UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Modulation of macrophage metabolism and function by the helminth peptide FhHDM-1

by Susel Mayra Loli Quinteros BSc. (Hon)

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

under the supervision of A/Prof Sheila Donnelly and A/Prof Bronwyn O'Brien

University of Technology Sydney
Faculty of Science

October 2023

Certificate of Authorship/ Originality

I certify that the work on this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as part of the collaborative doctoral degree and/or fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information source and literature used are indicated in the thesis.

Susel Mayra Loli Quinteros Production Note: Signature removed prior to publication. Date: 20/10/2023

This research has been supported by an Australian Government Research Training Program Scholarship.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisors, Associate Professor Sheila Donnelly and Associate Professor Bronwyn O'Brien for their constant guidance and incredible support throughout these challenging and fulfilling years. I truly appreciate this incredible opportunity and I am very thankful for your kindness and for being as passionate about my project as I am. I am really humbled and impressed by how passionate you both are as scientists, thank you for showing me how much you love science.

I would also like to thank the entire laboratory team for making this process incredibly enjoyable, especially a massive thank you to Joyce To, for the immense help and guidance with my experimental work, thank you so much for coming to my aid when I needed it the most. Another big thank you to Inah Camaya, Akane Tanaka, Iris Cheng, and Alison Ricafrente for your unwavering friendship and for sharing your invaluable knowledge in scientific techniques, thank you so much for making working in the laboratory fun and exciting. To Fiona Ryan and Lalit Overlunde, thank you for the massive amount of support towards becoming competent and highly confident with working with animals in research.

To my family and friends for their support, especially to my husband John, who has been helping me behind the scenes by keeping a constant supply of snacks and caffeinated drinks. To Allegra, you make me want to be the best possible version of myself, a single look at your little face and I am re-invigorated to keep working towards achieving my goals as a scientist. I hope that we can share many more exciting scientific achievements in the future.

Finally, I would like to thank the markers for taking time in their busy schedules to review my theses, and the Australian Commonwealth Government Department of Industry for the Australian Postgraduate Award Scholarship I received throughout my PhD candidature.

Publications in peer-reviewed journals arising from PhD research

Loli Quinteros *et al.,* 2022, 'Exploring the role of macrophages in determining the pathogenesis of liver fluke infection', *Parasitology.* 2022 Sep; 149(10): 1364-1373.

Loli Quinteros *et al.*, 2023*,* 'The helminth derived peptide FhHDM-1 redirects macrophage metabolism towards glutaminolysis to regulate the pro-inflammatory response, *Front Immunol.* 2023 Jan 25; 14:1018076.

Loli Quinteros *et al.,* 2023, 'The helminth derived peptide, FhHDM-1, reverses the trained phenotype of NOD bone marrow derived macrophages to regulate pro-inflammatory responses'. Currently under review.

Scientific presentations arising from PhD research

The parasite -derived peptide, FhHDM-1, alters the metabolic activity of macrophages to prevent pro-inflammatory responses.

Susel Loli Quinteros, Akane Tanaka, Joyce To, Bronwyn O'Brien, John Dalton, Sheila Donnelly. Poster presentation. The New Horizon Conference (36th Combined Health Science Conference) 2019.

The parasite-derived peptide, FhHM-1, alters the metabolic activity of macrophages to prevent pro-inflammatory responses.

Susel Loli Quinteros, Akane Tanaka, Joyce To, Nathaniel Snyder, Bronwyn O'Brien, John P. Dalton, Sheila Donnelly. Poster presentation. Foster Conference 2019.

The Parasite-Derived Peptide, FhHDM-1 regulates macrophage metabolism to support an anti-inflammatory phenotype.

Susel Loli Quinteros, Nathaniel Snyder, Bronwyn O'Brien, Sheila Donnelly. Poster presentation. European Macrophage and Dendritic Cell Society Conference 2020.

The parasite-derived peptide, FhHDM-1, reprogrammes macrophage metabolism to support a tolerant phenotype.

Susel Loli Quinteros, Nathaniel Snyder, Bronwyn O'Brien, Sheila Donnelly. Poster presentation. European Macrophage and Dendritic Cell Society Conference (31st Annual Conference) 2021.

Table of Contents

Certificate of Authorship/ Originality ii

Acknowledgements iii

Publications in peer-reviewed journals arising from PhD research iv

Scientific presentations arising from PhD research v

List of figures ix

List of tables xi

List of abbreviations xii

Abstract xvi

Chapter 1: Exploring the role of macrophages in determining the pathogenesis of liver fluke infection 1

1.1 Publication Statement 2

1.2 Abstract 3

1.3 Introduction 4

1.4 Life cycle of liver flukes 4

1.5 Polarization and functional activity of macrophages 7

1.6 Macrophage activation and function during liver fluke infection 9

1.7 Activation of macrophages by fluke-derived molecules 13

1.8 Concluding remarks 17

Chapter 2: The parasite-derived peptide FhHDM-1 reprogrammes the metabolic activity of macrophages 19

2.1. Publication Statement 20

2.2. Introduction 21

2.3. Materials and Methods 24

2.3.1. Production of Parasite-derived Peptides by Chemical Synthesis 24

2.3.2. Isolation and Culture of Bone Marrow-Derived Macrophages (BMDMs) 24

2.3.3. Isolation of Murine Peritoneal Macrophages 24

2.3.4. RNA extraction, cDNA Synthesis and qPCR 25
2.3.5. Analyses of Metabolic Pathways by Measuring Extracellular Flux 26
2.3.6. Normalization of Cell Number by CyQuant Cell Proliferation Assay....27
2.3.7. Acyl-CoA Mass Spectrometry 28
2.3.8. Measurement of Intracellular Metabolites 28
2.3.9. Statistical Analysis 29
2.4. Results 30
2.4.1. FhHDM-1 directed macrophages to utilise oxidative phosphorylation. .30
2.4.2. FhHDM-1 induced oxidative phosphorylation (OXPHOS) is fuelled by fatty acids. 34
2.4.3. The FhHDM-1 induced metabolic switch to oxidative phosphorylation (OXPHOS) in macrophages is associated with endogenous fatty acid synthesis concomitant with increased glutaminolysis. 37
2.5. Discussion.....40
Chapter 3: FhHDM-1 reprogramming of metabolic activity supports an anti-inflammatory phenotype in macrophages.....43
3.1. Publication statement.....44
3.2. Introduction 45
3.3. Materials and Methods 47
3.3.1. Methodology previously described 47
3.3.2. Quantification of TNF and IL-6 Protein Secretion by Macrophages.....47
3.3.3. Measurement of Hexokinase Activity.....47
3.3.4. Statistical Analysis 48
3.4. Results 49
3.4.1. FhHDM-1 treatment of macrophages suppressed the pro-inflammatory response and reduced glycolysis, independently of a switch to OXPHOS.....49
3.4.2. The regulation of macrophage inflammation by FhHDM-1 was dependent on the induction of glutaminolysis.....54

3.4.3. Increased glutaminolysis in FhHDM-1 treated macrophages was not associated with an increased abundance of succinate. 56

3.4.4. The regulation of inflammation by FhHDM-1 was dependent on fatty acid oxidation. 59

3.5. Discussion 61

Chapter 4: The helminth derived peptide, FhHDM-1, reverses the trained phenotype of NOD bone marrow derived macrophages to regulate pro-inflammatory responses 63

4.1. Introduction 64

4.2. Materials and Methods 66

4.2.1. Previously Described Methodology 66

4.3.2 Treatments of Mice with FhHDM-1 66

4.3.3 Western Blot Analyses 67

4.3.4 Statistical Analysis 67

4.4 Results 68

4.3.2 FhHDM-1 reprogrammed the metabolic and inflammatory activities of bone marrow derived macrophages *in vivo*. 68

4.3.3 Bone marrow derived macrophages from Non-Obese Diabetic (NOD) mice displayed the characteristics of a trained immune cell. 72

4.3.4 FhHDM-1 downregulated the epigenetic markers to regulate, but not fully suppress, the pro-inflammatory immune response of NOD bone marrow derived macrophages. 77

4.4 Discussion 82

Chapter 5: General Discussion 88

Bibliography 98

List of figures

Figure 1.1: Comparative life cycles and intra-mammalian migratory pathways of food-borne liver flukes……..6

Figure 1.2: Comparative activation of macrophages and pathological consequences of liver fluke infection……...11

Figure 2.1: Unlike IL-4, treatment of macrophages with FhHDM-1 enhanced oxidative phosphorylation (OXPHOS) without modulating glycolytic activity……..31

Figure 2.2: FhHDM-1 enhanced oxidative phosphorylation (OXPHOS) in peritoneal macrophages after a single i.p. injection……..32

Figure 2.3: FhHDM-1 did not alter the expression of typical M2 genes…….33

Figure 2.4: Oxidative phosphorylation (OXPHOS) induced by FhHDM-1 has a greater dependency on fatty acids than IL-4 driven metabolic activity……..….35

Figure 2.5: Oxidative phosphorylation (OXPHOS) induced by FhHDM-1 does not utilise exogenous fatty acids……..……..36

Figure 2.6: FhHDM-1 induced glutaminolysis and synthesis of endogenous fatty acids..…..38

Figure 2.7: Oxidative phosphorylation (OXPHOS) induced by FhHDM-1 is fuelled by glutaminolysis and the synthesis of endogenous fatty acids……39

Figure 3.1: FhHDM-1 inhibited the secretion of pro-inflammatory cytokines in response to LPS stimulation via a mechanism that is dependent on the inhibition of lysosomal vATPase……51

Figure 3.2: FhHDM-1 treatment of macrophages reduced LPS activation of the glycolytic pathway..…….52

Figure 3.3: The reduction in glycolysis in FhHDM-1 treated, LPS stimulated macrophages was not associated with an increase in oxidative phosphorylation (OXPHOS)……..53

Figure 3.4: The reduction in glycolysis and pro-inflammatory cytokine production in FhHDM-1 treated macrophages was dependent on the induction of glutaminolysis……55

Figure 3.5: The increased ratio of KG to succinate in FhHDM-1 treated macrophages did not correlate with the reduced expression of pro-inflammatory cytokine genes……58

Figure 3.6: The regulation of pro-inflammatory cytokine production by FhHDM-1 was dependent on fatty acid oxidation…….60

Figure 4.1: Treatment of mice with FhHDM-1 reduced glycolytic activity in bone marrow derived macrophages stimulated with LPS *ex vivo*……..…70

Figure 4.2 Treatments of mice with FhHDM-1 regulated the pro-inflammatory response of bone marrow derived macrophages to stimulation with LPS *ex vivo*…….……71

Figure 4.3: Lysine 3 on Histone 3 was mono- and tri- methylated in bone marrow derived macrophages from NOD mice……..74

Figure 4.4: Bone marrow derived macrophages from NOD mice had higher glycolytic activity than macrophages from BALB/c mice……..…...75

Figure 4.5: Bone marrow derived macrophages from NOD mice produce an increased pro-inflammatory response to LPS stimulation *ex vivo*, as compared to macrophages derived from the bone marrow of BALB/c mice……..……..76

Figure 4.6: Methylation of H3K4 was reduced in bone marrow derived macrophages from NOD mice that had been treated with FhHDM-1……..78

Figure 4.7: Treatment of NOD mice with FhHDM-1 significantly reduced the glycolytic activity induced by LPS stimulation of bone marrow derived macrophages *ex vivo*…….…..79

Figure 4.8: Treatment of NOD mice with FhHDM-1 reduced the production, but not gene expression levels of TNF and IL-6, in response to LPS stimulation of bone marrow derived macrophages *in vitro*…….....80

Figure 4.9: Treatment of NOD mice with FhHDM-1 prevented the enhanced (trained) pro-inflammatory response of macrophages to LPS……...81

Figure 5.1: Proposed mechanism of action of FhHDM-1 within macrophages.……93

List of tables

Table 1.1: The impact of macrophage polarization by treatments with liver fluke…….16

Table 2.1: qPCR primers specifically used within chapter 2……...25

Table 3.1: qPCR primers specifically used within chapter 3……...47

Table 4.1: qPCR primers specifically used within chapter 4……...66

List of abbreviations

UK5099	2-Cyano-3-(1-phenyl-1H-indol-3-yl)-2-propenoic acid
2-DG	2-deoxy-glucose
Acetyl-CoA	Acetyl coenzyme A
ATP	Adenosine triphosphate
FhPrx	Anti-oxidant peroxiredoxin of *Fasciola hepatica*
α-KG	Alpha-ketoglutarate
Arg-1	Arginase-1
ATP	Adenosine triphosphate
BafA1	Bafilomycin A1
BPTES	Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl) ethyl sulfide
BMDM	Bone marrow derived macrophage
BSA	Bovine serum albumin
Clec7a	C-type lectin domain containing 7A
CCA	Cholangiocarcinoma
EAE	Experimental autoimmune encephalomyelitis
cDNA	Complementary Deoxyribonucleic acid
CD	Cluster of differentiation
COX-2	Cyclooxygenase-2
FhCL	Cysteine proteases of Fasciola hepatica
^{o}C	Degrees Celsius
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
ER	Endoplasmic reticulum
EGF	Epidermal growth factor
FhES	Excretory/Secretory products of *Fasciola hepatica*
ECAR	Extracellular acidification rate
ERK	Extracellular signal-regulated kinase
FAO	Fatty acid oxidation
FAS	Fatty acid synthesis
FGF1	Fibroblast growth factor 1

