR^{-/-} animals (**Fig 6.25D-E**).

Next, to test whether WGP feeding impacted the epigenetic signature of these cell types I analysed the MFI of the common epigenetic training signature histone 3 (H3) lysine (K) tri-methylation (H3K4me3)²⁷⁰. Even though I did not see any changes in the relative proportion of T cells or neutrophils after WGP feeding, interestingly there were changes in epigenetic signatures. 4-weeks of WGP feeding significantly enhanced the MFI of H3K4me3 in T cells from wild type mice. However, this change did not occur in IFNAR^{-/-} animals (**Fig 6.26A**). Interestingly, this was also the case for neutrophils. 4-weeks of WGP feeding also significantly increased the MFI of H3K4me3 in circulating neutrophils in wild type animals but not in IFNAR^{-/-} (**Fig 6.26B**). This suggests that long term reprogramming of T cells and neutrophils via WGP feeding was dependent on type I IFN signalling.

I also wanted to see if there were similar changes in circulating monocytes. Monocytes were gated from live cells that were CD3⁻ Ly6g⁻ NK1.1⁻ CD11b⁺

Ly6c^{Hi}/Lo (**Fig 6.27A**). Similar to my previous results above 4-weeks of WGP feeding had no significant impact on the relative proportion of circulating monocytes in wild type animals (**Fig 6.27B**). However, interestingly in the IFNAR^{-/-} animals 4-weeks WGP feeding leads to a significant increase in the relative proportion of these cells (**Fig 6.27C**). This could suggest a regulatory role for type I IFN signalling during WGP feeding.

Having seen these results I wanted to investigate whether there were any changes in epigenetic modifications. First, I showed that all groups had significantly higher MFI of H3K4me3 than the FMO control (**Fig 6.27D**). Having shown that any changes in H3K4me3 were above the FMO I next investigated whether there were any effects with feeding. I saw that in circulating monocytes from mice fed WGP for 4-weeks there was a significant increase in the MFI of H3K4me3 in wild type animals (**Fig 6.27E**). However, in the IFNAR^{-/-} animals 4-weeks of WGP feeding had no overall significant impact on epigenetic changes (**Fig 6.27F**). This data suggests that even if 4-weeks of WGP feeding leads to a shift in monocyte proportions in IFNAR^{-/-} animals the overall epigenetic signature does not persist showing that type I IFN is vital in imprinting a long lasting immunological phenotype in monocytes.

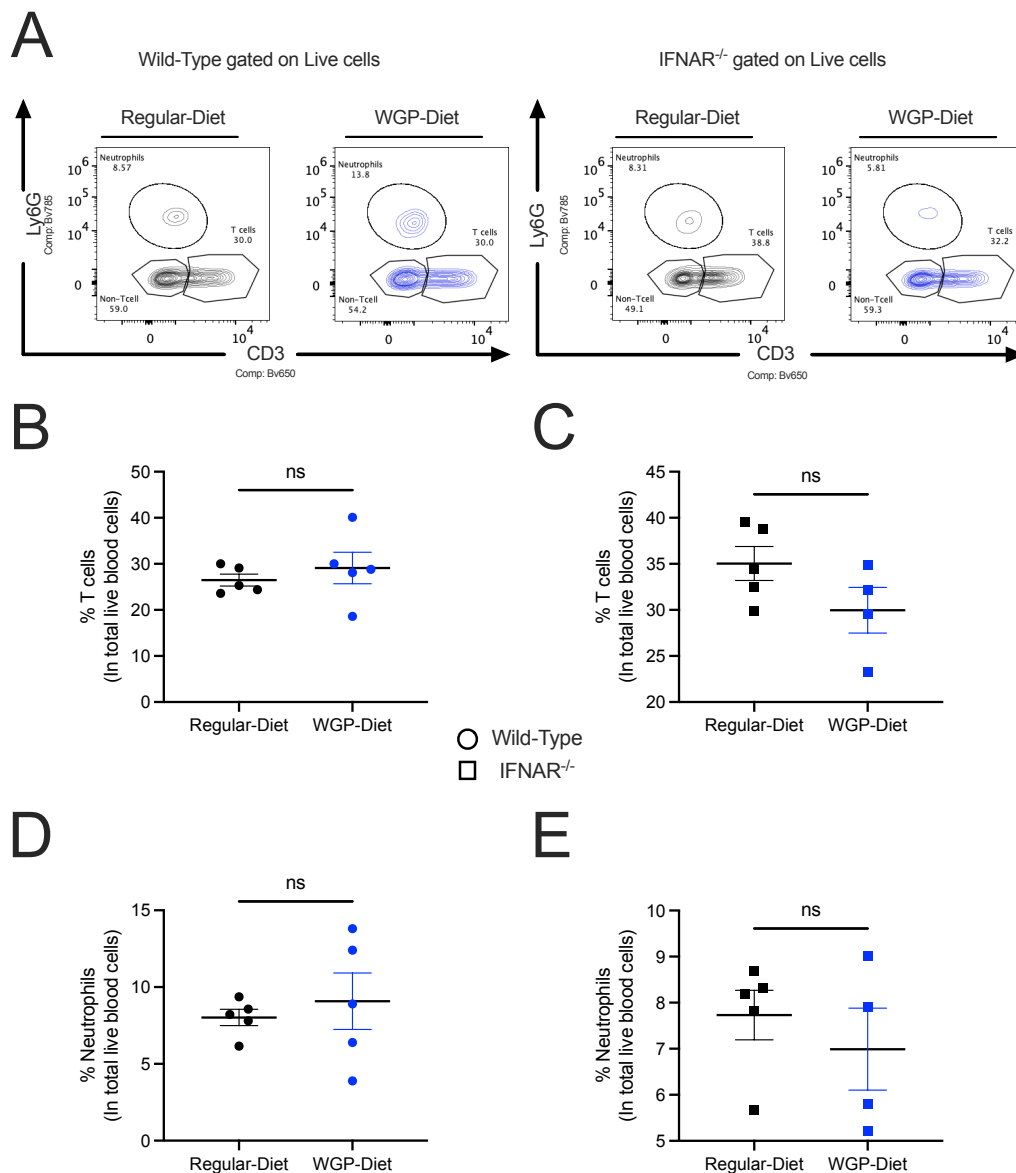


Figure 6.24 4-weeks WGP feeding has no impact on relative proportions of T cells or neutrophils in the circulation in either wild type or IFNAR^{-/-} animals

Wild-type and IFNAR^{-/-} mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. After the 4 weeks feeding period mice were culled according to protocol outlines in the material and methods section. 500μL of blood was collecting via syringe through the right ventricle of the heart. Blood was processed rapidly to avoid coagulation and red blood cells were removed before flow staining. (A) Graph shows gating strategy to isolate neutrophils and T cells from other cell types, (B) relative proportion of T cells in all live cells in the blood of wild-type animals, (C) relative proportion of T cells in all live cells in the blood of IFNAR^{-/-} animals, (D) relative proportion of neutrophils in all live cells in the blood of wild-type animals or (E) relative proportion of neutrophils in all live cells in the blood of IFNAR^{-/-} animals (Biological N = 5 or 4). NS = not significant; (Welch's t test post normalisation testing).

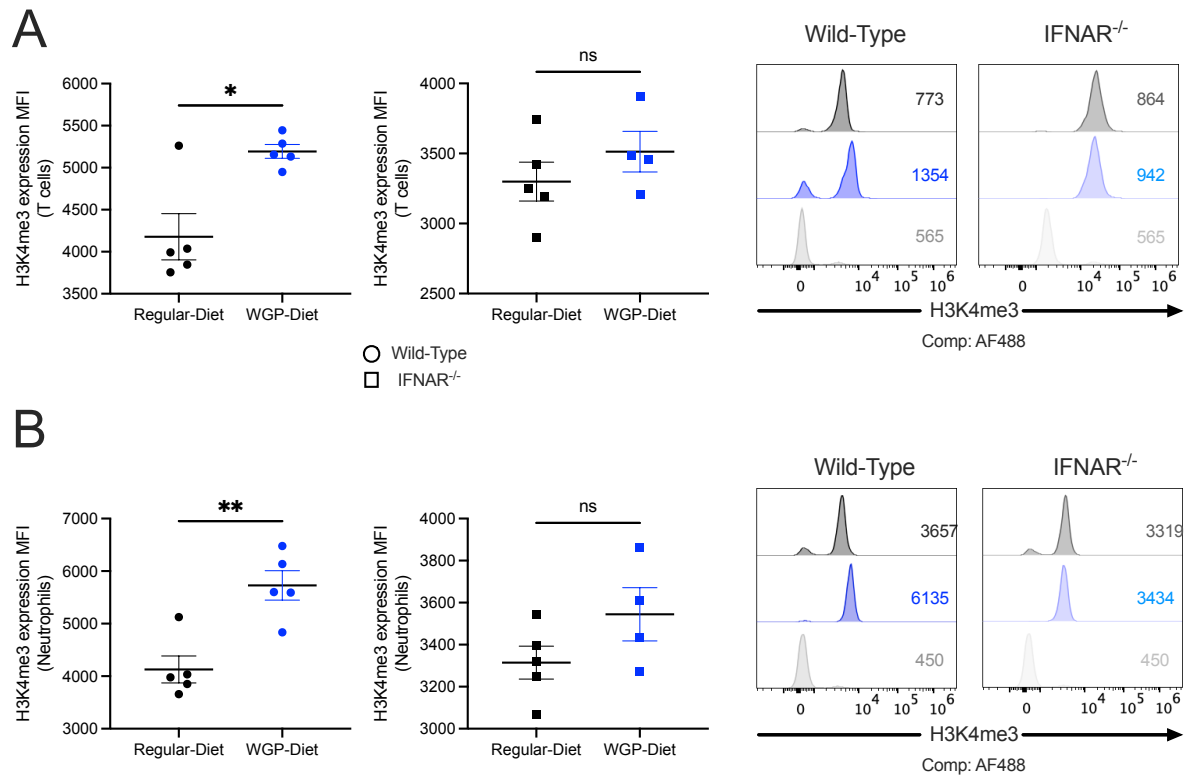


Figure 6.25 4-weeks WGP feeding leads to enhanced epigenetic modifications in circulating T cells and neutrophils in a type I IFN dependent manner

Wild-type and IFNAR^{-/-} mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. After the 4 weeks feeding period mice were culled according to protocol outlines in the material and methods section. 500μL of blood was collecting via syringe through the right ventricle of the heart. Blood was processed rapidly to avoid coagulation and red blood cells were removed before flow staining. Intranuclear staining was performed to investigate epigenetic modifications. (A) Graph shows MFI of H3K4me3 epigenetic modification in circulating T cells from wild-type and IFNAR^{-/-} animals and the representative histograms with FMO control and (B) MFI of H3K4me3 epigenetic modification in circulating neutrophils from wild-type and IFNAR^{-/-} animals and the representative histograms with FMO control. (Biological N = 5 or 4). NS = not significant, *P-value <0.05 or **P-value <0.005; (Welch's t test post normalisation testing).

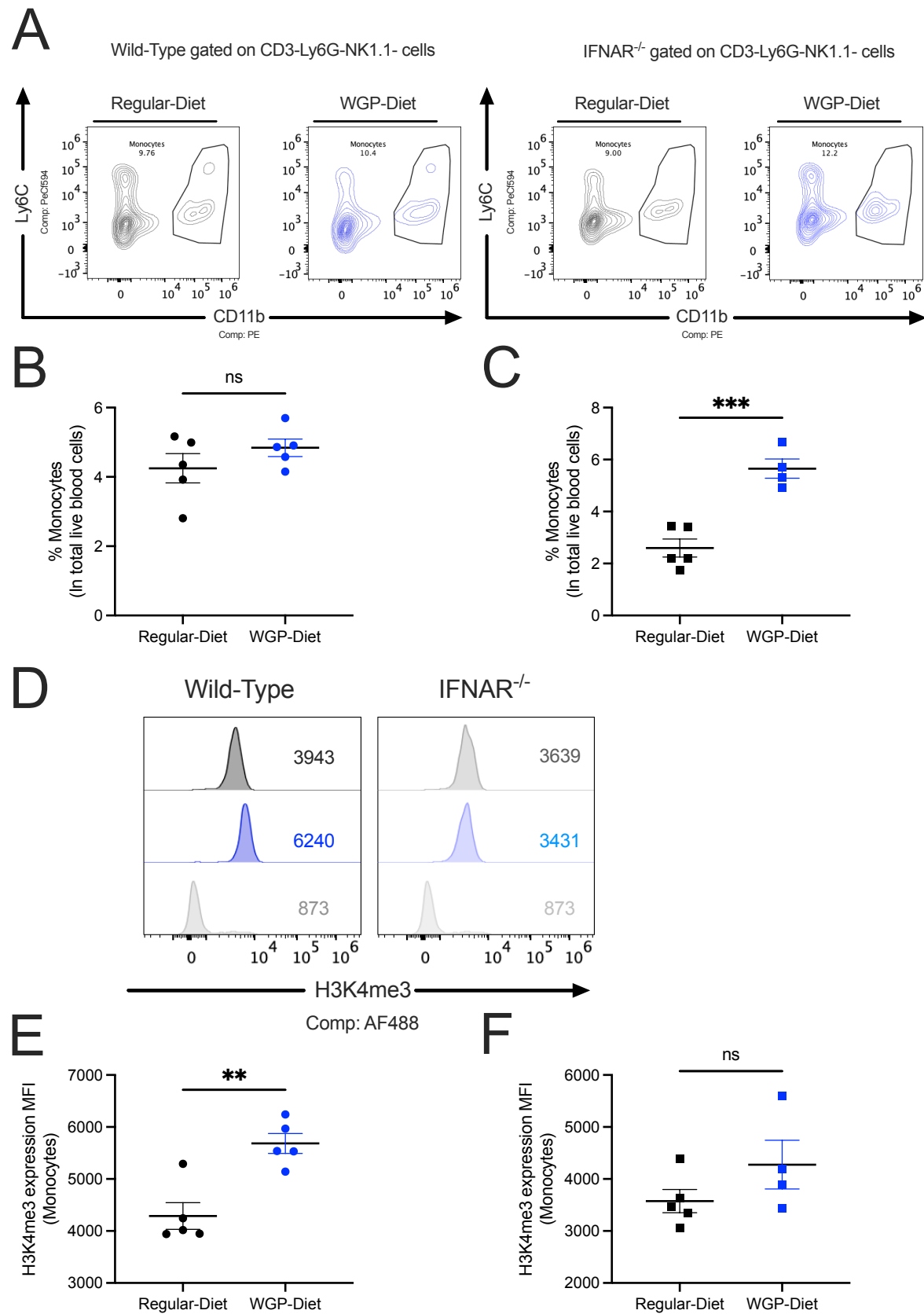


Figure 6.26 4-weeks WGP feeding leads no changes in circulating monocytes in wild type animals but leads to monocyte epigenetic modifications in a type I IFN dependent manner.

Wild-type and IFNAR^{-/-} mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. After the 4 weeks feeding period mice were culled according to protocol outlines in the material and methods section. 500μL of blood was collecting via syringe through the right ventricle of the heart. Blood was processed rapidly to avoid coagulation and red blood cells were removed before flow staining. Intranuclear staining was performed to investigate epigenetic modifications. (A) Graph shows gating strategy to isolate circulating monocytes from other cell types, (B) relative proportion of monocytes in all live cells in the blood of wild-type, (C) relative proportion of monocytes in all live cells in the blood of IFNAR^{-/-} animals, (D) histogram showing changes in H3K4me3 epigenetic modifications in wild-type and IFNAR^{-/-} monocytes +/- WGP feeding compared to FMO, (E) MFI of H3K4me3 epigenetic modification in circulating monocytes from wild-type animals and (F) MFI of H3K4me3 epigenetic modification in circulating monocytes from IFNAR^{-/-} animals. (Biological N = 5 or 4). NS = not significant, **P-value <0.005 or ***P-value <0.001; (Welch's t test post normalisation testing).

6.2.10 4-weeks WGP feeding leads to changes in subpopulations of alveolar macrophages within the lung dependent on type I IFN signalling

I have demonstrated that the relative proportion of monocytes and neutrophils do not change in the circulation of wild type animals when they have been fed a WGP diet for 4-weeks. However, since I also showed changes in the relative proportion of MPP and myeloid progenitor cells within the bone marrow after 1-week and 4-weeks of feeding, I hypothesized that these cells may have infiltrated the lung of WGP fed animals.

Since it has been shown that IP injection with β -glucan can impact neutrophil infiltration into the lungs in a type I IFN dependent manner I first wanted to explore whether this occurred during feeding³⁵. Therefore, I investigated whether 4-weeks WGP feeding impacts neutrophil infiltration into the lung. Cells were isolated from lung tissue as described in material and methods section and neutrophils were gated on live LIN⁻ (CD3-NK1.1-CD19⁻) cells that were positive for CD45 and Ly6G (**Fig 6.28A**). In wild type mice, 4-weeks of WGP feeding had no statistical impact on the relative proportion of these cells within the lung (**Fig 6.28B**). Similarly, when I investigated whether there were changes in IFNAR^{-/-} mice fed WGP for 4-weeks, I did not see any significant effects. Therefore, WGP feeding had no impact on neutrophil infiltration or recruitment in the lung tissue of either wild type or knock out mice.

Recently, there has been a growing interest of the ability of alveolar macrophages to undergo trained immunity, particularly in a type I IFN dependent manner¹⁸⁵. Therefore, since I did not see any significant changes in the relative proportion of neutrophils in either wild type or knock out animals fed WGP, I decided to investigate changes in the relative proportion of alveolar macrophages. I identified alveolar macrophages based on the fact that they were live LIN⁻ Ly6G⁻ and CD11C⁺ CD64⁺ SiglecF⁺ and CD11B_{low} or (-)ve (**Fig 6.29A**). I first investigated the impact of 4-weeks WGP feeding on wild type mice. Here I saw a slight but non-statistically significant increase in the relative proportion of alveolar macrophages in the lungs of animals that were fed WGP (**Fig 6.29B**, p-value = 0.29). However, in IFNAR^{-/-}

mice I saw an opposite effect with a slight but non-significant reduction in the frequency of alveolar macrophages from mice fed WGP (**Fig 6.29C**, p-value = 0.16).

Since I had seen these small but non-significant changes, I hypothesized that something may have occurred within the alveolar compartment during the 4-weeks of feeding. It has been shown recently that in different training models the alveolar compartment can become seeded with non-resident infiltrating monocytes that take on the characteristics of resistant alveolar macrophages⁹³. These cells are bone marrow derived and are referred to throughout the rest of this thesis as bone marrow monocytes derived alveolar macrophages (BMoAMs). The resident yolk-sac derived alveolar macrophages are referred to as foetal-yolk sac monocytes derived alveolar macrophages (FeMoAMs). My previous flow panel did not distinguish these sub-types, so to investigate these cells, I gated on the total alveolar macrophage population and split this population based on their expression of SiglecF and CCR2. It has been shown via transcriptomic profiling, CCR2 knock out and chimeric mouse models that FeMoAMs express high SiglecF and low to no expression of CCR2⁹⁴. In contrast BMoAMs have a lower expression of SiglecF and express CCR2, since they are derived from infiltrating monocytes. Using this information, I was able to split my alveolar macrophage population into two subgroups (**Fig 6.30A**). I also showed that BMoAMs expressed significantly higher CCR2 via CCR2 MFI (**Fig 6.30B**) as well as significantly less SiglecF via SiglecF MFI via flow cytometry (**Fig 6.30C**).