FCS	Foetal calf serum
FCCP	Carbonylcyanide-p-trifluromethoxyphenylhydrazone
GC	Glucocorticoids
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
FhHDM-1	Helminth Defence Molecule-1 of *Fasciola hepatica*
OvHDM	Helminth Defence Molecule of *Opisthorchis viverrini*
Hk1	Hexokinase-1
HIF1α	Hypoxia-inducible factor 1-alpha
iNOS	Inducible-nitric oxide synthase
Ic	Immune complexes
IFN-γ	Interferon Gamma
IL	Interleukin
IDH2	Isocitrate dehydrogenase
JNK	Jun N-terminal kinase
JMJD3	Jumonji domain-containing protein 3
KLH	Keyhole limped haemocyanin
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
M	Mole/ molar
M-CSF	Macrophage colony stimulating factor
M1	Macrophages associated with T-helper 1 immune response
M2	Macrophages associated with T-helper 2 immune response
MHC	Major histocompatibility complex
mTORC1	Mammalian/mechanistic target of rapamycin complex 1
µg/mL	Micrograms/millilitre
µL	Microliter
µm	Micrometer
µM	Micromole/ micromolar
mM	Millimole/millimolar
MS	Multiple sclerosis
H3K4	Mono- and tri-methylated lysine on histone 4
ng/mL	Nanograms/millilitre
NEJ	Newly excysted juvenile

NO	Nitric oxide
NLR	NOD-like-receptor
NLP3	NOD-like receptor protein 3
NOD	Non-obese diabetic mouse
NF- κβ	Nuclear factor kappa B
OXPHOS	Oxidative phosphorylation
OCR	Oxygen consumption rate
O/N	Overnight
Pen/Step	Penicillin/Streptomycin
PPP	Pentose phosphate pathway
PPARγ	Peroxisome proliferator-activated receptor γ
PBS	Phosphate buffered saline
Poly (I:C)	Polyinosinic:polycytidylic acid
PD-L2^{+}	Programmed death-ligand 2^{+}
PKA	Protein kinase A
ROS	Reactive oxygen species
R-MCSF	Recombinant macrophage colony stimulating factor
Rcf (g)	Relative centrifugal force
RT-PCR	Reverse Transcriptase-Polymerase chain reaction
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
Rote/AA	Rotenone/ Antimycin A
SD	Standard deviation
SEM	Standard error of the Mean
SOCS1	Suppressor of cytokine signalling 1
Stx5a	Syntaxin 5A
TRIF	TIR-domain-containing adapter-inducing interferon-β
Ct	Threshold cycle value
SMAD	Transcription factor small mothers against decapentaplegic
TGFβ	Transforming growth factor beta
TCA	Tricarboxylic acid cycle
T1D	Type 1 Diabetes

T2D	Type 2 Diabetes
Th1	T-helper 1 immune response
Th2	T-helper 2 immune response
Treg	T-regulatory immune response
TLR	Toll like receptor
TAM	Tumour associated macrophage
TEM	Tumour microenvironment
TNF	Tumour Necrosis Factor
vATPase	Vacuolar-type ATPase

Abstract

The mammalian immune system has evolved to protect against infection and injury, while also minimising tissue damage. However, with respect to parasite infection, the helminths themselves have evolved sophisticated strategies to circumvent and manipulate the immune responses of their host. This facilitates longevity for the parasite, while simultaneously minimising tissue damage for the host. As a result, the typical protective anti-microbial inflammatory responses are absent, which allows the parasites to establish chronic infections and complete their lifecycle. Accordingly, there is great interest in understanding these immune modulatory mechanisms to inform the development of strategies to prevent/control parasite infection. Of equal importance is the possibility that these same mechanisms offer novel approaches to abate/control the aberrant inflammatory responses that mediate diseases, such as type 1 diabetes (T1D), multiple sclerosis (MS), and non-alcoholic liver disease.

Macrophages are central to the immediate innate response to infection and injury. However, the pro-inflammatory/M1 phenotype, which provides functional anti-microbial activity, is largely inhibited during infection with most parasitic worms. This is not only fundamental to the ability of helminths to establish chronic infections, but also opens a therapeutic strategy for the prevention/treatment of immune-mediated diseases which, despite diverse pathogeneses, share the phenomenon of incorrect/unchecked activation of pro-inflammatory/M1 macrophages. A previous exploration of the biological activities of the excreted/secreted products of the liver fluke, *Fasciola hepatica*, identified a peptide (termed FhHDM-1) that selectively interacted with macrophages, and inhibited their pro-inflammatory immune response to stimulation. Furthermore, administration of FhHDM-1 prevented the onset and development of immune-mediated disease (T1D, MS and asthma) in murine models. This efficacy was presumably attributable to the effects of FhHDM-1 on macrophage function. The aim of this project was to investigate the intracellular mechanisms by which FhHDM-1 was regulating the activation and functionality of macrophages.

With emerging evidence linking the metabolism of immune cells to their functionality, the metabolic activity of macrophages treated with FhHDM-1 was first examined. This revealed a dependence on oxidative phosphorylation (OXPHOS) rather than glycolysis, which was reliant on the oxidation of endogenous fatty acids. Further exploration revealed that FhHDM-1 treatment of macrophages induced glutaminolysis, which provided the citrate necessary for the synthesis of endogenous fatty acids needed to fuel the OXPHOS. This

metabolic profile is characteristic of an anti-inflammatory/M2 phenotype rather than a pro-inflammatory/ M1 macrophage, the latter being reliant on glycolysis for the metabolites that fuel pro-inflammatory and antimicrobial functions. However, FhHDM-1 treated macrophages did not express any of the genetic markers that characterise an M2 phenotype.

Therefore, it was possible that the induction of fatty acid oxidation and OXPHOS mediated by FhHDM-1 was supporting an anti-inflammatory functionality, independent of a switch to an M2 phenotype. To examine this possibility, FhHDM-1 treated macrophages were stimulated with a pro-inflammatory ligand, bacterial lipopolysaccharide (LPS). While macrophages exposed to LPS displayed a predominance of glycolysis, pre-treatment with FhHDM-1 prevented this, and reduced the production of the pro-inflammatory cytokines, IL-6 and TNF. The reduction in glycolytic activity and the prevention of activation of the M1 phenotype by FhHDM-1 was dependent on the induction of glutaminolysis, and was associated with the resultant accumulation of the metabolite, alpha ketoglutarate (α-KG).

α-KG is a co-factor for multiple demethylases and has been shown to regulate the induction of trained immunity, which raised the possibility that the modulation of macrophage metabolism by FhHDM-1 may be altering histone marks, to create a stable regulation of inflammatory responses. To test this hypothesis, the bone marrow derived macrophages (BMDMs) from non-obese diabetic (NOD) mice (model of T1D) were first examined for the characteristics of a trained immune response. This revealed that, unlike a non-diabetic immune competent mouse strain (BALB/c), the BMDMs from NOD mice displayed an enhanced pro-inflammatory response to stimulation, exhibited increased glycolytic activity, and showed mono- and tri-methylated lysine on histone 4 (H3K4). These features are all characteristic of a trained immune response. Remarkably, the administration of FhHDM-1 to NOD mice, using the same treatment regime previously shown to permanently prevent T1D, removed the methylation marks on H3K4 in BMDMs isolated 3 weeks after the final injection of FhHDM-1. In addition, these changes in methylation status were associated with a concomitant reduction in glycolytic activity, and a significant decrease in the production (but not expression) of TNF and IL-6 in response to stimulation with LPS *ex vivo*. Importantly, this reduction in pro-inflammatory cytokine production did not represent a total suppression, but rather a reduction in levels to the same quantities produced by BMDMs from BALB/c mice. This indicated that FhHDM-1 was capable of fine-tuning macrophage function, to regulate, but not fully suppress, pro-inflammatory immune responses.

The collective outcomes of this PhD project have revealed new insights into the mechanisms by which FhHDM-1 modulates the response of macrophages to inflammatory stimulation and explains the disease protective effect previously observed in murine models of immune mediated disease. The data presented here has established that FhHDM-1 offers a unique therapeutic approach that would be efficacious across the multitude of human diseases that are mediated by chronic/aberrant inflammation. Examining the biological activity of this unique peptide, which evolved within a worm to modulate the mammalian immune response to support an adaptation to endemic parasitic exposure, has unveiled a new paradigm of innate immune regulation.

Chapter 1: Exploring the role of macrophages in determining the pathogenesis of liver fluke infection

1.1 Publication Statement

This chapter has been published as an invited review in ***Parasitology***: Quinteros, S., O'Brien, B., & Donnelly, S. (2022). Exploring the role of macrophages in determining the pathogenesis of liver fluke infection. *Parasitology, 149*(10), 1364-1373. doi:10.1017/S0031182022000749

The text presented here is the accepted version of the manuscript. Numbering of sections, style of referencing, numbering of tables and figures are altered to align with the formatting of the thesis.

Author Contribution:

Susel Loli Quinteros (graduate student) is the first author of this article. In consultation with her supervisors, she conceived and planned the review. Susel Loli conducted the literature search, designed, and wrote the first draft of the manuscript. Her supervisors, Sheila Donnelly and Bronwyn O'Brien contributed to the editing of the final manuscript.

Signatures:

Susel Loli Quinteros (graduate student) Production Note: Signature removed prior to publication.

Sheila Donnelly (PhD supervisor) Production Note: Signature removed prior to publication.

Bronwyn O'Brien (PhD supervisor) Production Note: Signature removed prior to publication.

1.2 Abstract

The food-borne trematodes, *Opisthorchis viverrini* and *Clonorchis sinensis*, are classified as group 1 biological carcinogens: definitive causes of cancer. By contrast, infections with *Fasciola hepatica*, also a food-borne trematode of the phylum Platyhelminthes, are not carcinogenic. This chapter explores the premise that the differential activation of macrophages during infection with these food-borne trematodes is a major determinant of the pathological outcome of infection. Like most helminths, the latter stages of infection with all 3 flukes induce M2 macrophages, a phenotype that mediates the functional repair of tissue damaged by the feeding and migratory activities of the parasites. However, there is a critical difference in how the development of pro-inflammatory M1 macrophages is regulated during infection with these parasites. While the activation of the M1 macrophage phenotype is largely suppressed during the early stages of infection with *F. hepatica*, M1 macrophages predominate in the bile ducts following infection with *O. viverrini* and *C. sinensis*. The anti-microbial factors released by M1 macrophages create an environment conducive to mutagenesis, and hence the initiation of tumour formation. Subsequently, the tissue remodelling processes induced by the M2 macrophages promote the proliferation of mutated cells, and the expansion of cancerous tissue. This chapter will also explore the interactions between macrophages and parasite-derived signals, and their contributions to the stark differences in the innate immune responses to infection with these parasites.

1.3 Introduction

The liver flukes comprise of 2 families of food-borne trematodes that cause diseases in humans and animals: *Opisthorchiidae* (which includes *Clonorchis sinensis* and *Opisthorchis viverrini*) and *Fasciolidae* (which includes *Fasciola hepatica*). These parasites cause infection via the consumption of contaminated raw fish, crustaceans or vegetation. While infections are generally asymptomatic, higher worm burdens and/or continuous reinfection can cause severe liver disease (1-4). Infection with *C. sinensis* and *O. viverrini* can result in the mineralization of bile ducts (cholangitis), formation of bile duct stones and a sub-type of liver cancer: cholangiocarcinoma (CCA), which is an adenocarcinoma with poor prognosis (5-7). Fasciolosis is similarly associated with the development of cholangitis. However, despite being phylogenetically related to *Opisthorchiidae*, and ultimately residing in the same tissue of their mammalian hosts, infection with *F. hepatica* is not associated with the development of any cancers (8-10).

1.4 Life cycle of liver flukes

As with most trematodes, the liver flukes have a complex life cycle, requiring both intermediate and primary hosts (Figure 1.1). The eggs of these parasites discharge with faeces from their primary mammalian host, and then progress through several developmental stages in an aquatic snail intermediate host. Free-swimming cercariae, which are released from the snails, then encyst as metacercariae; the stage that is infectious to the definitive mammalian hosts. For *C. sinensis* and *O. viverrini*, this encystment occurs within the muscles or under the scales of freshwater fish, thereby making fish the secondary intermediate host and the vehicle for human infection. The metacercariae of *F. hepatica* encyst on aquatic vegetation (such as watercress) and are transmitted to human and animal hosts after ingestion of infected plants.

Differences in the intermediate hosts underpin the distinct global distribution for each of these food-borne trematodes. Specifically, while the snail hosts for *F. hepatica* (*Lymnaeidae* family) are found in almost every country worldwide, the snail hosts for *Clonorchis* and *Opisthorchis* have a more restricted global distribution (11). In addition, the eating habits of populations around the world confer different susceptibilities to infection. For example, consuming raw (dried, fermented or salted) or undercooked fish is a common practice throughout Asia, and in the far eastern regions of the former Soviet Union (12). In contrast, the consumption of aquatic plants by animals and humans occurs worldwide. Because of these

dietary variations, *F. hepatica* has been found in all inhabited continents (11, 13), whereas infection with *C. sinensis* is only endemic in China, Korea and Vietnam, and *O. viverrini* is predominantly found in Thailand, Lao People's Democratic Republic, Cambodia and central Vietnam (11, 12). *Opisthorchis felineus* is the predominant species found in Siberia, and like *O. viverrini* it is also suspected of being a group 1 carcinogen (14). This variation in dietary practices also introduces additional risk factors for the development of CCA, which may influence the differential outcome to infection with liver flukes. Salted and fermented fish contain high levels of nitrosamines, which are classified as carcinogenic factors. Their consumption may create a microenvironment that is more favourable to the development of malignancies, such as CCA, following infection with *Clonorchis* or *Opisthorchiidae* (15).

After ingestion of the metacercariae by mammalian hosts, a series of stimuli (including temperature, pH and bile salts) in the digestive tract activates the excystment of the newly excysted juvenile (NEJ) worms (16-19). At this developmental stage, *Opisthorchiidae* migrate to the ampulla of Vater and ascend into the bile ducts where the parasites mature (Figure 1.1). The adults of these parasites typically reside in the intrahepatic bile ducts for 10 years (20, 21). Although the destination of the *Fasciola* parasites is also the bile duct, their migratory route is quite different (Figure 1.1). After penetrating intestinal epithelia, the NEJs migrate through the peritoneal cavity to the liver, where over several weeks, they tunnel a path towards the bile duct (22). Like *C. sinensis* and *O. viverrini*, the adult *F. hepatica* parasites can also remain in the bile ducts for up to 12 years after infection (23, 24). This review examines the possibility that variations in the migratory patterns of the liver flukes differentially influence the polarization of macrophages, which consequently mediates the distinct pathological outcomes to infection.

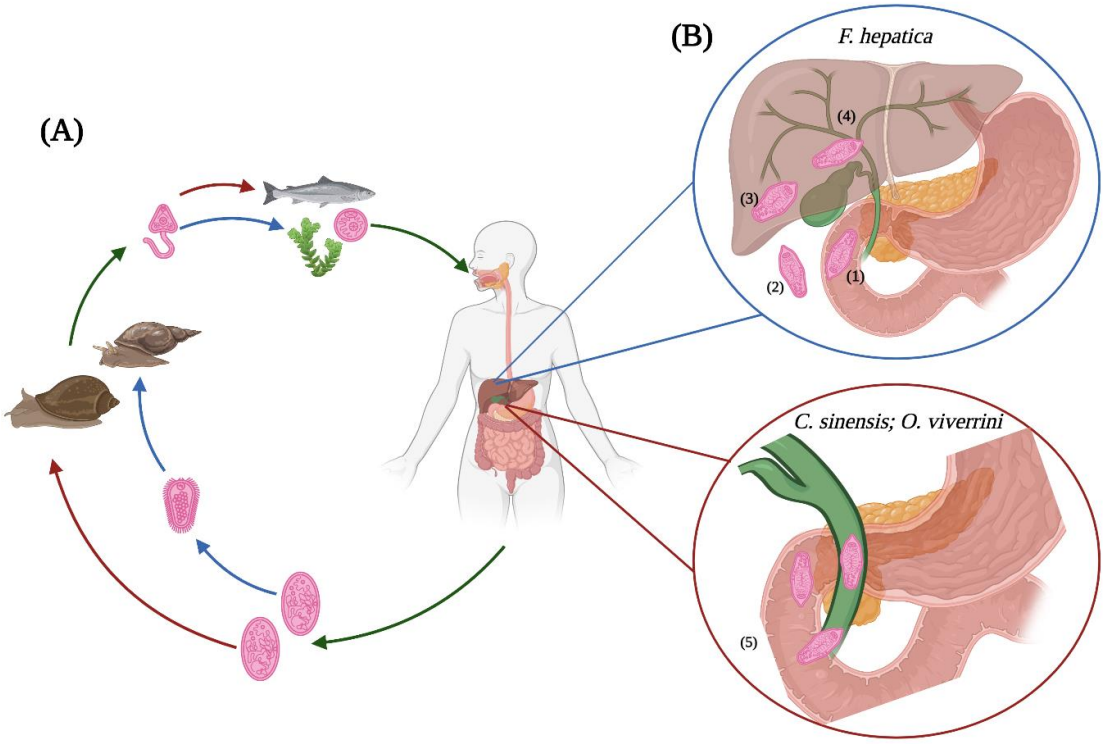

Figure 1.1: Comparative life cycles and intra-mammalian migratory pathways of food-borne liver flukes. (A) All adult flukes produce eggs that are passed with faeces from the mammalian host. For *Fasciola hepatica*, these eggs become embryonated in fresh water to release miracidia, which then invade a suitable snail host. In contrast, for *Opisthorchis viverrini* and *Clonorchis sinensis* this intermediate stage begins when the snail ingests the unembryonated eggs. Once inside the snail, the parasite undergoes several developmental stages before emerging as cercariae. These cercariae must encyst (on vegetation for *F. hepatica*; within freshwater fish for *O. viverrini* and *C. sinensis*) to become metacercariae; the infective stage for mammalian hosts. (B) After ingestion, the environment within the digestive system of the mammalian host promotes excystment of the metacercariae: (1) the emergent juvenile flukes begin the migratory journey to the bile duct. To achieve this, (2) the *F. hepatica* flukes penetrate the intestinal epithelia to enter the abdominal cavity. (3) After a period of days, the juvenile flukes begin to tunnel through the liver parenchyma. Once inside this tissue the parasites spend time feeding and maturing, (4) finally reaching the bile ducts approximately 12 weeks after infection. (5) In contrast, *O. viverrini* and *C. sinensis* travel a more direct route to their destination. After emerging from the metacercariae, the juvenile flukes ascend directly to the bile ducts from the duodenum, via the ampulla of Vater. There, after a period of 3–4 weeks they mature into egg laying adults, thereby restarting the parasite life cycle. Image created using BioRender.

1.5 Polarization and functional activity of macrophages

Macrophages are both tissue resident and infiltrating immune cells that are critical for the innate immune response, repair of damaged tissue, systemic metabolism, cold adaptation and tissue homoeostasis and development (25). The initiation of each of these biological activities occurs in response to the composition of the local environment in which the macrophages reside, and the type of pathogen or injury to which macrophages are exposed (26).

Changes in the tissue microenvironment stimulate the polarization of differentiated tissue-resident macrophages into a diverse range of functional phenotypes (25, 27). When numbers of resident macrophages are insufficient to satisfy the functional demands within a tissue, the population can be expanded by local proliferation or recruitment. In addition, monocytes (macrophage precursors) can be recruited from the circulation to differentiate into functional macrophages within the affected tissues (25).

The polarization of macrophages into different phenotypes is a dynamic process, which occurs continuously throughout the inflammatory response to infection or tissue damage. This plasticity is a hallmark of macrophages and enables them to continuously respond to a changing microenvironment. This differentiation of macrophages requires an accurate regulation of gene transcription that is dependent on epigenetic modifications (28). Dynamic and reversible changes of epigenetic markers at the promoters and enhancers of signal-sensitive genes are critical for the quick reprogramming of macrophage polarization and give macrophages the ability to switch rapidly between cellular programmes (29). In addition, some signals (host and pathogen) can induce a more persistent 'epigenetic memory', which endows macrophages with a long-lasting capacity to respond more strongly to future challenges (30). Thus, monocytes/macrophages emerging from the bone marrow are functionally conditioned to execute an enhanced (trained) or restricted (tolerant) response to subsequent restimulation (31).

Each phenotype of macrophage is inherently linked to the activation of specific metabolic and molecular pathways, which ultimately determine the biological function of each subtype of macrophage (32, 33). Early investigations to determine the mechanisms underlying macrophage polarization used models of dichotomous macrophage phenotypes, namely pro-inflammatory M1 or anti-inflammatory M2. This binary classification of macrophage phenotype was based on *in vitro* observations that macrophages treated with the type 1 T helper (Th1) cytokine, interferon gamma (IFNγ), or the Th2 cytokine, interleukin 4 (IL-4), exhibited distinct genetic expression patterns, termed M1 and M2, respectively (34).

Generally, M1 macrophages are characterized by the upregulation of tumour necrosis factor (TNF), inducible nitric oxide synthase (iNOS) and IL-1β expression, which mediate antimicrobial innate immune responses (35). Production of IL-12 and IL-23 further supports the inflammatory state by promoting the differentiation and expansion of Th1 and Th17 cells, respectively (34). In addition, M1 macrophages adopt a metabolic signature that favours glycolysis, thereby rapidly producing adenosine triphosphate (ATP) that supports pro-inflammatory signalling (36). In contrast, a typical M2 macrophage is associated with the resolution of inflammation and mediation of the healing process and is characterized by increased expression levels of different genetic markers, notably Arg1, Retnla, Ym1 and Ear11 (37). For M2 macrophages, oxidative phosphorylation (OXPHOS) is the predominant metabolic activity resulting in a delayed, but more prolific production of ATP, which is needed to support the specific functional demands of the M2 phenotype, as compared to the anti-microbial activities of M1 macrophages (38, 39). More recent studies of macrophage metabolism have revealed that while the anti-inflammatory activity of macrophages solely requires oxidative metabolism (40, 41), the expression of characteristic M2 genes induced by IL-4 simply requires a threshold of ATP, which can be reached via glycolysis or OXPHOS (42). These observations uncover a disconnect between the original, binary paradigm of macrophage phenotypes (characterized by a simple genetic signature), and the more recent functional characterizations of macrophage subsets according to metabolic preferences (43).

The need for a model for the activation of multiple macrophage phenotypes has become increasingly evident after the identification of numerous factors that drive the activation of macrophages into distinct phenotypes, which are characterized by distinct genetic and biological profiles (37, 44, 45). Thus, macrophage populations cannot always be appropriately assigned to either the M1 or the M2 phenotype (46, 47). Accordingly, the notion that macrophage phenotypes lie along a spectrum between the polarized functional states of M1 (typically pro-inflammatory) and M2 (typically immune suppressive) is now widely accepted (37, 48).

This fluidity in polarization along a continuum of functional states is particularly evident for macrophages found within the tumour microenvironment (TME). These tumour-associated macrophages (TAMs) account for the largest fraction of the myeloid infiltrate in most human malignancies, including CCA (49), and display a high degree of functional plasticity to adapt to the changes occurring during tumour progression and across different regions of the TME (50, 51). As a result, TAMs are phenotypically heterogeneous, comprising

the spectrum extremes of M1 and M2 along with other, yet to be characterized, phenotypes (28, 52, 53). Like the macrophage subtypes identified during an inflammatory response to infection/injury or to the processes of tissue repair, this diversity in phenotypes is associated with distinct metabolic profiles. Switches in metabolic activity between glycolysis and OXPHOS) direct the functional response within the tumour, with respect to rates of angiogenesis, tumour growth, metastasis and immune cell activation (54). It has been proposed that the heterogeneity of macrophage phenotypes within the TME reflects a dual role of TAMs in tumours. The functional activity of M1/pro-inflammatory macrophages creates a mutagenic microenvironment that supports tumour initiation, while M2/wound-healing macrophages promote malignancy progression (55-58).

Collectively, these observations demonstrate that conversions between macrophage phenotypes, which are largely induced by changes in metabolic flux, are a major determinant of macrophage function. In turn, this becomes a principal regulating factor, not only for the resolution of infection and removal of danger signals, but also in the initiation, progression and termination of several human diseases, including pathologies associated with helminth infection.

1.6 Macrophage activation and function during liver fluke infection

Macrophages dominate the immune responses to helminths. Due to their spectra of functional phenotypes, macrophages play a central and pleiotropic role in the host response to infection with helminths (59, 60). It has been recently proposed that the functional roles of macrophages during helminth infection can be allocated to 3 categories (61). Immediately after infection, an increase in tissue alarmins, released due to the migrating parasites, combined with the presence of helminth secreted molecules, signals the presence of invading parasites that causes the macrophages to ‘react’ and produce a range of effector molecules that drive anti-helminth activities and recruit other immune cells to the infection site. Many parasites evade and/or modulate this initial host protective response, which supports their ability to survive and establish chronic infections. During these long-term infections, the feeding and migration behaviours of parasites cause extensive tissue damage, which is counteracted by the ‘repair’ activities of macrophages. These cells mediate the wound-healing response to prevent prolonged haemorrhaging and translocation of microbiota. However, this activity must be carefully balanced to prevent excessive tissue remodelling, which may cause fibrosis. Thus,

macrophages enter a final ‘resolve’ phase during which they perform potent immune-regulatory activities.

The react, repair, and resolve phases of macrophage activation are evident during infection with *F. hepatica* (Figure 1.2). Immediately after infection, there is an influx of immune cells, of which macrophages are the most predominant, into the peritoneal cavity signalling the host’s reaction to the migration of NEJs from the intestine (62, 63). However, despite the presence of migrating parasites in combination with perforations to the intestinal epithelium and the likely translocation of intestinal microbiota (19, 64), there is no evidence of a pro-inflammatory M1 phenotype, which is characteristic of the mammalian protective immune response. Peritoneal macrophages isolated from infected animals (sheep and mice) show low expression levels of major histocompatibility complex-II (MCH-II), and no significant increase in the production of pro-inflammatory cytokines (TNF, IL-12, IFNγ) or anti-microbial effectors [iNOS, nitric oxide (NO)] (63, 65). These observations led to the hypothesis that the Fasciola NEJs possess mechanisms to actively suppress the ability of the host to activate an immediate protective pro-inflammatory innate response. This would ensure the survival of the parasite and support its safe passage from the intestine, across the peritoneal cavity and on to the liver (19). Support for this premise is evident from animal studies, which have shown a correlation between the activation of the M1 phenotype (and associated Th1 type immune responses) and experimentally acquired or naturally occurring resistance to *F. hepatica* infection (66-68).