Having structured a gating strategy to identify the different myeloid populations, I investigated this in my WGP feeding model (**Fig 6.31A**). I first investigated whether WGP could impact the relative proportions of these populations within the lung in wild type mice. After 4-weeks of feeding WGP led to a significant increase in the relative proportion of BMoAMs in the total alveolar macrophage population (**Fig 6.31B**). In contrast during feeding in IFNAR^{-/-} even with a slight increase WGP was not capable of significantly increasing the relative proportions of BMoAMs within the alveolar macrophage populations (**Fig 6.31C**). Similarly, when I investigated the impact this had on the proportion of FeMoAMs I saw that WGP feeding led to a significant reduction in FeMoAMs (**Fig 6.31D**). In IFNAR^{-/-} animals, there again was no significant change (**Fig 6.31E**). Taken together this data indicates that WGP

expands the relative proportion of BMoAMs in wild-type animals but has no significant effect in IFNAR^{-/-} again suggesting a role for type I IFN signalling in WGP feeding mediated changes. Finally, to further empathize the impact of recruited cells to the lung during WGP feeding, I measured the MFI of CCR2 within the BMoAM populations between diets. There was a trend towards a significant increase in CCR2 MFI of BMoAMs in wild type animals fed WGP perhaps suggesting recent recruitment of monocytes into the alveolar space, although more work will be needed to fully prove this (**Fig 6.31F**). In contrast this did not occur in IFNAR^{-/-} mice with no significant differences between both diets.

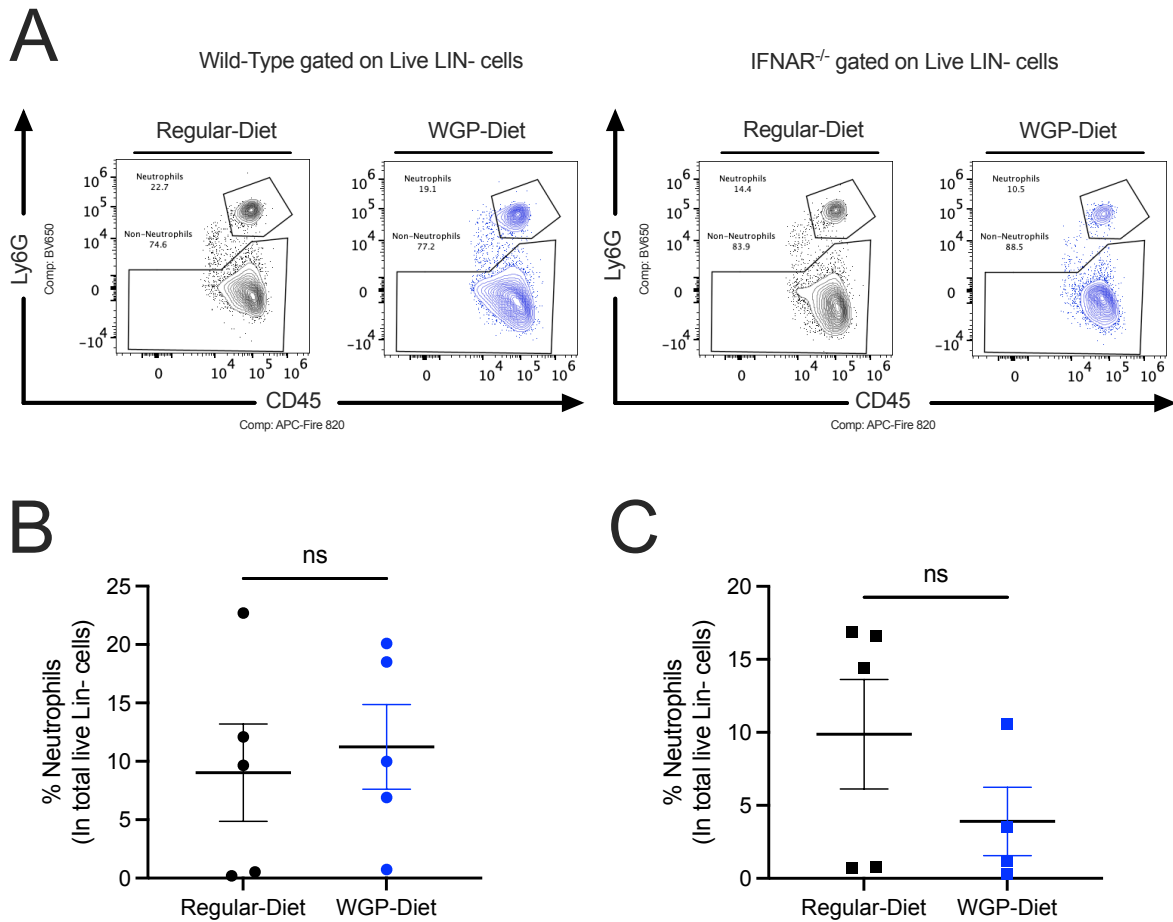


Figure 6.27 4-weeks WGP feeding has no impact on relative proportions of neutrophils in the lung

Wild-type and IFNAR^{-/-} mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. After the 4 weeks feeding period mice were culled according to protocol outlines in the material and methods section. Lungs were processed and stained for flow cytometry. (A) Graph shows gating strategy to isolate neutrophils from other cell types, (B) relative proportion of neutrophils in all live LIN⁻ cells in the lungs of wild-type animals or (C) relative proportion of neutrophils in all live LIN⁻ cells in the lungs of IFNAR^{-/-} animals (Biological N = 5 or 4). NS = not significant; (Welch's t test post normalisation testing).

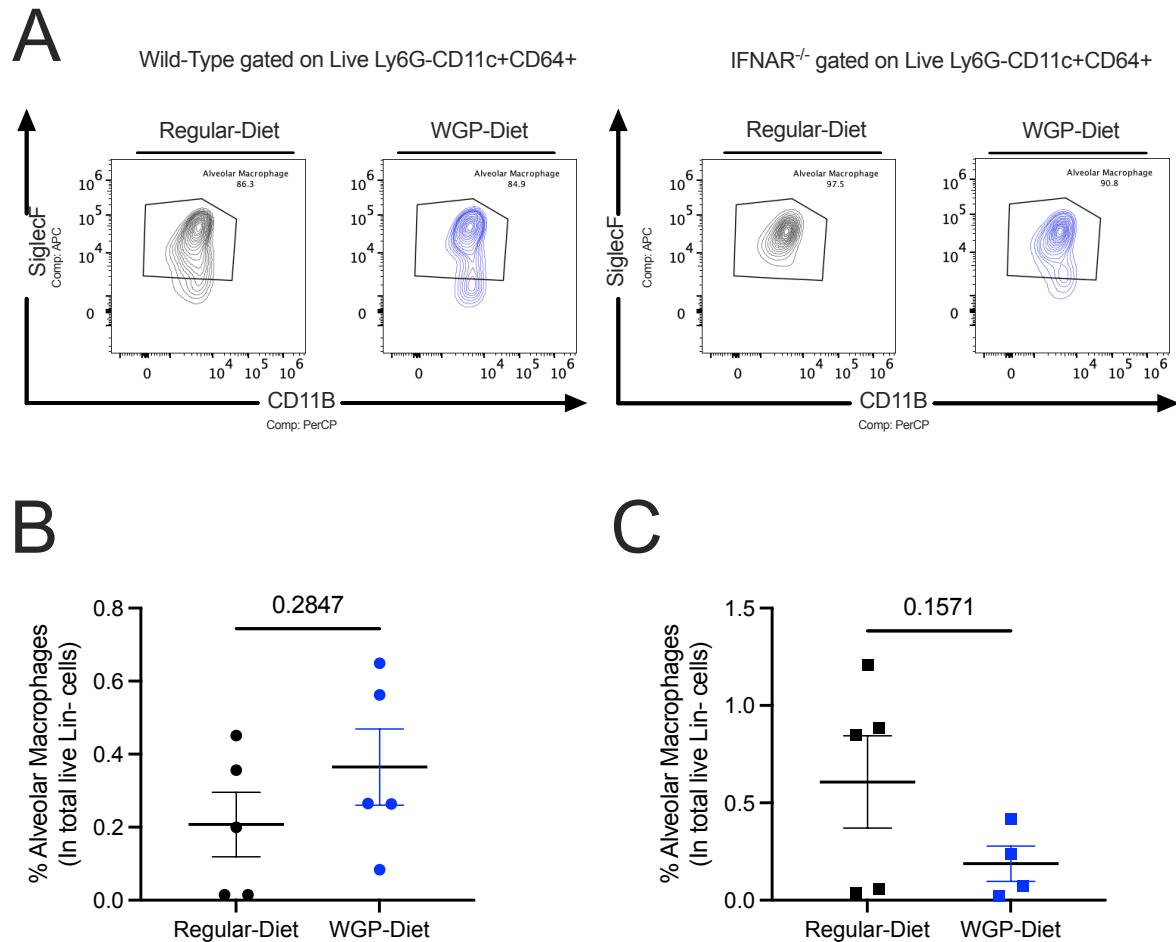
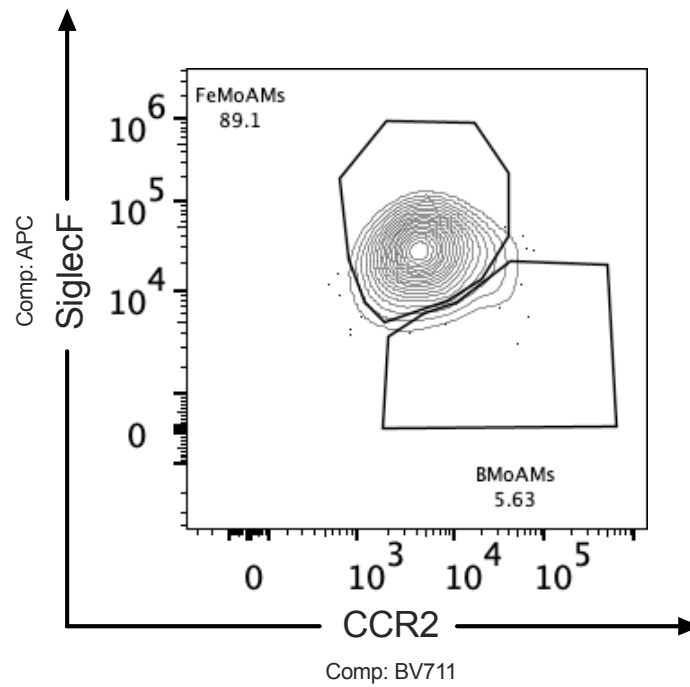


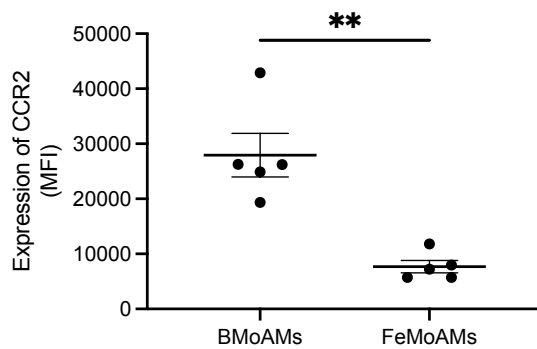
Figure 6.28 4-weeks WGP feeding has no significant impact on relative proportions of total alveolar macrophages in the lung

Wild-type and IFNAR^{-/-} mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. After the 4 weeks feeding period mice were culled according to protocol outlines in the material and methods section. Lungs were processed and stained for flow cytometry. (A) Graph shows gating strategy to isolate alveolar macrophages from other cell types, (B) relative proportion of alveolar macrophages in all live LIN⁻ cells in the lungs of wild-type animals or (C) relative proportion of alveolar macrophages in all live LIN⁻ cells in the lungs of IFNAR^{-/-} animals (Biological N = 5 or 4). NS = not significant; (Welch's t test post normalisation testing).

A



B



C

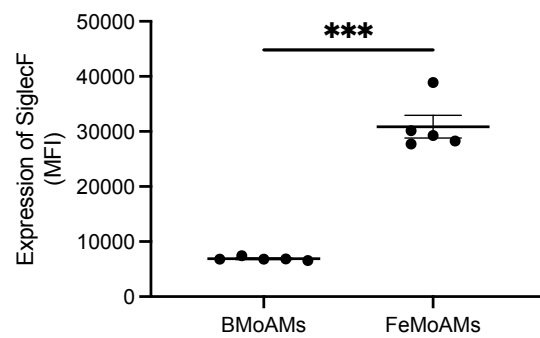


Figure 6.29 Alveolar macrophages can be split into two unique populations based on their expression of CCR2 and SiglecF

Wild-type mice were culled according to protocol outlines in the material and methods section. Lungs were processed and stained for flow cytometry. Graph shows gating strategy to isolate alveolar macrophages subpopulations: (A) Bone marrow monocyte derived alveolar macrophages (BMoAMs) and resident foetal-yolk sac monocyte derived alveolar macrophages (FeMoAMs) comparison between cell types of CCR2 MFI (B) and comparison between cell types of SiglecF MFI (C). (Biological N = 5). *P-value <0.05, **P-value <0.005; (Welches T test post normalisation testing).

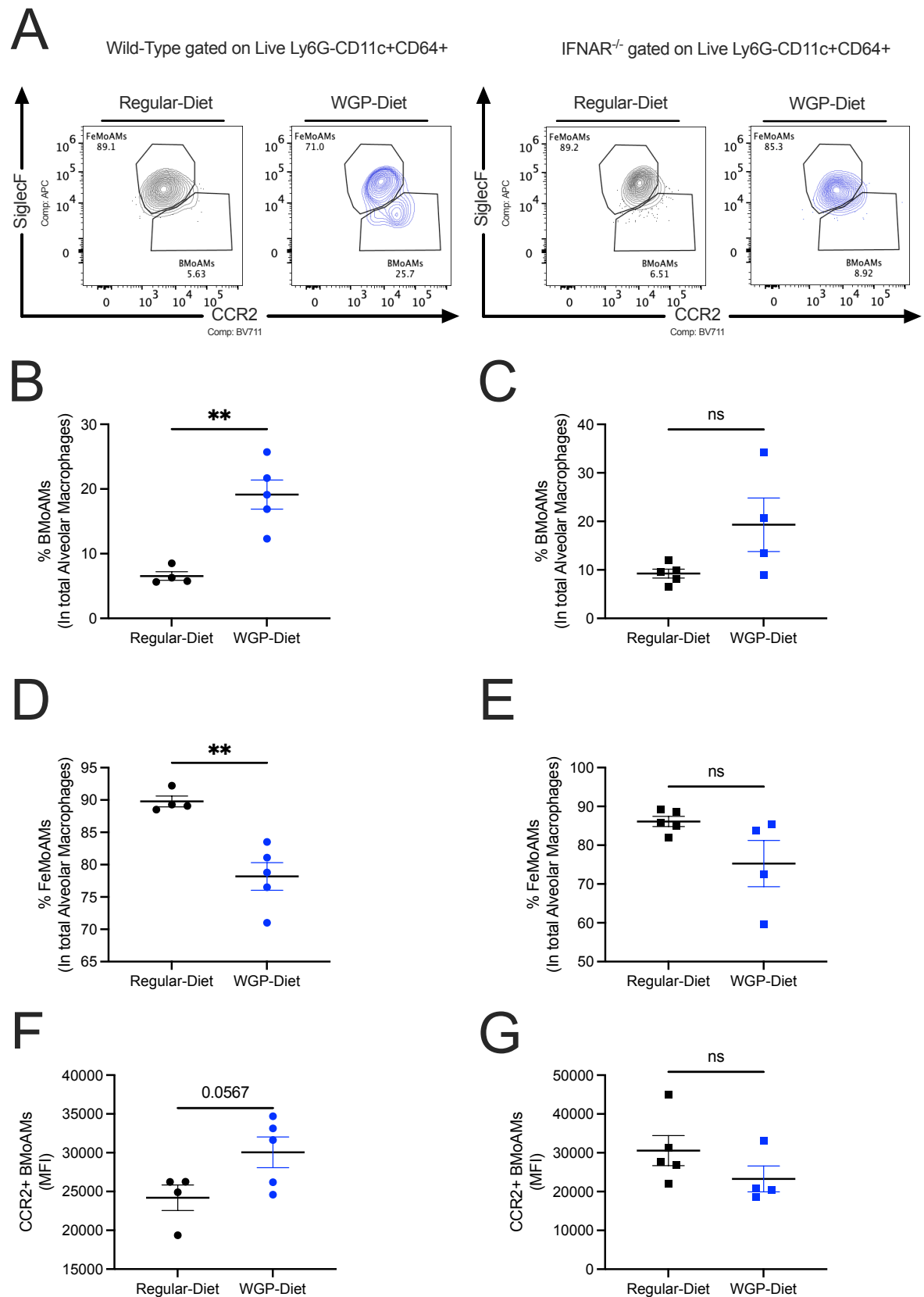


Figure 6.30 4-weeks WGP feeding leads to a switch in the relative proportion of alveolar macrophage subpopulations in the lung dependent on type I IFN signalling

Wild-type and IFNAR^{-/-} mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. After the 4 weeks feeding period mice were culled according to protocol outlines in the material and methods section. Lungs were processed and stained for flow cytometry. (A) Graph shows gating strategy to isolate alveolar macrophage sub-populations from other cell types, (B) relative proportion of BMoAMs within the alveolar macrophage population in the lungs of wild-type animals (C) relative proportion of BMoAMs within the alveolar macrophage population in the lungs of IFNAR^{-/-} animals, (D) relative proportion of FeMoAMs within the alveolar macrophage population in the lungs of wild-type animals, (E) relative proportion of FeMoAMs within the alveolar macrophage population in the lungs of IFNAR^{-/-} animals, (F) MFI of CCR2⁺ BMoAMs within the lung of wild-type animals or (G) MFI of CCR2⁺ BMoAMs within the lung of IFNAR^{-/-} animals. (Biological N = 4 or 5). NS = not significant, **P-value <0.005 or exact P value shown; (Welch's t test post normalisation testing).

6.2.11 Mice fed WGP diet for 4-weeks have enhanced early IL-6 responses to intranasal pIC challenge, even after 1 week reversal to regular diet

Having seen that WGP feeding causes immunological changes in-vivo that are lost in IFNAR^{-/-} animals I next wanted to test whether these animals had enhanced responses to viral ligand challenges. Since my previous experiments have focused on analysing the phenotypical response of different organs to WGP feeding with constant exposure of the animal to the training stimulus, I decided to remove WGP from the diet for 1-week. to test whether any immunological responses observed were due to priming or immunological innate memory. After this wait period, mice were intranasally (IN) injected with pIC and bronchoalveolar lavage fluid (BALF) was extracted after 6hrs (**Fig 6.32A**).

One issue I theorised that may impact the response of mice to viral ligands were changes in food consumption or weight gain during feeding²⁷¹. Therefore, before IN delivery mice were monitored for food consumption as well as weight gain throughout the feeding and reversal period. At the start of WGP feeding mice seemed to consume less food per mouse, however, throughout the feeding period this was not significantly altered and by reversal mice consumed the same amount of chow as the mice on a regular diet (**Fig 6.32B**). Therefore, mice on WGP diet had no significant increase or reduction in food consumption. Similarly, the weight of mice increased equally over the 5-week period, with no significant changes in mouse weight between diets (**Fig 6.32C**). Therefore, I concluded that any results observed were due to changes induced by components of the diet itself (WGP) and not due to changes in weight gain or changes in calorie consumption.