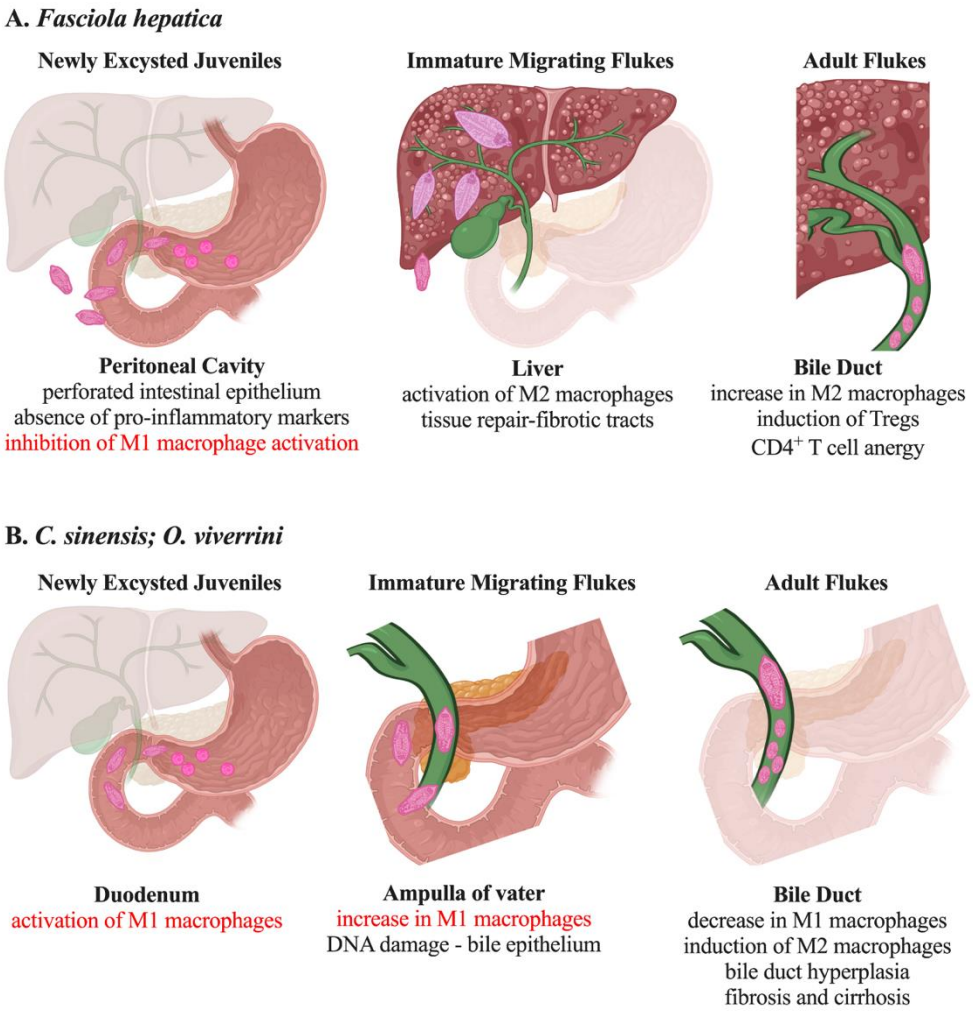

Figure 1.2: Comparative activation of macrophages and pathological consequences of liver fluke infection. (A) During infection with *F. hepatica* the ability of macrophages to polarize towards a pro-inflammatory M1 phenotype is actively inhibited. In contrast, the activation of M2 macrophages, which occurs coincident with the parasite migration to the liver tissue, is actively promoted to mediate tissue repair and the regulation of effector T-cell responses. (B) Immediately after infection with either *O. viverrini* and *C. sinensis*, M1 macrophages are abundant and correlate with the presence of DNA damage within the bile duct epithelium. As infection progresses, this population of macrophages decreases, although is never totally absent, and M2 macrophages become the abundant phenotype coincident with the appearance of fibrosis and cirrhosis.

The penetration of the liver capsule by the NEJs is coincident with the appearance of an M2 phenotype of macrophage, as characterized by an increase in the expression levels of Arg1 and Ym1 (69). The primary role of macrophages at this stage of infection is the promotion of tissue repair, rather than anti-helminth responses. A lack of programmed death-ligand 2^+ (PD-$L2^+$) M2 macrophages in Fasciola infected mice had no effect on worm burden or size but led to exaggerated liver damage resulting in premature host death (70). The immune-mediated repair of liver tissue was evidenced by the visible formation of fibrotic tracts and granulomas, both of which are characteristic liver pathologies associated with fasciolosis (71). During the later stages of liver migration and the final life stage in the bile duct, Fasciola infection stimulates the expansion of regulatory and anergic T-cell populations, which are important in reducing the severity of tissue pathology and sustaining the suppression of parasite-specific effector immune responses. The observation that PD-$L2^+$ M2 macrophages induced by *F. hepatica* stimulated the differentiation of forkhead box $p3^+$ T regulatory cells and anergy in $CD4^+$ T cells *ex vivo* (72, 73) suggests a critical role for macrophages in the initiation and perpetuation of these adaptive immune responses. However, additional *in vivo* studies with specific depletion of PD-$L2^+$ macrophages will be required to definitively characterize their functional role in the promotion of host immune responses and the control of pathological outcomes.

Like the host response to *F. hepatica*, macrophages are the predominant immune cell population within the bile duct immediately after infection with *O. viverrini* and *C. sinensis* (74, 75), and remain abundant for several months after infection (76). However, in contrast to Fasciola, the expression levels of iNOS in macrophages is significantly increased as early as 3 days after infection in animal models [(75), Figure 1.2). The proportion of these $CD16/32^+$/$iNOS^+$ M1 macrophages steadily increases in the liver and bile duct over the next 18 days of infection (75, 77). Then, coincident with the maturation of the parasite and subsequent egg production, this population of macrophages begins to decrease, but does not return to uninfected basal levels. At the same time, a population of $CD206^+$/$Arg1^+$ macrophages emerges, indicating the presence of an M2 macrophage phenotype (75, 77). The wound-healing activity of macrophages, as described above, is correlated with their increased abundance during the fibrotic and cirrhotic stages of infection, when they become localized adjacent to the areas of collagen deposition within tissues (78). Moreover, increasing the number of M2 macrophages in tissue exacerbated bile duct hyperplasia, and examination of tissue sections

revealed an association between macrophages and cancer-associated fibroblasts (79, 80). These observations highlight a significant role for M2 macrophages in the progression of CCA.

A comparison of macrophage phenotypes induced in response to liver fluke infections emphasizes that the induction of M1 macrophages is the primary difference in the innate immune response to *O. viverrini* and *C. sinensis* versus *F. hepatica* (Figure 1.2). With an understanding that pro-inflammatory M1-like macrophages have been suggested to drive the mutagenesis that supports tumour initiation, it is plausible that the induction of this phenotype of macrophage contributes to the carcinogenic effect of *Opisthorchiidae* infection, and a reason for the differential pathology between liver fluke infections. Supporting this proposition, it has been shown that the production of NO and the superoxide anion radical, O_2^-, by M1 macrophages caused an increase in oxidative and nitrosative DNA damage, and accumulation of proliferating cell nuclear antigen (PCNA), in the epithelium of bile ducts of hamsters infected with *O. viverrini* (81). Notably, repeat infections with *O. viverrini* resulted in an earlier and enhanced production of iNOS by macrophages and led to increased DNA damage (81). This finding corroborates evidence that the chronic inflammation due to reinfection with *O. viverrini* or *C. sinensis* is the primary risk factor for the development of CCA (82).

1.7 Activation of macrophages by fluke-derived molecules

The premise that parasite-derived factors interact with host macrophages to influence their functional activity is supported by analyses of tissue from animals infected with *F. hepatica* and *O. viverrini*. For both parasites, their antigens commonly co-localize with inflammatory cell infiltrates, notably macrophages, even at sites that are distant from the flukes (83, 84). Exploring this hypothesis, many groups have used an 'omics' approach to characterize the proteins of the food-borne flukes within their excretory/secretory products, as these would be the most likely to interact with host immune cells and mediate the pathogenesis of infection (18, 85-87).

Proteomics comparisons of the excretory/secretory products of the liver flukes reveal a slight variation in the proteome for each parasite, which likely reflects the different biological activities required to support their distinct migratory paths through the host tissue. For example, proteolytic enzymes were underrepresented among the secreted proteins of *O. viverrini*, as compared to those of *F. hepatica* (88). A more detailed examination of peptidase expression in the flukes revealed that while *O. viverrini* and *C. sinensis* produced high levels of cathepsin F

(89-91), *F. hepatica* secreted a variety of cathepsin L and cathepsin B enzymes with no cathepsin F detected (92). This stark variation clearly relates to the different routes each parasite takes through host tissue. Fasciola requires the collagenolytic activities of the cathepsin L and B enzymes to disrupt the interstitial matrix to cross the intestinal wall and to subsequently penetrate the liver. However, this enzymatic activity is not required by *O. viverrini* or *C. sinensis* as they reach the bile ducts from the duodenum by simply ascending the hepatopancreatic duct (ampulla of Vater). However, this difference in migratory patterns may also impact the host immune responses. For example, cathepsin L enzymes secreted *by F. hepatica* selectively inhibit several pro-inflammatory signalling pathways in mammalian macrophages, which prevent the development of an M1 phenotype (93).

In fact, the secreted proteins of *F. hepatica* typically skew the phenotype of macrophages away from a pro-inflammatory M1 phenotype and/or towards a wound-healing/regulatory M2 phenotype. In contrast, the secreted proteins of *O. viverrini* and *C. sinensis* predominantly promote the development of pro-inflammatory M1 macrophages (Table 1.1). These reported differences in protein activity could be explained by differences in the production and purification methods used across different research groups. Traditional recombinant production using bacterial cells can result in the presence of residual bacterial lipopolysaccharide (LPS), which will contaminate the recombinant protein and skew the macrophage response towards an M1 phenotype. However, as this differential activation of macrophages by fluke-derived proteins is consistent across multiple research groups, and with both native secretions and recombinant/synthetic proteins it seems plausible that these differences in biological activities reflect a specific functional adaptation for each parasite.

This discrepancy in the immune modulating activity of the parasite-derived proteins may be attributed to the initiation of distinct protective host responses associated with different anatomical locations. As described previously, the induction of antimicrobial pro-inflammatory M1 macrophages in the peritoneal cavity is characteristic of a protective immune response against *F. hepatica* (66). In contrast, the expulsion of intestinal helminths is mediated by a Th2 immune response (94). Rather than utilizing direct anti-microbial mechanisms, this immune phenotype drives an accelerated epithelial cell turnover, which effectively dislodges the parasites resulting in their clearance (95). Importantly, the expulsion of worms from the intestinal lumen is mediated by intestinal contractility, which is regulated by M2 macrophages (96). Of relevance to the liver flukes, susceptibility to infection with the intestinal fluke, *Echinostoma caproni*, is linked to the induction of a Th1 type immune response, which slows

epithelial cell turnover and promotes tissue hyperplasia. This allows parasites to more stably attach to host cells, which favours the establishment of chronic infections (97). Putatively, a similar protective mechanism is initiated in the bile duct in response to infection with *Clonorchis* and *Opisthorchis*. This would explain the broad capacity for their secreted proteins to drive M1/Th1 type immune responses.

An alternate/additional benefit of inducing M1 macrophages may be to balance the development of M2 macrophage populations that mediate wound healing in the bile duct, as excessive fibrotic scar tissue would prevent the parasites effectively accessing the epithelium for feeding. This notion is supported not only by the evident induction of M1 macrophages after infection, but also by the observation that this population is sustained, albeit at a reduced proportion, throughout the course of infection (75, 77). Furthermore, the manipulation of innate immune signalling pathways in mice infected with *C. sinensis*, which resulted in a reduction of M1 macrophages and an increase in M2 macrophages, was associated with aggravated peribiliary fibrosis (80). This functional role for M1 macrophages in the context of a parasite infection is further strengthened by studies of mice infected with the blood fluke, *Schistosoma mansoni*. For this helminth, the manipulation of cytokines to create a bias towards the development of M1 macrophages resulted in reduced collagen deposition and smaller granuloma formation around parasite eggs deposited in the liver (98, 99). Furthermore, the resulting mixed immune response, with a slight predominance of Th1 and M1 cells, was most effective as it afforded sufficient protection from tissue damage caused by the parasite while simultaneously minimizing fibrosis (100).

Consideration of the pathogenesis of *S. mansoni* is of relevance when comparing the biological activity of the helminth defence molecules (HDMs), which are peptides secreted by all trematode parasites (101). Remarkably, the HDM secreted by *F. hepatica* (FhHDM-1) inhibits the development of M1 macrophages, but despite being classified in the same family of peptides, the HDM secreted by *C. sinensis* (CsHDM) induces an M1 phenotype (Table 1.1) (102-104). Analysis of the phylogenetic relationship of the HDM peptide family shows that while CsHDM is on the same branch as FhHDM, it is evolutionarily closer to another branch of HDM peptides, the Sm16 peptides, found exclusively in Schistosoma (105). Characterization of the Sm16 from *S. mansoni* showed that it was primarily expressed by cercariae and eggs, and like the CsHDM induces a pro-inflammatory response in macrophages. These findings thus suggest a functional role in the management of wound-healing responses associated with the predominance of M2 macrophages.

Table 1.1: The impact of macrophage polarization by treatments with liver fluke.

Parasite	Protein	Macrophage origin	Biological Activity	Reference
Fasciola hepatica	Native glutathione *S*-transferase (nFhGST) extracted from soluble extract of adult fluke	Murine (C57BL6) bone marrow derived	Prevented LPS induced NF-κB dependent production of TNF and IL-1β	(106)
	Peroxiredoxin (Prx/Trx); *Escherchia coli* recombinant	Murine (BALB/c) peritoneal; murine RAW 264.7 cell line	Increased expression of M2 markers (Arg1, Ym1, Fizz1) and increased production of IL-10 and prostaglandin E2	(69)
	Native fatty acid binding protein (Fh12) purified from adult fluke extract	Murine (C57BL6) bone marrow derived	Suppressed phosphorylation of ERK, p38 and JNK to inhibit LPS-induced expression of IL-12, TNF, IL-6 and IL-1β	(107)
		Human monocyte derived	Induced the expression of M2 markers (Arg1, Ym1)	(108)
	Fatty Acid Binding Protein; *E. coli* recombinant	Murine (C57BL6) bone marrow derived	Inhibited LPS-stimulated production of TNF and IL-1β	(109)
	Cathepsin-L1; *Pichia pastoris* recombinant	Ex vivo murine (BALB/c) peritoneal	Inhibited TLR3-dependent cytokine production by LPS via cleavage of TRIF	(93)
	Transforming growth factor-like molecule (FhTLM); *E. coli* recombinant	Bovine blood derived	Activated SMAD2/3 signalling to induce a regulatory phenotype expressing high levels of IL-10, Arg1 and PD-L1 and low levels of IL-12 and NO	(110)
	Helminth defence molecule (FhHDM-1); synthetic peptide	Murine (C57BL6) bone marrow derived; *ex vivo* murine Non-Obese Diabetic mice (NOD) peritoneal	Reduced production of TNF in response to LPS; inhibition of lysosomal vATPase prevented activation of the NLRP3 inflammasome and thus production of IL-1β	(102, 103, 111)
Clonorchis sinensis	Lysophospholipase A (csLysoPLA); *E. coli* recombinant	Murine RAW 264.7 cell line	Stimulated IL-25 expression via the PKA-dependent B-Raf ERK1/2 signalling pathway	(112)
	Excretory/Secretory proteins of adult flukes	Murine (BALB/c) hepatic	Increased production of pro-inflammatory cytokines TNF and IL-6	(77)
	Helminth Defence Molecule (CsMF6p/HDM); *E. coli* recombinant	Murine RAW 264.7 cell line	Increased production of pro-inflammatory cytokines via NF-κB dependent MAPK pathways	(104)
Opisthorchis viverrini	Whole worm homogenate of adult flukes	Murine RAW 264.7 cell line	Enhanced expression of TLR2, leading to activation of NF-κB-mediated expression of iNOS and COX-2	(113)
	Excretory/secretory products of adult flukes; whole worm homogenate of adult flukes	Human U937 cell line	Increased expression of myristoylated alanine-rich C kinase substrate (MARCKS) which has been implicated membrane cytoskeleton alterations that underlie LPS-induced macrophage responses	(114)
	Adult Flukes	Human peripheral blood mononuclear cells	Increased pro-inflammatory cytokines, cell adhesion molecules and chemoattractant chemokines	(115)

LPS, lipopolysaccharide; NF-κβ, nuclear factor kappa B; TNF, tumour necrosis factor; IL, interleukin; Arg-1, arginase-1; Ym1, chitinase-like protein 3; Fizz1, resistin-like molecule alpha 1; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; TLR, Toll-like receptor; TRIF, TIR-domain-containing adapter-inducing interferon-β; SMAD, transcription factor small mothers against decapentaplegic; PD-L1, programmed death-ligand 1; NO, nitric oxide; v-ATPase, vacuolar-type ATPase; NLRP3, NOD-like receptor protein 3; PKA, protein kinase A; MAPK, mitogen-activated protein kinase; SOCS1, suppressor of cytokine signalling 1; Clec7a, C-type lectin domain containing 7A; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase.

1.8 Concluding remarks

Helminths have evolved multiple mechanisms to modulate the immune response of their mammalian hosts to support the establishment of chronic infections, and to ensure sustained reproductive success. The development of immune modulatory mechanisms is informed by different phenomena within the host, the most evident of which is the migratory pathways of each parasite. The analyses of macrophage phenotypes presented here highlight a remarkable difference in host immune responses to carcinogenic flukes, as compared to non-carcinogenic *F. hepatica*. Evidence supports the likelihood that this phenomenon reflects the specific requirements associated with each parasite's distinctive migration patterns and final anatomical location within the mammalian host.

The influence that anatomical location has on the immune mediated pathological outcome to infection with helminths can be extended to infection with Schistosoma. While the hepatic schistosomes of humans, *S. japonicum* and *S. mansoni*, do not cause cancer, *S. haematobium*, a parasite that resides in the bladder, is carcinogenic, with infection associated to the development of squamous cell carcinoma (116). A comparison of the immune and fibrotic response to the deposition of *S. haematobium* eggs in the bladder, compared to the response of *S. mansoni* eggs in the liver revealed distinct collagen-remodelling pathways associated with each anatomical location (117). This likely reflects the specific adaptation of host immune responses to infection within the bladder, which has been characterized as a highly polarized Th2 type immune response that directs the rapid re-epithelialization, and thus prioritizes the regeneration of tissue at the expense of preventing re-infections (118). While there was no specific analysis of macrophage populations, analysis of gene expression within the tissues revealed that like the carcinogenic liver flukes, the immune response for *S. haematobium* reflected a greater mix of type 1 and type 2 immune responses, compared to the predominance of M2 macrophages and a Th2 type immune response associated with *S. mansoni* infection (117).

Therefore, while multiple factors likely modulate susceptibility to fluke-induced cancer, the evidence from both flukes and schistosome infections would support a scenario in which the differential regulation of an M1/pro-inflammatory type of macrophage is a major contributory factor in the pathological outcome to infection with liver flukes. However, while the studies reviewed here classified macrophage phenotypes as M1 or M2, in the most part these characterizations were based on the expression of only a small number of characteristic genes (Arg1, Ym1, PD-L2) or cytokines (TNF, IL-6, IL-10). As macrophages are more likely

to be present as multiple functional variants throughout the different stages of parasite infection, further longitudinal and more detailed characterization of the genotype and functional phenotype of these macrophages will be critical to fully understand how the biological activities of macrophages modulate the host responses to infection with liver flukes.

Chapter 2: The parasite-derived peptide FhHDM-1 reprogrammes the metabolic activity of macrophages

2.1. Publication Statement

The experimental data presented in this chapter and in Chapter 3 contribute to a research article, which has published in *Frontiers in Immunology*:

Quinteros, S., von Krusenstiern, E., Snyder, NW., Tanaka A., O'Brien, B., & Donnelly, S. (2022). The helminth derived peptide FhHDM-1 redirects macrophage metabolism towards glutaminolysis to regulate the pro-inflammatory response. *Front Immunol*. 2023 Jan 25; 14:1018076.

Author Contributions:

Susel Loli Quinteros (graduate student) designed, performed, and analysed the data presented here. In addition, she was actively involved in the writing of the initial draft, and the iterative editing and review process. Eliana von Krusenstiern and Nathaniel W. Snyder performed the mass spectrometry to quantify cellular metabolites and uptake of ^{13}C in macrophages, as a blinded analysis of samples provided by Susel Loli Quinteros. Akane Tanaka provided technical assistance in the quantification of cytokines secreted by macrophages (presented in Chapter 4). Sheila Donnelly and Bronwyn O'Brien made substantial contributions to the conception of the work, critical analyses of data, writing and editing of the manuscript.

The introduction and discussion presented here differ from the published manuscript to better position the experimental work and data within the context of the PhD project and written thesis. In addition, numbering of sections, style of referencing, and numbering of tables and figures have been edited to align with the formatting of the thesis.

2.2. Introduction

As reviewed in Chapter 1, helminths stealthily invade their hosts, by initially inactivating innate detection systems, and then manipulating the immune response such that the host becomes tolerant of the parasites presence (119-121). Consequently, the typical anti-microbial immune response that is characteristic of infection with most other pathogens (viral and bacterial), is not activated. In fact, a consistent feature of mammalian helminth infection is that complete expulsion or killing of all parasites is rarely achieved, there is little evidence of protection against re-infection, and, for the most part, there is no effective vaccine strategy (122, 123). Understanding how these pathogens manipulate host immune responses to create an environment that promotes their survival, is necessary to develop intervention strategies that will target these immunomodulatory mechanisms.

Fasciola hepatica is a major production limiting disease of ruminant livestock. Infection with the parasite results in substantial delays in animals reaching slaughter weight, with increased levels of fluke burden in the liver directly correlating with reduced growth rates. To date, all vaccine trials have focused primarily on targeting the adult parasite (68). However, at this stage of the infection the parasite is actively destroying the liver tissue as it feeds and migrates towards the bile duct. Burden models suggest that even with as few as 10 parasites in the liver, an animal's body weight increases at a significantly slower rate, as compared to uninfected animals (124). Therefore, a more effective preventative strategy would be one that targets the early migratory stages of the parasite, with the primary aim of blocking the penetration of the liver capsule by the parasites.

During this pre-hepatic phase of infection animals display no clinical signs of diseases, no inflammatory changes are observed in the intestine wall, and pathological findings are rare (95). In addition, microarray analyses of peritoneal macrophages during early *F. hepatica* infection of mice revealed a significant reduction (> 20 fold) in the expression levels of all genetic markers of M1 macrophage activation, including TNF, IL-6 and IL-12 (125). These observations are corroborated by studies of the macrophages from infected sheep and cattle (71). This data suggests that immediately after infection, *F. hepatica* is employing a mechanism(s) to disarm the host's innate 'early-response' immune system to prevent parasite detection at a vulnerable time in the invasion process, and to ensure a safe migration from the intestine, through the peritoneal cavity, and onto the liver. Proteins with immune regulatory activity are secreted by parasites, and these are responsible for regulating the host's immune

response. Understanding the mechanisms of action of these parasite excreted/secreted products is critical for the development of effective anti-helminth therapies.

The concept of immunometabolism has proposed that reprogramming the metabolic activity of macrophages offers a mechanism to selectively, and specifically, regulate inflammation and immunity (126-129). This process may be effectively used by parasitic worms. Indeed, the initial understanding of how macrophage phenotypes were regulated by different metabolic pathways emerged from studies of M2 macrophages induced by IL-4, secreted during helminth infection. These M2 macrophages displayed increased OXPHOS metabolism. It was initially thought that the maintenance of this metabolic pathway and the M2 phenotype was dependent on fatty acid oxidation (FAO), fuelled by lysosomal lipolysis of fatty acids (130). However, it is now evident that while the IL-4 induced expression of M2 genes simply requires a threshold level of ATP, which can be reached by OXPHOS or glycolysis (42), the anti-inflammatory functional activity of macrophages is mediated only by OXPHOS, which can be fuelled by acetyl-CoA or glutamine (41, 131, 132). The administration of IL-4 to mice fails to fully replicate the metabolic reprogramming of macrophages observed during helminth infection. This observation supports the hypothesis that additional parasite derived factors also have the capacity to reprogram the metabolic signature of macrophages to induce a functional phenotype, which can prevent inflammation (133).

A combined proteomic and immunological approach has facilitated the characterisation of the regulatory proteins within the excretory/secretory products of the liver fluke, *Fasciola hepatica* (134). Biological characterisation of these proteins led to the identification of a single peptide, the *Fasciola hepatica* Helminth Defence Molecule (FhHDM-1), which has the capacity to modulate host inflammatory responses *in vivo* (72). Structurally, FhHDM-1 is comprised of an alpha helix with an amphipathic C- terminal region. Subsequent analysis of the genomes of all species of worms and mammals showed that the HDM peptides are uniquely expressed by flatworms (101). Phylogenetic analysis further revealed the existence of two distinct branches, one which has been classified as *Fasciola*-like HDMs, and the second, which exclusively contains a group of HDMs that are larger in molecular weight, termed the Sm16-like peptides, and are only produced by the Schistosomatoidea superfamily (105). The *Fasciola*-like HDM branch, currently contains HDMs from *Fasciola hepatica*, *Echinostoma caproni*, *Clonorchis sinensis,* and *Opisthorchis vivverrini*, which cluster together. It also contains HDMs from various species of the Schistosomatoidea superfamily, although these are found on a separate extended branch (105).

Analysis of cellular binding specificity of FhHDM-1 revealed a preferential interaction with macrophages and monocytes *in vivo* and *in vitro* (murine splenocytes and human peripheral blood monocytes [PBMCs]) (102, 135). Subsequent *in vitro* analyses revealed that after interacting with the macrophage membrane, FhHDM-1 was actively internalized, and then localized to endolysosomes where it inhibited vacuolar ATPase (vATPase), an enzyme central to the regulation of lysosomal pH (111). Consequently, functional activities that are dependent upon an acidic lysosomal pH, such as NOD-like-receptor (NLR)-P3 activation, were inhibited (103). Given that the activation of NLRP3 is tightly regulated by cellular metabolism (136), this chapter aimed to determine whether changes in the utilisation of specific metabolic pathways by macrophages underpins the biological activity of FhHDM-1. Such an outcome would present a new opportunity to specifically, and selectively, regulate the inflammatory behaviour of macrophages during infection with a helminth parasite.

2.3. Materials and Methods

2.3.1. Production of Parasite-derived Peptides by Chemical Synthesis

Synthetic peptides corresponding to the sequence of the mature full-length native FhHDM-1 (101), was synthesised to 95% purity and verified to be endotoxin free (GLBiochem, Shanghai, China). The peptides were solubilised in sterile, endotoxin-free water (Baxter), aliquoted, and stored at -80 °C until use.

2.3.2. Isolation and Culture of Bone Marrow-Derived Macrophages (BMDMs)

Bone marrow was harvested from 6-week-old C57BL/6 mice and cultured at 37ºC/5% CO_2 in RPMI 1640 Medium (Gibco) containing 10% v/v heat-inactivated FCS (Gibco), 2-mercaptoethanol (Sigma-Aldrich) and 5% penicillin/streptavidin (Life Technology). The cells were supplemented with 50ng/ml recombinant macrophage colony stimulating factor (MCSF; Miltenyi Biotec) on days 1 and 3 of culture to stimulate the differentiation of monocytes to macrophages. At day 6, differentiated BMDMs were harvested and used for experiments. Culture and stimulation conditions for each experiment are described in the respective figure legends. Ethics approval for these studies was granted by the University of Technology Sydney (UTS) Animal Care and Ethics Committee (Approval Number: ETH18-2257), and experiments were conducted in accordance with the approved guidelines to be compliant with 'The Australian Code for the Care and Use of Animals for Scientific Purposes'.

2.3.3. Isolation of Murine Peritoneal Macrophages

C57BL/6 mice (6 weeks old) were administered either 10µg of FhHDM-1 in 100µl sterile saline, or 100µL saline, by an intraperitoneal (i.p.) injection. One hour post injection, peritoneal cells were harvested by lavage with 5ml sterile saline, and peritoneal macrophages were isolated by negative selection using the peritoneum macrophage isolation kit according to the manufacturer's instructions (Miltenyi Biotec; cat # 130-110-434). The purified macrophages were seeded overnight in a Seahorse XFe24 cell culture microplate at $1x10^5$ cells per well ($0.275cm^2$) before analysis of metabolic activity. Ethics approval for these studies was granted by the University of Technology Sydney (UTS) Animal Care and Ethics Committee (Approval Number: ETH21-5823) and experiments were conducted in accordance with the approved guidelines to be compliant with 'The Australian Code for the Care and Use of Animals for Scientific Purposes'.