Having seen this, mice were then IN injected with PBS or pIC and allowed to respond to the stimulus over a 6hr period. I analysed the cytokines that were released into the alveolar space in the BALF. I measured both classical pro-inflammatory cytokines, IL6 and TNF, as well as anti-viral cytokines, IFN β and IP10. There were no significant differences in baseline cytokine levels observed between untrained and trained mice that had been challenged with PBS control. However, after pIC challenge untrained mice produced some IL6, but trained mice produced

significantly more IL6 in response to the IN injected pIC (**Fig 6.33A**). I next measured TNF responses. Similar to the IL6 response there were no significant differences between baseline TNF responses in either diet. More interestingly, unlike increased IL6 responses, TNF was not significantly different compared to the untrained animals (**Fig 6.33B**). Taken together this data indicates a specificity for trained mice to upregulate IL6 production in response to IN pIC injection, without impacting proinflammatory cytokines such as TNF. Next, I decided to analyse antiviral cytokine responses. Again, there was no significant differences in IFN β levels in between diets in the PBS control. Surprisingly, I did not observe any changes in the ability of trained mice to upregulate IFN β response to pIC challenge relative to the untrained controls (**Fig 6.33C**). This was also similar to IP10 responses with no significant increase in the ability of trained animals to enhance their responses to pIC (**Fig 6.33D**). Overall, this data suggests that trained animals can enhance their production of IL6 in response to IN injected pIC, whereas TNF, IFN β and IP10 levels do not change.

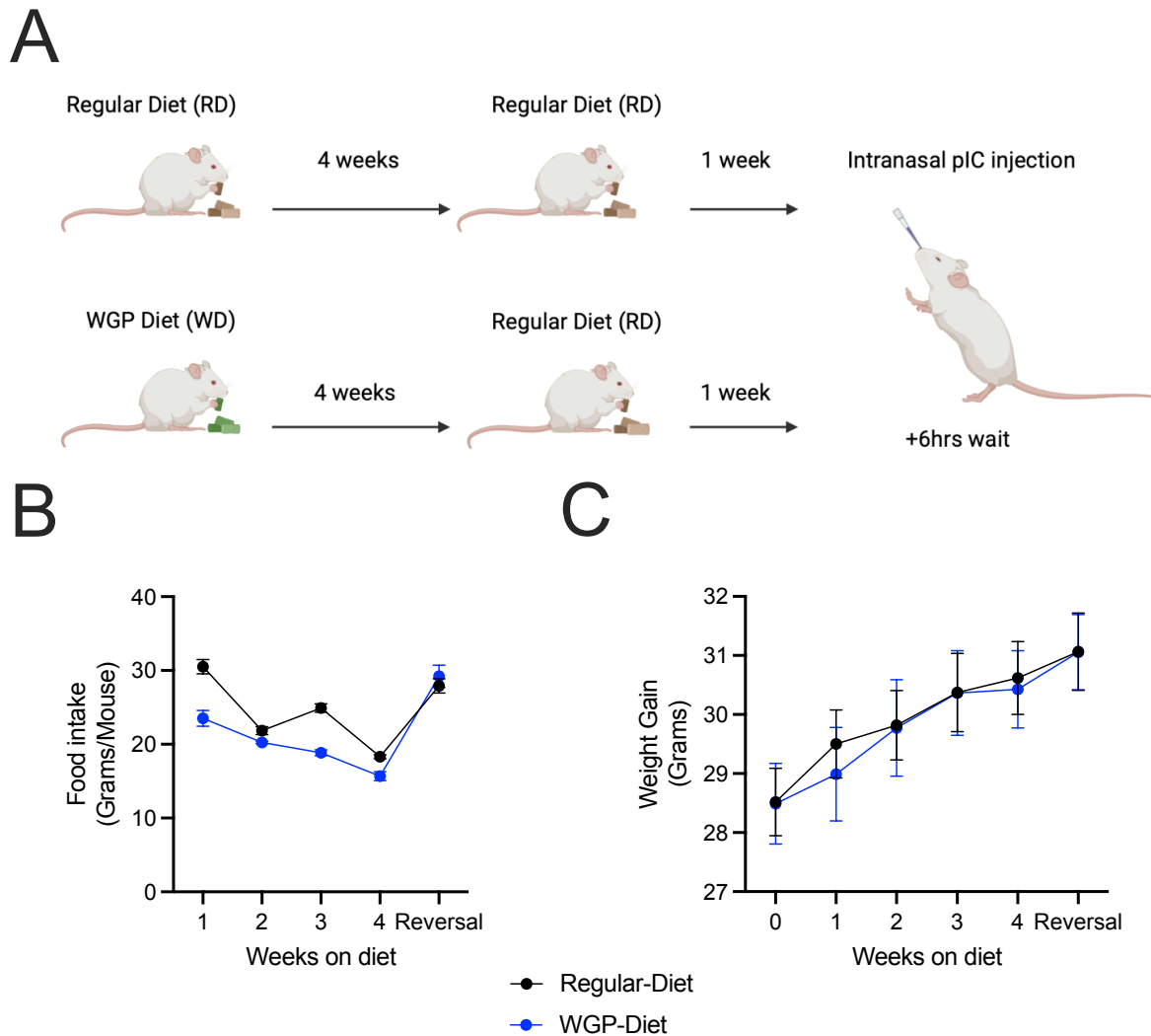


Figure 6.31 4-weeks WGP feeding and a return to regular diet has no significant impact on food consumption or weight gain in mice

Graph shows representation of experimental procedure and effects on food intake and weight gain of mice. Wild-type mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. (A) After 4-weeks mice were returned to a normal chow diet and intranasally injected with 1mg/Kg pIC for 6hrs to test for enhanced cytokine responses to viral ligands. The weight of food was measured over a weekly basis and (B) food consumption (intake) was calculated based on the weight of the food at the start of the week minus the weight of food by the end of the week divided by the average weight of the mice in each cage and mice were individually weighed each week and graph shows the gain of weight of mice on diets (C).

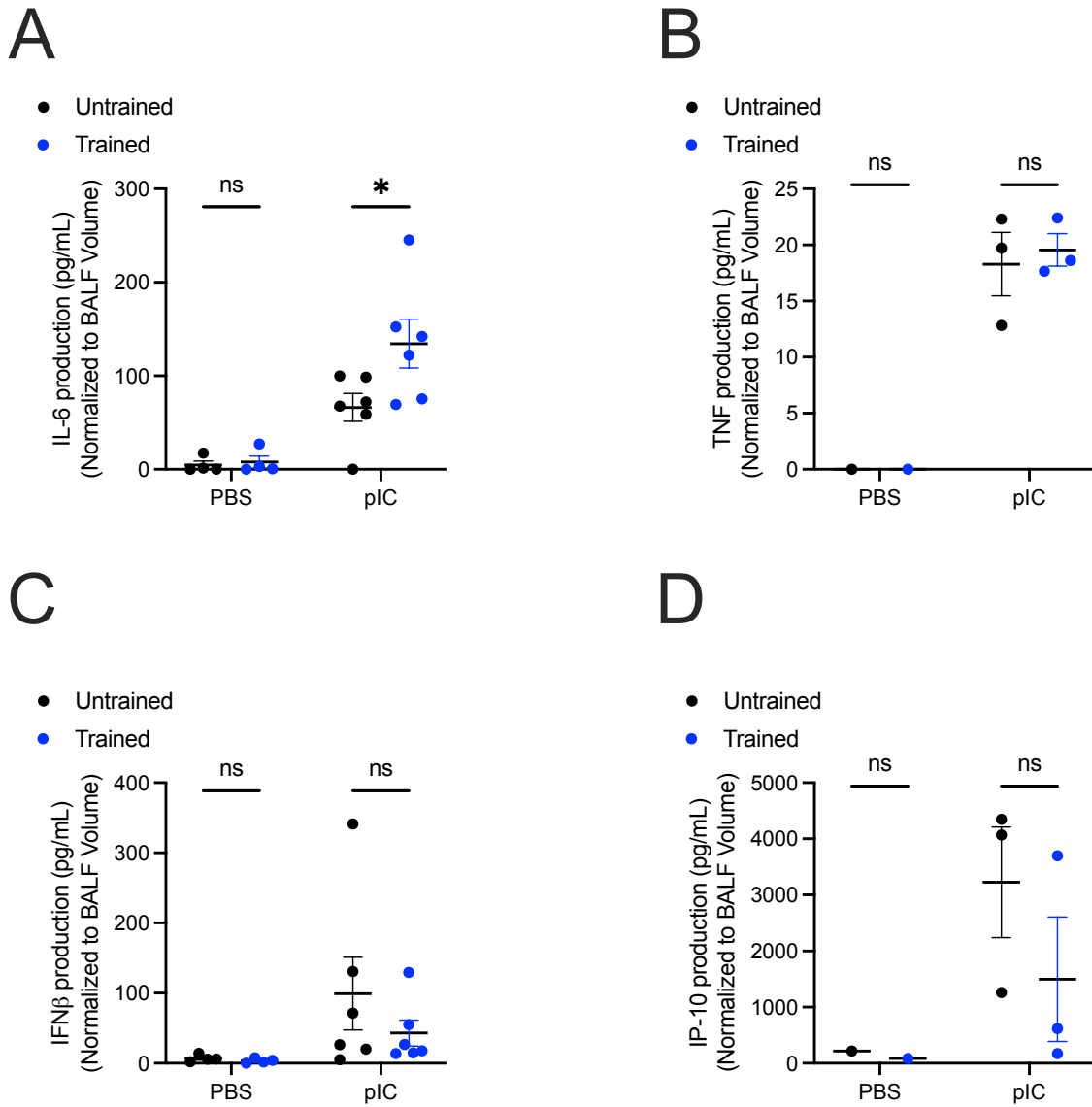


Figure 6.32 4-weeks WGP feeding leads to significant IL-6 responses to intranasal pIC challenge after a return to regular diet for 1-week

Wild-type mice were fed 0.05% W/W WGP enriched diet or standard control diet for a period of 4-weeks. After 4-weeks mice were returned to a normal chow diet and intranasally injected with 1mg/Kg pIC for 6hrs to test for enhanced cytokine responses to viral ligands. Bronchoalveolar lavage fluid (BALF) was taken from animals after CO₂ asphyxiation and levels of fluid obtained was noted. Cytokines were measured via ELISA and results were normalised relative to the amount of BALF retrieved per sample. Graph shows IL-6 levels (A), TNF levels (B), IFN β levels (C) or IP-10 levels (D) in PBS or pIC IN treated mice either on regular diet (untrained) or reversed from WGP diet (trained).