2.3.4. RNA extraction, cDNA Synthesis and qPCR

Total RNA was isolated from macrophages using the Isolate II RNA mini kit (Bioline/ Life Science), according to the manufacturer's instructions. cDNA was reverse transcribed using a mixture of random hexamer (Life Technologies) and 10mM dNTP (Life Technologies). After 5 min in an Eppendorf Mastercycler at 65 °C, 10 µL of reverse transcriptase master mixture (10X RT buffer, 25mM $MgCl_2$, 0.1M DTT, RNAse OUT™ and SuperScript III Reverse Transcriptase - Superscript III First Strand Synthesis Kit [Thermo Scientific]) was added to samples. qRT-PCR analysis was performed using TaqMan gene expression master mixture and TaqMan primers (Applied Biosystems). The reaction was run on a Quant Studio™ 12K Flex machine for 40 cycles.

The software programmes GeNorm (137), NormFinder (138) and Best-Keeper (139) were used to identify the optimal housekeeping gene to calculate the relative expression levels of genes by qRT-PCR. For this, the raw C_t values were transformed to different input formats for GeNorm and NormFinder analyses. For analysis using BestKeeper software, raw C_t values were used. Based on this analysis, differential gene expression was calculated after normalization to the *Stx5a* (Applied Biosystems) housekeeping gene (140).

Table 2.1: qPCR primers specifically used in Chapter 2.

Name	Interrogated Sequence	Source
Stx5a (housekeeping)	NM_001167799.1	TaqMan® Mm00502335_m1
Arg1	NM_007482.3	TaqMan® Mm00475988_m1
Retnla	NM_020509.3	TaqMan® Mm00445109_m1
Ym1	NM_00989.2	TaqMan® Mm00657889_mH

2.3.5. Analyses of Metabolic Pathways by Measuring Extracellular Flux

The Seahorse XF24 Extracellular Flux Analyzer (Agilent Technologies) was used to measure the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR). The Seahorse Flux analyser was used to determine the metabolic activity of macrophages. This system measures oxygen consumption rate (OCR) and extracellular acidification (ECAR) rate of live cells in a multi-well plate, which quantifies mitochondrial respiration and glycolysis, respectively. Cellular respiration and glycolysis cause measurable changes to the concentrations of dissolved oxygen and free protons in the well, which are measured in real-time every few seconds by solid state sensor probes.

The Agilent Seahorse Glycolysis Stress Test kit measures the capacity of the glycolytic pathway after glucose starvation. This is accomplished by driving cells toward glycolysis (by the addition of glucose) and assessing their ability to increase glycolytic activity to meet metabolic and bioenergetic demands. The assay measures basal glycolysis (following the addition of glucose) and glycolysis capacity upon inhibition of mitochondrial ATP production by the addition of oligomycin. Glycolysis is calculated by subtracting the last rate measurement before the glucose injection from the maximum rate measurement before the oligomycin injection. The glycolytic capacity is measured by subtracting the last rate measurement before the glucose injection from the maximum rate measurement after the oligomycin injection.

The Agilent Seahorse XFp Cell Mito Stress Test measures key parameters of mitochondrial function by directly measuring the OCR of cells following the sequential addition of modulators of respiration to cells within wells during the assay to reveal the key parameters of mitochondrial function. The modulators are Oligomycin, Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), Rotenone, and Antimycin. Following basal measurements, oligomycin is injected to inhibit ATP synthase (complex V). This decreases electron flow, resulting in a reduction in OCR. Next, cells are treated with FCCP, an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential, consequently electron flow through the ETC is uninhibited, and OCR reaches its maximum. The FCCP-stimulated OCR can then be used to calculate spare respiratory capacity, defined as the difference between maximal respiration and basal respiration. Spare respiratory capacity is a measure of the ability of the cell to respond to increased energy demand or under stress. The final injection is a mixture of rotenone and antimycin A. This combination shuts down mitochondrial respiration and enables the calculation of non-mitochondrial respiration driven by processes outside the mitochondria. Following treatment with LPS, IL-4, or HDM peptides

(as described in the figure legends) BMDMs ($1x10^5$ cells per well) were rinsed twice with either (i) glycolysis stress test media [Seahorse Base Media w/o phenol red (Agilent Technologies) supplemented with 2mM L-glutamine]; (ii) mito stress test media [Seahorse Base Media w/o phenol red supplemented with 10mM D-Glucose, 2mM L-glutamine and 2mM Na-Pyruvate (Life Technologies)]; or (iii) XF-mito fuel stress test media [Seahorse Base Media w/o phenol red supplemented 10mM D-Glucose, 2mM L-glutamine and 1mM Na-Pyruvate] before incubation with assay medium for 1 h at 37 °C in a non-CO_2 incubator. Plates were then treated and assessed using the XF Glycolysis Stress Test Kit (Agilent, SEA103020100), the XF Cell Mito Stress Test Kit (Agilent, SEA103015100) or the XFp Mito Fuel Flex Test Kit (Agilent, SEA103260100), according to the manufacturers' instructions. Briefly, primary murine macrophages were analysed in response to subsequent injections of 10mM glucose, 2µM oligomycin and 2-deoxy-glucose (2-DG) for the glycolysis stress test (ECAR); and 2µM oligomycin, 2µM fluoro-carbonyl cyanide phenylhydrazone (FCCP), and 0.5µM Rotenone/ Antimycin A (Rote /AA) for the mitochondrial stress test (OCR). For the assessment of fatty acid oxidation (FAO) dependency, the OCR of macrophages were analysed in response to subsequent injection of a fatty acid inhibitor (4µM Etomoxir, long chain fatty acid inhibitor), and a combination of glutamine (3µM Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl) ethyl sulfide, BPTES, glutamate inhibitor) and glucose (2µM 2-Cyano-3-(1-phenyl-1H-indol-3-yl)-2-propenoic acid, UK5099, pyruvate carrier blocker) inhibitors. The percentage of maximal capacity that is dependent upon FAO was calculated from the maximal metabolic rate from each treatment. For the determination of FAO capacity, the OCR was measured from cells responding to a combination of glucose (2µM UK5099) and glutamine (3µM BPTES) inhibitors, followed by a long fatty acid inhibitor (4µM of Etomoxir). The fuel oxidation capacity was determined by the dependency to oxidize FA, and flexibility to use glucose (GLC) or glutamine (GLN).

2.3.6. Normalization of Cell Number by CyQuant Cell Proliferation Assay

A CyQuant cell proliferation assay kit (Invitrogen) was used to quantify cell numbers at the endpoint of live metabolic assays, following the manufacturer's instructions. XFe-24 microplates containing cells were frozen at -80 °C for 24 h upon completion of the metabolic flux assays. Prior to the quantification of cell numbers, the plates were allowed to reach room temperature before 200µL of CyQuant/cell-lysis buffer solution was added to each well.

Fluorescence intensity was then measured using the Infinity M200 pro plate reader at ~480 nm excitation and ~520 nm emission maxima.

2.3.7. Acyl-CoA Mass Spectrometry

To assess TCA cycle fuel utilization, BMDMs were treated with LPS (20ng/mL), IL-4 (20ng/mL), or FhHDM-1 (2.5µM) for 24h at 37 °C and 5% CO_2 followed by 1h of treatment with nutrient labelled/non-labelled media (100µM $^{13}C_{16}$-potassium palmitate (Cambridge Isotope Laboratories) + 5mM glucose (Chem-supply), 100µM potassium palmitate (Sigma-Aldrich) + 5mM $^{13}C_6$-glucose (Cambridge Isotope Laboratories), 100µM potassium palmitate + 5mM glucose in DMEM no glucose, no glutamine, no phenol red). Acyl-CoA analysis was performed by liquid chromatography- high resolution mass spectrometry (LC-HRMS) as previously described by collaborators Nathaniel Snyder and Eliana von Krusenstiern (141). Briefly, samples and calibration standards containing commercially available acetyl-CoA (Sigma-Aldrich) in 10% (w/v) trichloroacetic acid in water were spiked with $^{13}C_3{}^{15}N_1$-acyl-CoA internal standards, sonicated for 12 × 0.5 s pulses, protein was pelleted by centrifugation at 17,000 ×g from 10 min at 4 °C, then supernatants were extracted by solid-phase extraction using Oasis HLB 1cc (30 mg) SPE columns (Waters). Columns were washed with 1 mL methanol, equilibrated with 1 mL water, loaded with supernatant, desalted with 1 mL water, and eluted with 1 mL methanol containing 25 mM ammonium acetate. The purified extracts were evaporated to dryness under nitrogen then resuspended in 55 µL 5% (w/v) 5-sulfosalicylic acid in water. Samples, 5 µL in 5% SSA, were analyzed by injection of an Ultimate 3000 Quaternary UHPLC coupled to a Q Exactive Plus (Thermo Scientific) mass spectrometer in positive ESI mode using the settings described previously (142). Quantification of acyl-CoAs was via their predominant $[(M-507) + H]^+$ product ions. Data were integrated using Tracefinder v4.1 (Thermo Scientific) software. Isotopic enrichment in tracing experiments was calculated by normalization to unlabelled control samples using the FluxFix calculator (143).

2.3.8. Measurement of Intracellular Metabolites

BMDMs were cultured overnight with either LPS (20ng/mL), IL-4 (20ng/mL) or FhHDM-1 (2.5µM). After removal of the supernatants, cells were washed 3 times with cold PBS. Cells were then harvested and snap frozen. Pellets were extracted by 80/20 methanol/water polar metabolite extraction. LC-HRMS was performed as previously described by collaborators Nathaniel Snyder and Eliana von Krusenstiern, with minor modifications (144). Briefly, an

Ultimate 3000 UHPLC equipped with a refrigerated autosampler (at 6 °C) and a column heater (at 55 °C) with a HSS C18 column (2.1 × 100 mm i.d., 3.5 µm; Waters, Milford, MA) was used for separations. Solvent A was 5 mM N,N-diisopropylethylamine (DIPEA) and 200 mM hexafluoro-2-propanol (HFIP) and solvent B was methanol with 5 mM DIPEA and 200 mM HFIP. The gradient was as follows: 100 % A for 3 min at 0.18 mL/min, 100 % A at 6 min with 0.2 mL/min, 98 % A at 8 min with 0.2 mL/min, 86 % A at 12 min with 0.2 mL/min, 40 % A at 16 min and 1 % A at 17.9 min-18.5 min with 0.3 mL/min then increased to 0.4 mL/min until 20 min. Flow was ramped down to 0.18 mL/min back to 100 % A over a 5 min re-equilibration. For MS analysis, the UHPLC was coupled to a Q Exactive HF mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a HESI II source operating in negative mode. The operating conditions were as follows: spray voltage 4000 V; vaporizer temperature 200 °C; capillary temperature 350°C; S-lens 60; in-source CID 1.0 eV, resolution 60,000. The sheath gas (nitrogen) and auxiliary gas (nitrogen) pressures were 45 and 10 (arbitrary units), respectively. Single ion monitoring (SIM) windows were acquired around the $[M-H]^-$ of each analyte with a 20 *m/z* isolation window, 4 *m/z* isolation window offset, $1e^6$ ACG target and 80 ms IT, alternating with a Full MS scan from 70-950 *m/z* with 1e6 ACG, and 100 ms IT.

2.3.9. Statistical Analysis

In all cases, data are presented as means ± standard error of the means (SEMs) of multiple biological replicates. The comparison of data was determined by either an unpaired, two tailed Welch's t-test, or a one-way ANOVA with Tukey's multiple comparison test, using GraphPad Prism 7 software (GraphPad). Statistical significance was considered as a p-value <0.05.

2.4. Results

2.4.1. FhHDM-1 directed macrophages to utilise oxidative phosphorylation.

To initially assess the effect of FhHDM-1 on the metabolic activity of macrophages, extracellular flux analysis was used to quantify the mitochondrial oxygen consumption rates (OCR), indicating oxidative metabolism, and the extracellular acidification rate (ECAR) as a measure of glycolysis. For comparison to the broadly characterised functional metabolism of M1 and M2 phenotypes, bone marrow derived macrophages (BMDMs) were also stimulated with bacterial LPS, or recombinant IL-4, respectively. In addition, samples of macrophages were untreated, thus representing a resting, unstimulated M0 phenotype.

This approach revealed that macrophages treated *in vitro* with FhHDM-1 displayed increased OCR, as compared to untreated cells, or cells treated with LPS (Figure 2.1A). Furthermore, the increase in maximum respiration (MR) observed in FhHDM-1 treated macrophages was equivalent to that seen in IL-4 induced M2 macrophages (Figure 2.1B). In contrast, examination of the extracellular acidification rate (ECAR) as a measure of glycolysis, suggested that, unlike IL-4, FhHDM-1 had no effect on the glycolytic activity (Figure 2.1C). FhHDM-1 treated macrophages exhibited the same maximum glycolytic activity as resting untreated cells, which was significantly less than values observed in cells treated with either IL-4 or LPS (Figure 2.1D). Validating this finding and verifying that the alteration to macrophage metabolism by FhHDM-1 occurs *in vivo*, peritoneal macrophages isolated from mice 1h after an i.p. injection of FhHDM-1 also showed a similarly significant increase in OCR as compared to macrophages from mice that received saline only (Figure 2.2).

Despite this biased metabolic profile of cells treated with FhHDM-1 being indicative of an M2 phenotype, there was no evidence of any expression of the characteristic M2 markers; Arg-1, Retnlα or Ym1 (Figure 2.3).