6.3 Discussion

So far throughout the thesis I have described the cellular responses to β -glucan from initial interaction, metabolic and epigenetic changes to anti-viral responses and the immunometabolic process via which macrophages facilitate enhanced IFN β production. In this chapter I sought to combine this in-vitro knowledge with my observations in with dietary feeding of WGP. As previously mentioned in this thesis, WGP is sold as an oral food supplement under the brand name Wellmune®. Therefore, I wanted to explore the immunological changes that WGP has in-vivo via an oral route. Most studies carried out with β -glucan have focused on IP injection route, which do not have a great physiologically relevant translatability to human models^{100, 101, 262}. It has been shown previously that gut macrophages, and not monocytes, can engulf and take up orally delivered β -glucan leading to increased resistance to infectious challenge^{272, 273}. Even though the ability of oral β -glucan delivery to induce trained immunity is well described, the mechanism via which this occurs is debated in the literature. Therefore, the goal of this chapter was to identify a functioning in-vivo mechanism via which oral delivery of β -glucan could induce cellular changes.

I first showed that upon oral delivery WGP was recognised by colon lamina propria macrophages. There were two possible hypotheses that could have been explored here. The first, was the potential that WGP could enter the gut and travel to the bone marrow via the blood stream and induce haematopoiesis. However, from the DTAF experiments there was no detection of fluorescent WGP in either blood or bone marrow macrophage niches. Therefore, the second hypothesis was explored, which was gut macrophages recognising WGP would induce a cytokine response. It is well known that gut macrophages can crosstalk with the bone marrow niche during stress²⁷⁴. Using data developed from previous chapters type I IFN was identified as a possible candidate for this process. Interestingly, type I IFN and downstream genes have been identified in multiple training models and diseases^{185, 213}. After 1-week of feeding with WGP I measured the levels of IFN in the blood serum as well as in the bone marrow aspirate. Although not significant there seemed to be low levels of IFN detectable in both the serum and aspirate of animals fed WGP for 1-

week. Here, I encountered my first real limitation in this chapter; reliably measuring low IFN levels. As of yet there is a need to develop a successful assay for the measurement of low cytokine levels in animals, especially in tissues such as blood and bone marrow. In humans the ultrasensitive single-molecule enzyme-linked immunosorbent assay (SIMOA) has been successfully used to measure baseline and low-grade IFN levels in humans^{275, 276}. To overcome this limitation, I used different techniques that are available for mouse work to show a consistent IFN signature across my animal work. I first utilised different concentrations of recombinant mouse IFN to train BM cells and see if this could induce a trained effect on the mature cells in-vitro. Here I saw that similar dosages to what I measured in-vivo were sufficient to drive a trained effect in-vitro in BMDMs.

I also explored the production of type I IFN, IFN α , via flow cytometry. It has been shown that the changes observed in the bone marrow, mainly haematopoiesis, caused by IP delivery of β -glucan stem from enhanced IFN signalling by bone marrow pDCs and CD169+ macrophages^{119, 262}. It has also been shown that type I IFN can also impact the expression and expansion of CD169+ macrophages in the bone marrow²⁷⁷. However, in my model there were no changes in cell number or percentage of pDCs or CD169+ macrophages in the bone marrow potentially indicating that direct sensing of β -glucan does not impact mature cell numbers in the bone marrow. However, after 1-week of feeding there was a percentage increase in the proportion of CD169+ macrophages producing IFN α in the bone marrow niche. This was not accompanied by an overall increase in IFN α MFI. However, this data just shows that overall, there are more cells producing IFN α , but each individual cell just produces the same relative amount. This overall was an exciting finding since it has never been shown that dietary feeding of any sort can impact IFN producing cells in the bone marrow.

Next, I wanted to explore these effects further. Expansion of bone marrow stem and progenitor cells known as HSPCs are a classical hallmark of trained immunity in-vivo^{228, 262}. Our lab has previously focused on the effects on bone marrow progenitors 4-weeks post feeding⁹⁹. However, for this work I wanted to investigate whether 1-week of WGP feeding was sufficient to alter HSPC compartments. I

showed significant changes in multiple HSC and progenitor populations showing potentially that in order to see nicer IFN responses earlier time points should be explored. This was a second limitation of this work. Multiple timepoints could, and should, be explored but for the focus of my PhD this was not feasible. However, this work is on-going for future publications. The third vital evidence to support interferon production as a source of WGP feeding mediated trained immunity came in analysing Sca-1 MFI. Sca-1 has been identified as an ISG²⁶⁵ in the bone marrow and 1-week WGP feeding significantly increased its expression (MFI) on LT-HSCs, MPPs and CMPs. This pointed to the role of IFN specifically in myeloid cells but also in exploring interferon levels at an earlier timepoint.

It has been shown throughout the literature that consistent exposure to type I IFN can lead to inhibition of bone marrow proliferation²⁷⁸. At the same time, it has been shown that type I IFN can also force bone marrow maturation due to loss of quiescence and self-renewal capacity²⁷⁹. I have already shown the results of this acute response with increased relative proportions of ST-HSCs, MPPs and other progenitors and a reduction in the proportion of LT-HSCs which are typically self-renewing and quiescent. However, I also showed that these cell type also lower their expression (MFI) of the glucose transporter, glut-1, in response to WGP feeding. This was accompanied by slightly lower puromycin uptake which a pseudo readout for metabolism. However, progenitors such as the total LKS, ST-HSCs, MPPs and CMPs were more sensitive to glucose metabolism inhibition potentially indicating a metabolic rewired phenotype. This trend has been shown to occur in type I IFN induced training of mature alveolar macrophages¹⁸⁵, so potentially this also points to a hallmark of type I interferons being induced by WGP feeding driving bone marrow changes and again potentially an earlier timepoint would show effects in more detail. Another limitation of this work was not exploring these responses in an IFNAR deficient animal or using anti-IFNAR antibodies. However, I am still optimizing these techniques and will hopefully introduce those concepts soon. One thing to note here is the novelty of exploring HSPC metabolism at the cellular level. This is poorly understood in the training field with most of the emphasis being put on mature cells. However, for the first time I have demonstrated that each progenitor cell type has slightly different metabolic responses to dietary feeding with WGP.

The initial part of this chapter dealt with changes that occur during early exposure to WGP, 3 days to 1-week. However, as I have mentioned before WGP is sold under the brand name Wellmune®. The concentration W/W that we feed to the mice is equivalent to the recommended dose as sold to humans and each box contains enough for 4-weeks supply. Therefore, I wanted to investigate how 4-weeks feeding would impact the mammalian immune system systemically. Firstly, IFN β levels had significantly elevated after 4-weeks within the bone marrow, without impacting inflammatory cytokines and chemokines such as IP-10. Similarly to our 1-week experiments, 4-weeks of feeding also led to an expansion of the relative proportion of LKS⁺ cells within the bone marrow in wild type mice. However, since I was interested in the impact of IFN signalling, I investigated this effect within IFNAR^{-/-} animals and saw no significant expansion. Throughout the thesis I split the graphs between wild type and knock out due to the differences in variance in the knockouts at the later stage of the results section. I did not deem it appropriate to run Two-Way ANOVA in this case, since this test assumes equal variance between groups (not the case with the knockouts) and with the low N numbers used, would lead to high type I errors. Therefore, before comparing via T-tests with Welch's correction, which does not assume equal variance between groups, I log transformed the data and ran Two-Way ANOVAs to test for significance between groups. Here I still saw significant differences between wild type and knock outs and therefore I was able to conclude that loss of IFN signalling significantly impacted the ability of WGP feeding to regulate bone marrow haematopoiesis. This isn't surprising, however, since other groups have shown the importance of IFN in driving bone marrow expansion^{35, 262}. However, importantly this shows the impact that systemic type I interferon levels I saw at the early timepoint has during 4-weeks of continuous feeding. These results were accentuated by further analysis of the progenitor populations within the LKS. Unlike at 1-week when all the relative proportions of these progenitors were increased, except the LT-HSCs, 4-weeks of feeding led to significant reduction in LT-HSCs and ST-HSCs in wild type mice. Again, this did not occur within the IFNAR^{-/-}. I hypothesize that IFN signalling pushes LT-HSCs and ST-HSCs out of their quiescent state and forces them to differentiate and mature. This was seen with the increase in the relative proportion of MPP cells within the LKS in wild type animals that was not seen within the knockouts.

Because of these results I hypothesised that more mature progenitor cell populations as well as circulating cells would increase in the wild type. However, neither the more mature progenitor cells, CMPs, GMPs or CLPs, nor the circulating cells, monocytes, neutrophils and T cells, were increased in wild type animals. Here I predict that I missed the time point for when these cells egress from the bone marrow and enter the circulation. This was similar in the IFNAR^{-/-}, although I did see that WGP increased the proportion of monocytes in the circulation. However, this might just be due to lower frequency of other cell types within the blood. Importantly however, WGP feeding lead to a significant increase in markers of epigenomic changes linked to trained immunity incirculating cells, which was not seen in the IFNAR^{-/-}. This could suggest that IFN signalling imprints epigenetic signatures on bone marrow progenitors which is then carried onto mature immune cell populations. It has been shown that type I IFN can induce epigenetic changes in B cells postinfection²⁸⁰ and has been shown to imprint chromatin marks on fibroblasts and macrophages¹⁸⁴. My future goal here is to explore these epigenetic marks and explore whether they stem from a bone marrow origin. In conjunction with this, I was able to show that bone marrow progenitors differentiated ex-vivo into mature BMDMs maintained an epigenetic profile and enhanced their IFN responses to viral challenge. Therefore, it would be interesting to explore whether these mature circulating cells also had enhanced responses to viral challenges.

To try and answer this question I investigated changes in the lung compartment. Although I did not see significant changes in the relative proportion of the cellular landscape, there was a slight but not specific changes in alveolar macrophages, again dependent on interferon signalling. This was due to a change in the origin of the alveolar macrophages with an increase in infiltrating BMoAMs. It has been shown that these cells accumulate after viral infection, such as influenza (IAV) infection, and have a greater cytokine potential than resident FeMoAMs that can protect mice from subsequent bacterial infections⁹³. Interestingly, these BMoAMs differ in their ability to produce IL6, with most other cytokines not changing when faced with acute bacterial ligand or live infection⁹³. This is an interesting result since in response to IN injection of pIC, which would impact the alveolar space, mice fed WGP and reverted to a standard diet for 1-week had enhanced IL6 responses. In

terms of anti-viral immunity this is a fascinating result. IL6 has been shown to be vital in suppressing IAV responses in the lung by modulating antibody and complement responses and enabling the generation of T follicular helper (Tfh) cells²⁸¹. IL6 has also shown to be important by preventing IAV mediated neutrophil cell death enabling for proper clearance of IAV from the lungs by neutrophils^{282,281}. IL6 has also shown to be important in preventing IAV mediated neutrophil cell death enabling for proper clearance of IAV from the lungs by neutrophils²⁸². I only investigated short term cytokine responses to pIC (6hrs) with IL6 being the only significantly different cytokine. However, it would be interesting to explore which cell types are producing the IL6 and are there any changes in cytokine levels at later timepoints. This will be vital in understanding the impact WGP feeding might have on protection against a live viral infection since sustained IL6 itself can lead to lung damage through cytokine storm²⁸³. However, from my in-vitro data it looks as though trained mature cells have a balanced response to viral infection, that is, they rapidly increase cytokine during the early phase and then switch off genes more rapidly entering a more repair phase. Therefore, I predict this will also be the case for mature trained cells in-vivo.

In conclusion, I have shown in this chapter that dietary feeding of WGP leads to rapid bone marrow changes accompanied by systemic IFN levels, ISG changes in the bone marrow and metabolic reprogramming making stem cells more sensitive to glucose metabolism. Further feeding leads to increased IFN in the bone marrow aspirate, expansion of MPP subsets and epigenetic changes in circulating cells dependent on type I IFN signalling. Maturation of bone marrow cells ex-vivo leads to mature macrophages that maintain an epigenetic signature and respond better to viral challenge. Finally, feeding leads to expansion of BMoAMs in the alveolar spaces, which when challenged with a viral ligand increase IL6 production, potentially mediated through these cell types.

Chapter 7. General Discussion

7. 1 Discussion

Since the dawn of humankind we have been afflicted by numerous plagues that have shaped our evolution. The development of antibiotics has greatly halted the threat of bacterial pandemics, with the last major global bacterial pandemic occurring during the “Black Death”. However, since the turn of the 21st century global health authorities have already had to face 3 different emerging pandemic viruses, Severe-acute respiratory syndrome Corona virus (SARS-CoV), H1N1 Influenza A virus (IAV) and SARS-CoV2). This is coupled with global health fighting against existing viruses with pandemic potential, such as Zika virus (ZIKV) and Ebola virus (EBOV), as well as dealing with the ramifications of the impact previous viral pandemics have caused on global health and social prejudice, such as the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS). Due to the rapid and unpredictable nature of the spread of these diseases, oftentimes going undetected for months to years before discovery^{284, 285}, it can be difficult to develop quick and successful vaccines. However, due to the non-specific nature of trained immunity many have hypothesized using different training agents as a “stop-gap” between emerging viral pathogens and successful vaccines or therapies^{286, 287}. The overarching hypothesis from this thesis was that yeast β -glucan - because of its more amenable nature as a dietary supplement - could induce trained immunity that could be harnessed against viral infections. This was then furthered upon by exploring the molecular mechanisms that underpin this response, before finally assessing its importance on the mammalian immune system in-vivo.

Work from this thesis identified *S. cerevisiae* derived whole glucan particle (WGP) β -glucan as an effective training agent, due to its' ability to activate the Dectin-1 receptor and drive immunometabolic changes that enhanced epigenetic modifications. WGP has already been shown to work well against tumours, driving enhanced survival in mice when given as an IP injection¹⁰⁰. The work in this paper highlights the importance of these metabolic alterations, specifically the role of mitochondrial metabolism which I demonstrate as a key feature of WGP-induced trained immunity. Mitochondrial ROS is a driver of the anti-tumour response & although I did not explore it in the context of anti-tumor immunity, in this thesis,

others from our lab have recently highlighted its' importance. Secondly, I also demonstrated the ability of WGP to enhance pro-inflammatory cytokine responses upon secondary challenges highlighting its' ability to be used in several different contexts. For example, WGP has been shown to enhance the effectiveness of PD-1/PDL-1 checkpoint blockade for immunotherapy through the inhibition of FoxP3+ T regulatory cells and in human patients enhanced cytokine levels in the serum in a synergistic manner with immunotherapy²⁸⁸. These results highlight the power of WGP's non-specific nature through direct effects, metabolism and cytokine synergy, without even touching on epigenetic and long-term memory responses.

Further, it has recently been shown that fungal ligands, such as chitin, can induce protective anti-tumour immunity via a cGAS-STING-IFNAR mediated axis²⁸⁹. In this way, cGAS is activated due to mitochondrial stress that induces release of mitochondrial nucleic acids that then activates IFNAR. The sustained IFN response leads to better maturation and antigen presentation of DCs to activate CD8+ T cell responses. In our in-vitro setting, we similarly see that WGP stimulation of macrophages lead to mtRNA release which can activate and sustain the IFN response in macrophages. My RNA-seq analysis reveals that both the NF- κ B responses are activated by WGP leading to a balanced pro-inflammatory and IFN response. It would be interesting to explore whether WGP stimulation of dendritic cells could drive a similar or more potent IFN response, maybe even working as the mechanism via which WGP enhances anti-tumour responses.

However, in this thesis I focused on the ability of WGP to drive anti-viral immune responses. I saw early on that treatment with WGP enhanced trained responses to viral ligands, particularly TLR3 and MDA5/RIG-I responses. While developing this mechanism, I considered whether enhanced anti-viral responses were the result of the metabolic alterations I had seen or whether enhanced metabolic responses were in response to changes in IFN signalling. Firstly, in response to type I IFNs DCs enhance oxidative metabolism, in particular fatty acid oxidation (FAO)¹⁶⁴. In this work the authors show that increased metabolism is induced via import of pyruvate as well as enhanced FAO dependent on PPAR α . In my work I mainly investigated the role of glucose metabolism, either through glycolysis or through

pyruvate import into the TCA cycle. My isotope tracing results showed that 3-days post WGP training, only three metabolites were significantly different, fumarate ($p = 0.08$), succinate and itaconate. However investigating the isotopologues, I did not see any increases in overall glucose flux. I did see that WGP treated macrophages increased their overall up take of glucose, however, coupled with the inhibitor data showing 2DG inhibition does not impact trained responses, I do not believe increased glycolysis is vital for WGP mediated trained responses. This may rather represent a bystander effect of initial activation. This has actually been confirmed in other contexts using conditional knock outs of raptor and HIF-1 α , showing that peritoneal macrophages trained with WGP still maintain their ability to enhance TNF responses¹⁰⁰. However, I have not explored other fuel types such as glutamine or fatty acids which is something I am keen to explore moving forward.

However, I was successfully able to show that IFN was not the driver of metabolic responses. IFNAR knock out BMDMs still maintained their ability to ramp up glycolysis and OxPhos as well as changes in TCA cycle genes in response to WGP-stimulation. Therefore, I turned my attention to how metabolic changes can impact the trained responses, specifically enhanced IFN. It has been previously shown that changes in glutamine metabolism and fumarate can lead to trained immunity²³⁹. Similarly, here I showed that derivatives of fumarate, dimethyl fumarate (DMF), was sufficient in enhancing anti-viral trained responses. Moving forward I want to investigate whether fumarate training causes similar epigenetic signatures as WGP and whether fumarate is the leading metabolite linking metabolic changes and epigenetic ones. However, I also focused my attention on how anti-viral signalling occurs after WGP treatment, since not much is known about anti-fungal innate responses and interferon signalling. others have shown that changes in TCA cycle gene expression, *Fh* and *Irg1*, are associated with changes in IFN expression I explored these changes in my in-vitro training model^{243, 248}. I saw that WGP significantly enhanced *Irg1* expression and reduced *Fh* expression independent of IFN signalling and similarly lead to changes in mitochondrial polarisation and release of mtRNA into the cytosol. Since exogenous fumarate, but not itaconate, also led to similar trained responses, I hypothesize that changes in fumarate levels may link classical training to our proposed novel interferon dependent training (IDT)

mechanism. To further touch on this mechanism it has been shown that fumarate accumulation is linked to changes in the aspartate-argininosuccinate shunt^{248, 249}. Since I also saw that WGP stimulation leads to changes in the expression of argininosuccinate synthetase 1 (ASS1) I predict that this pathway enables trained macrophages to have non-specific responses as well as epigenetic changes, since this enables fumarate to accumulate outside of the mitochondria and thus enable for interactions with chromatin. However, these experiments unfortunately could not be completed in time for this thesis but will be explored in the future. I therefore propose that the downregulation of *Fh* is a key step in WGP induced trained immunity with potential changes in the argininosuccinate shunt allowing for accumulation of fumarate in the cytosol^{248, 290, 291}, which is known for its importance in driving epigenetic changes²³⁹. Coupled with this classical training effect of fumarate accumulation, reduction in *Fh* leads to mitochondrial hyper polarisation which causes stress leading to the release of mt nucleic acids^{248, 291}. These can be recognised by cytosolic PRRs, such as MDA-5 and RIG-I which lead to a primed anti-viral response through type I IFN signalling. I further propose that coupled with this priming effect, IFN can directly impact chromatin “opening” anti-viral genes for a more robust anti-viral response²⁹²