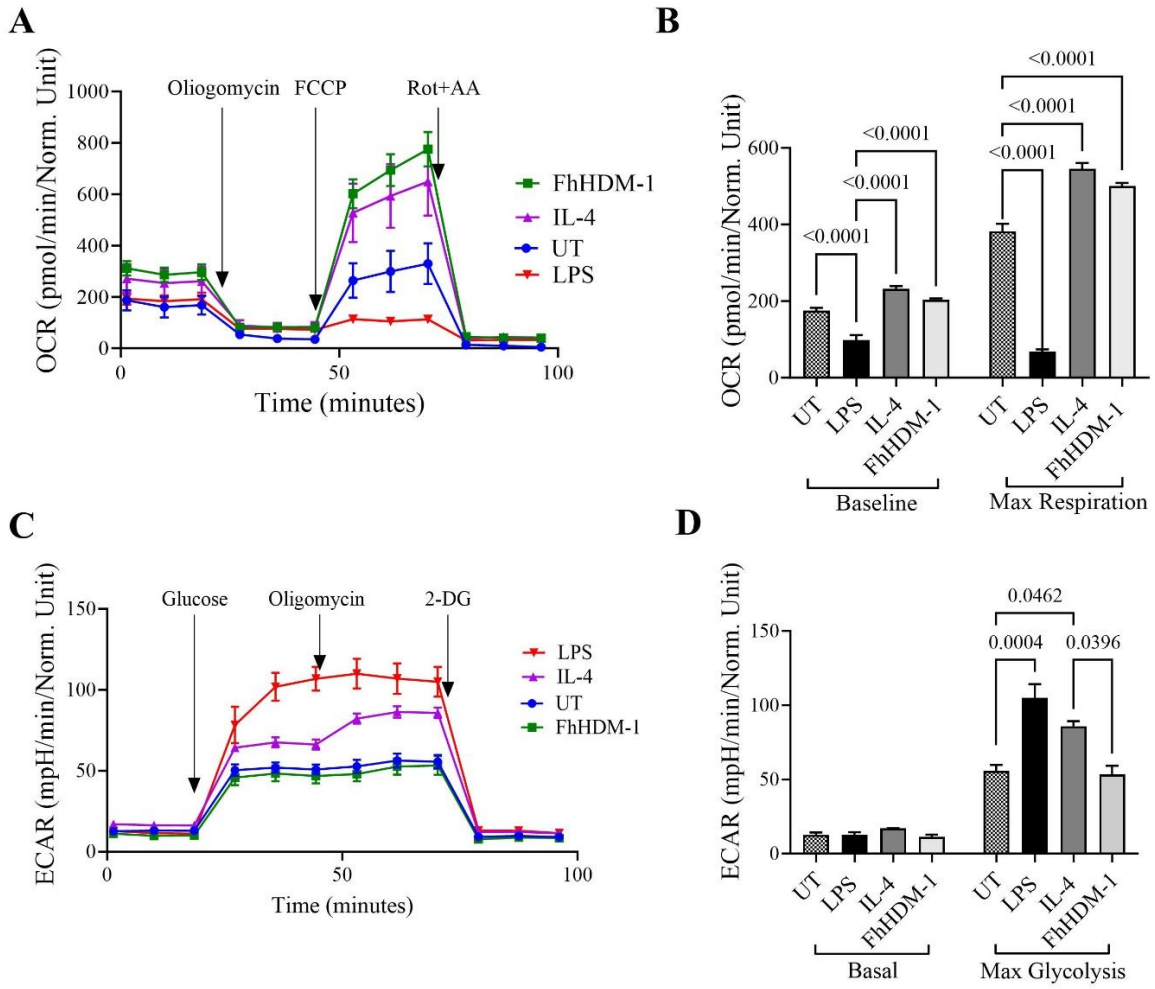

Figure 2.1: Unlike IL-4, treatment of macrophages with FhHDM-1 enhanced oxidative phosphorylation (OXPHOS) without modulating glycolytic activity. BMDMs were either untreated (UT) or treated with LPS (20ng/ml), IL-4 (20ng/ml), or FhHDM-1 (2.5µM) for 24h (n=5). (A) The oxygen consumption rate (OCR) was measured at basal levels and following sequential treatments with oligomycin, FCCP, and rotenone/antimycin A (Rot+AA) to determine (B) the maximum respiratory capacity of cells. Glycolytic activity was determined by measuring (C) the extracellular acidification rate (ECAR) and (D) maximum glycolytic activity as cells were treated with glucose, oligomycin and 2-DG (2-deoxy-glucose). Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test. Data is representative of five independent experiments and is presented as means ± SEMs.

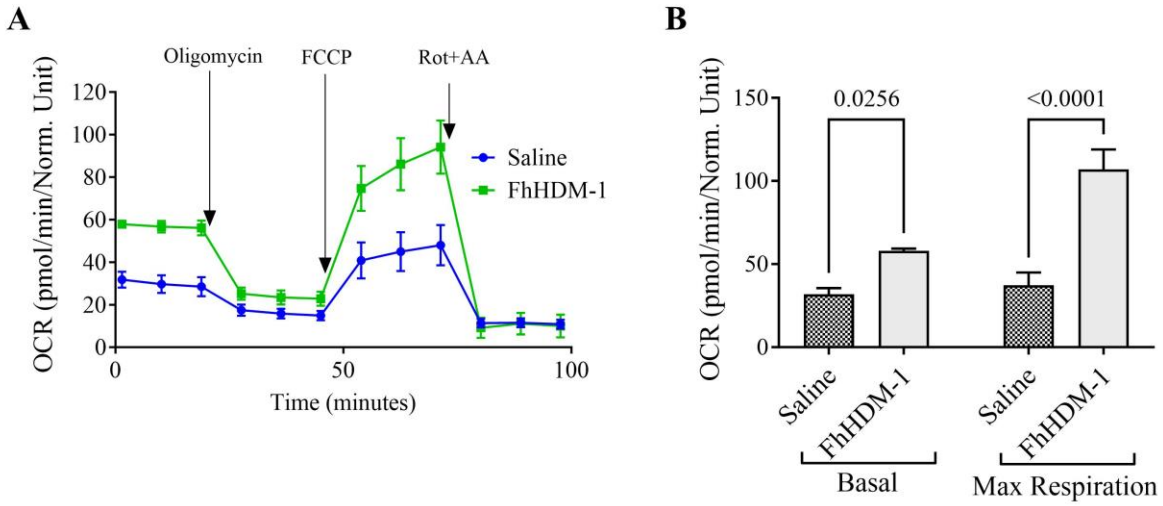

Figure 2.2: FhHDM-1 enhanced oxidative phosphorylation (OXPHOS) in peritoneal macrophages after a single i.p. injection. (A) OCR was measured at basal levels and following sequential treatments with oligomycin, FCCP, and rotenone/antimycin A (Rot+AA) in peritoneal macrophages harvested from mice (n=10) that had received a single i.p. injection of either FhHDM-1 or saline. This data was used to (B) determine the maximum respiratory capacity of cells. Data is presented as means ± SEMs. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

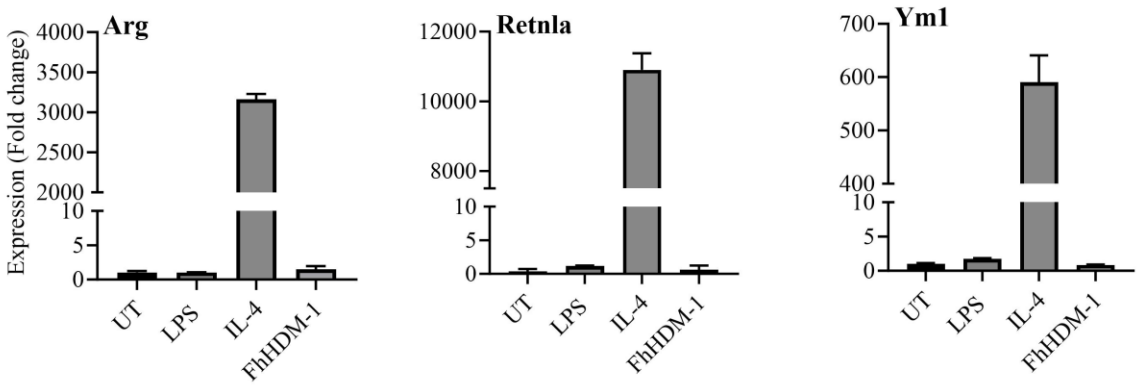

Figure 2.3: FhHDM-1 did not alter the expression of typical M2 genes. The differential expression of Arg1, Retnlα, and Ym1 was measured by qRT-PCR in cell lysates of BMDMs that were either untreated or cultured with LPS, IL-4, or FhHDM-1 for 6h (n=3). Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test. Data is representative of three independent experiments and is presented as means ± SEMs.

2.4.2. FhHDM-1 induced oxidative phosphorylation (OXPHOS) is fuelled by fatty acids.

Independent of a switch to an M2 phenotype, which can be mediated by either glycolysis or OXPHOS, the anti-inflammatory functional activity of macrophages is mediated only by OXPHOS, which can be fuelled by FAO or glutamine. This suggested that FhHDM-1 treated macrophages, although not classified as M2, may still have possessed the metabolism required for anti-inflammatory functionality.

To investigate this, the preference and flexibility of macrophages to use various fuels was assessed. Accordingly, the dependency of BMDMs treated with either FhHDM-1 or IL-4 on different fuels was determined by quantifying changes to the OCR after the combined inhibition of glycolysis and glutaminolysis, followed by the inhibition of FAO (Figure 2.4A). This revealed that, like IL-4 treated cells, FhHDM-1 treated macrophages had a strong dependency for FAO, illustrated by the decrease in OCR following the addition of etomoxir, an FAO inhibitor (Figure 2.4A). In fact, FhHDM-1 treated macrophages were significantly more dependent on FAO to drive OCR than IL-4 treated cells (Figure 2.4B) Aligning with this increased dependence on fatty acids, and the lack of enhanced glycolysis in FhHDM-1 treated macrophages (Figure 2.1), these cells also showed a reduced flexibility to use other to fuels, as compared to IL-4 treated cells (Figure 2.4C).

To support this analysis, the incorporation of ^{13}C, derived from fatty acids or glucose, into the central metabolite acetyl-CoA in macrophages was traced by mass spectrometry. As expected, unlike LPS or IL-4, which both induced an increase in glycolysis, treatment of macrophages with FhHDM-1 did not increase the metabolism of glucose above levels seen in resting, unstimulated cells (Figure 2.5A). However, surprisingly this analysis also showed that FhHDM-1 treated macrophages did not utilise the carbon provided by exogenous fatty acids (Figures 2.3B). Furthermore, the addition of exogenous palmitate did not augment the metabolic respiration induced by FhHDM-1 treatment (Figure 2.5C).

Although seemingly contradictory to the measure of enhanced OCR in response to FhHDM-1 (Figure 2.1), and the clear dependency on FAO (Figure 2.4), these results suggested that the metabolic activity of macrophages induced by FhHDM-1 may be reliant on the oxidation of endogenous fatty acids.

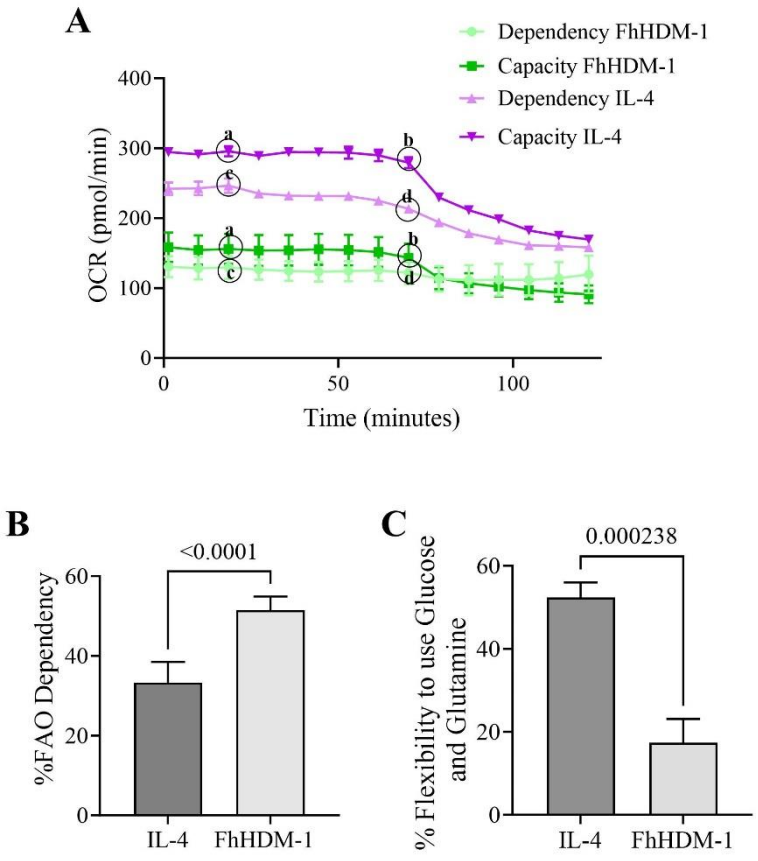

Figure 2.4: Oxidative phosphorylation (OXPHOS) induced by FhHDM-1 had a greater dependency on fatty acids than IL-4 driven metabolic activity. (A) The dependency and capacity for different fuels to support mitochondrial respiration was determined by measuring OCR. BMDMs were treated with IL-4 (20ng/mL) or FhHDM-1 (2.5µM) for 24h (n=5) and oxidative metabolism was measured after treatment with combination of BPTES and UK5099 (a) followed by treatment with Etomoxir (b) to determine the metabolic capacity, or with Etomoxir (c) followed by BPTES and UK5099 (d) for fatty acids (FA) dependency. (B) The FAO dependency was calculated from the cells ability to sustain mitochondrial respiration after exposure to etomoxir to inhibit fatty acids. (C) The percentage of flexibility to use glucose and glutamine was calculated by the fuel capacity minus the dependency to oxidise FA. Data is representative of three independent experiments and is presented as means ±SEMs. Statistical significance was determined by an unpaired parametric student t-test (two tailed, Welch's correction).