More importantly, however, was the fact that dietary feeding of WGP not only lead to changes in the immune phenotype around the mammalian body, but also led to IFN signalling changes, specifically in bone marrow CD169+ macrophages. I propose the following mechanism for how this occurs (**Fig 7.1**). Since macrophages in the gut recognise WGP during dietary feeding coupled with the in-vitro data generated I hypothesize that these macrophages produce type I IFN, potentially through FH reduction, which feeds back onto the bone marrow leading to sustained IFN production by CD169+ macrophages to trigger maturation and loss of stemness of LT-HSCs which drives haematopoiesis. Changes in bone marrow metabolism and structure leads to epigenetic changes in immature immune cells which exit the bone marrow and seed peripheral tissue such as the lungs. Here these cells have enhanced responses, particularly IL6, in response to viral ligand challenge. Unfortunately, I was not able to test this mechanism against a live viral challenge in-vivo, however, this has since been planned and with the evidence I have available I believe dietary WGP feeding could be used as a useful tool in bridging the gap

between emerging viral pathogens and successful vaccination or therapeutic strategies.

However, a potential limitation to the application of this proposed mechanism relates to the potent ability of IFNs to drive pathology in the literature. Type I IFNs although originally discovered as anti-viral cytokines vital for immunology have also been linked to numerous autoimmune diseases²⁹³. For example, in certain cases of systemic lupus erythematosus (SLE) gain-of-function mutations in TLR7 leads to excessive type I IFN worsening pathology²⁹⁴. In both the adult and juvenile form of dermatomyositis (DM) type I IFN correlates with how effected organs are²⁹⁵. Similarly, monocytes from patients with primary Sjögren's syndrome (pSS) have enhanced IFN signature as well as increased infiltration of pDCs into effected organs^{296, 297}. There is also already literature evidence suggesting a role for IDT in these diseases with groups showing that patients with SLE have monocytes which express lower *Fh* than monocytes from healthy controls, potentially linking metabolic changes with abhorrent IFN levels in these patients²⁴⁸. Similarly, many patients suffering from these types of diseases have an increased risk of developing cardiac issues, through the development of athrosclerosis^{298, 299}. Groups have started to identify the role of trained immunity in the development of atherosclerosis in these patients, however, I believe we may have uncovered the metabolic mechanism underpinning these changes²¹³.

Another possibility is the length and strength of the type I IFN induced. Recent work has shown that tonic sustained IFN signalling is required for maintaining innate cells in a "ready" state, primed for infection. Here, the authors showed that certain microbes, specifically *Clostridium scindens*, can produce factors like deoxycholic acid which can pass through the epithelial layer of the gut and prime pDCs and monocytes to help contain viral infections³⁰⁰. In preliminary work our Lab has shown that WGP feeding does not impact the diversity of the gut microbiome, however, it would be interesting to investigate whether bacterial derived factors and metabolites may have changed which could help drive a tonic and low-grade IFN signature. Therefore, it would be interesting to explore the impact of feeding in mice that have been administered antibiotics, adding clinical relevance to our studies if the dietary supplementation is to be taken to the clinic.

In contrast, in the pathologies described above chronic high-grade IFN signalling may be the root cause of inflammatory conditions. Adaptive immune cells, particularly T-cells, are sensitive to chronic IFN recognition. Groups have shown that sustained IFN can lead to increased expression of co-inhibitory molecules on T-cells, such as PD-1 and LAG-3³⁰¹, both markers of T-cell exhaustion. In SLE, type I IFN from APCs causes dysregulated Treg function. These effects were highlighted ex-vivo with healthy human donor APCs rescuing the function of SLE+ Tregs, yet APCs from SLE patients inhibited the function of healthy donor Tregs³⁰². The authors showed that the ability of APCs to suppress Treg function correlated with the levels of IFN produced, therefore implicating chronic high-grade IFN in pathologies. Therefore, I predict that WGP feeding induce a “Goldilocks” IFN signature, enough to drive immune cell expansion, epigenetic changes and trained immunity, but not enough to drive pathologies. It would be interesting to further explore whether sustained feeding may have negative impacts on immune cell functions or to explore feeding in the context of autoimmune patients.

Similarly, the fact that WGP can be given as an oral supplement raises interesting possibilities. For example, as of yet there have been no effective vaccine for diseases that affect the gut, such as *H. pylori*³⁰³. This is in part due to the tolerogenic nature of gut microenvironment and the difficulty in directing adjuvants, which help activate the innate immune response for effective vaccines, to this area as well. Since I have shown that WGP causes changes in immune populations in the tolerogenic environment such as the lung it would be interesting to investigate if gut innate immune cell populations are altered. Preliminary data from the lab (not shown in this thesis) shows that WGP increases the numbers of monocytes and macrophages in the gut after 4-weeks of feeding, making oral delivery of WGP and attractive oral adjuvant which could be paired with novel vaccines. Coupled to this idea of WGP’s role in vaccine development is it’s use as a tool for protecting vulnerable people who may be vaccine hesitant or “anti-vax”. These people may be more likely to agree to oral supplementation over injection strategies, which would help increase general immunity in the population.

Taken together the data from this thesis not only identifies a novel approach for boosting responses to viral infection but also provides a hypothetical metabolic mechanism underpinning type I interferonopathies and subsequent co-morbidities. Therefore, further study of this pathway could identify key targets for therapeutic potential which could help target one of the biggest threats to developed countries, heart disease³⁰⁴.

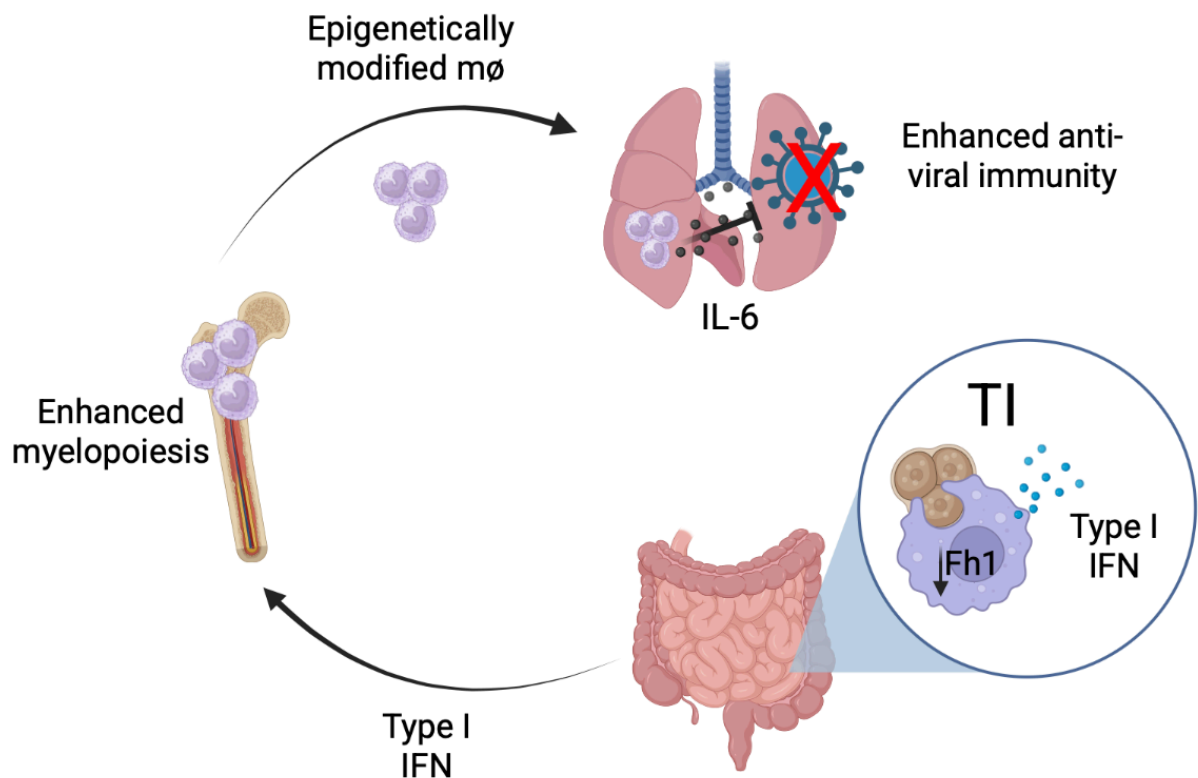


Figure 7.1 Proposed hypothetical model for in-vivo trained responses to WGP dietary feeding

WGP is fed to mice as previously described and is taken up by gut resident macrophages or monocytes. In-vitro evidence points towards FH changes in these macrophages leading to reduced expression. Even though I have shown that macrophages respond with weak IFN responses to WGP, the changes in FH could lead to a feedforward loop enhancing IFN levels which can impact the bone marrow. Once in the bone marrow type I IFN can trigger further IFN responses by CD169+ macrophages leading to loss of stemness of LT-HSCs and maturation down the haematopoietic tree. Changes in metabolism of these cells may lead to epigenetic differences which is maintained in monocytes and immune cells in the circulation, dependent on type I IFN. These cells seem to seed the lung compartment, which causes enhanced IL6 responses to viral ligand challenge.

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