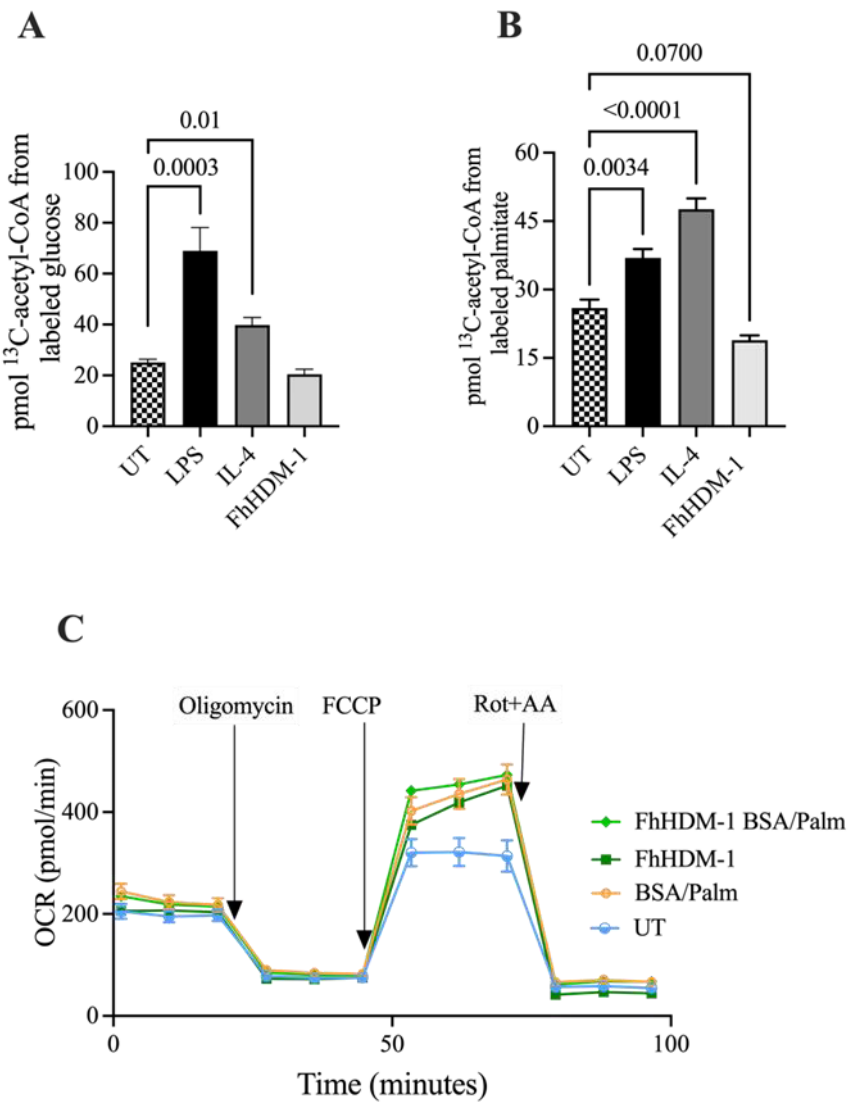

Figure 2.5: Oxidative phosphorylation (OXPHOS) induced by FhHDM-1 does not utilise exogenous fatty acids. (A, B) The incorporation of ^{13}C into acetyl-CoA was determined by mass spectrometry analysis of BMDMs that had been untreated (UT) or cultured with FhHDM-1 (2.5µM), IL-4 (20ng/mL), or LPS (20ng/mL) for 24h followed by 1h in the presence of $^{13}C_{16}$-potassium palmitate of $^{13}C_{16}$-glucose (n=6). (C) The ability of cells to utilise exogenous fatty acids was determined by measuring the OCR levels in BMDMs cultured with FhHDM-1 (2.5µM) in the presence of BSA/palmitate (n=4). Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test. Data is representative of 2 and 5 independent experiments respectively and is presented as means ± SEMs.

2.4.3. The FhHDM-1 induced metabolic switch to oxidative phosphorylation (OXPHOS) in macrophages is associated with endogenous fatty acid synthesis concomitant with increased glutaminolysis.

M2 (IL-4 induced) macrophages have the capacity to utilise glutamine to support their metabolic demands, as illustrated by the data presented here (Figure 2.4) and by a report that one-third of TCA carbons in these cells are glutamine derived (145). The increased glutaminolysis induced by IL-4 supports the *de novo* synthesis of fatty acids, in the absence of exogenous fatty acids (130). Glutaminolysis generates α-ketoglutarate (α-KG), which is converted to citrate either via the citric acid (TCA) cycle or by reductive carboxylation. Cytoplasmic citrate is then converted to acetyl-CoA for fatty acid synthesis (142, 146).

Therefore, to investigate the hypothesis that FhHDM-1 may be inducing the synthesis of fatty acids in macrophages to support FAO to fuel a switch to OXPHOS, alterations in the abundance of different components of this pathway were measured. Macrophages that were treated with FhHDM-1 had significantly increased levels of α-KG and citrate, as compared to LPS treated cells (Figure 2.6A, B). This observation may reflect a greater dependence on FAO because FhHDM-1 treated macrophages had significantly higher quantities of citrate, as compared to IL-4 treated cells. In addition, there was a correlative dose dependent decrease in α-KG following FhHDM-1 treatment, as compared to IL-4 treatment, although this did not reach significance (p=0.06 for HDM treatment [15μM]). The increased abundance of α-KG and citrate was coupled with a significant increase in the gene expression levels of two key enzymes involved in fatty acid synthesis (fatty-acid synthase [*fasn*] and ATP citrate lyase [*acly*]) (Figures 2.4B, C). This finding supports the hypothesis that endogenous fatty acids were being synthesized for subsequent oxidation.

Finally, the likelihood that FhHDM-1 was reprogramming macrophages to utilize glutamine as a major carbon source to fuel the synthesis and oxidation of fatty acids was further supported by the observation that the OXPHOS induced by FhHDM-1 in macrophages (which is dependent on FAO) was significantly enhanced under glutamine replete conditions (Figures 2.4D, E). Furthermore, both glutaminolysis and the increased expression levels of *fasn* and *acly* were reversed in FhHDM-1 treated macrophages by the addition of the glutaminase inhibitor, bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl) ethyl sulphide (BPTES) (Figure 2.5).

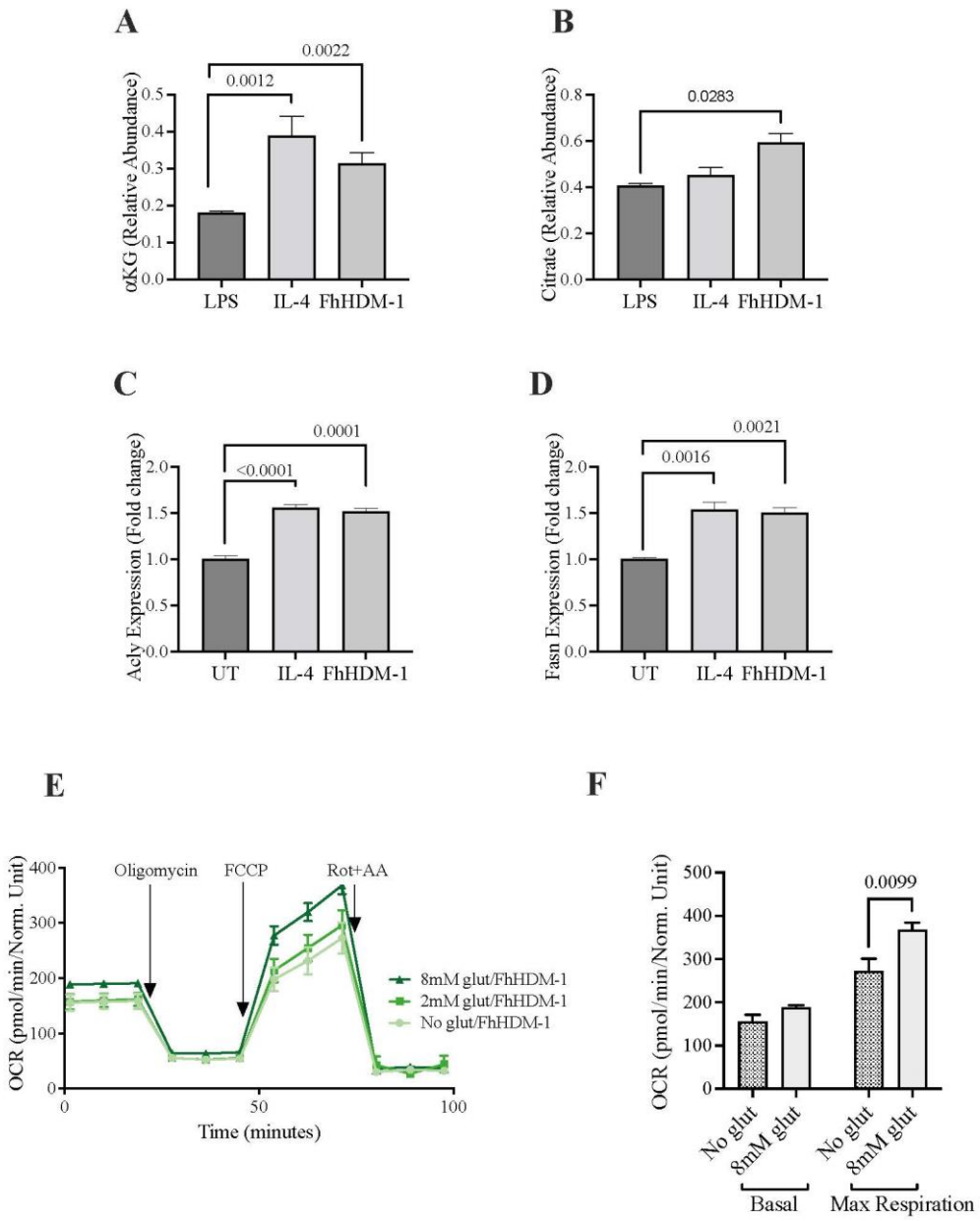

Figure 2.6: FhHDM-1 induced glutaminolysis and synthesis of endogenous fatty acids. (A, B) BMDMs were treated with LPS (20ng/mL), IL-4 (20ng/mL) or FhHDM-1 (2.5µM). The intracellular levels of citrate and α-KG were quantified by mass spectrometry after metabolite extraction (n=6). (C, D) The differential expression of *acly* and *fasn* was quantified by qRT-PCR in lysates of cells (n=3) that were untreated or treated with IL-4 or FhHDM-1(2.5µM), for 6h. (E) The OCR was measured in BMDMs treated with FhHDM-1 (2.5µM) for 6h in glutamine replete (2mM or 8mM) or depleted media for 18h (n=4). The basal oxygen consumption rate (OCR) was initially determined, and then measured following sequential treatments with oligomycin, FCCP, and rotenone/antimycin A (Rot+AA) to determine (E) the maximum respiratory capacity of cells. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test. Data is representative of three independent experiments and is presented as means ± SEMs.

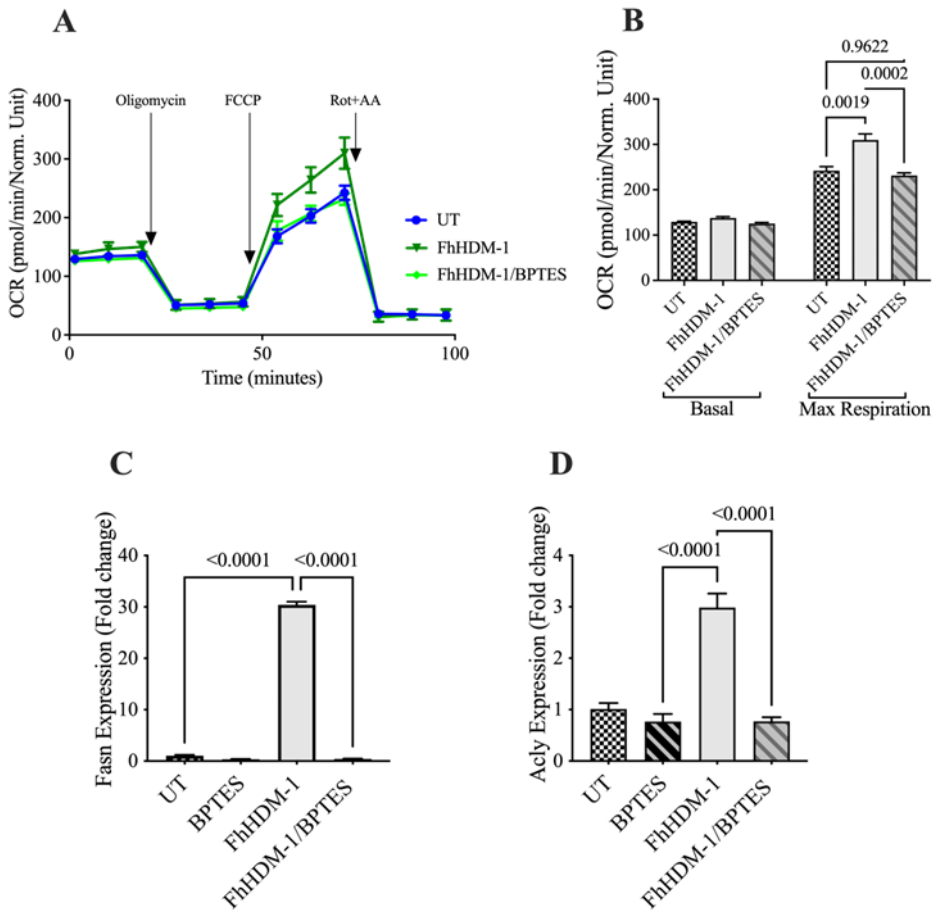

Figure 2.7: Oxidative phosphorylation (OXPHOS) induced by FhHDM-1 was fuelled by glutaminolysis and the synthesis of endogenous fatty acids. (A, B) The requirement for glutamine by FhHDM-1 treated cells was validated by measuring the (A) OCR and the (B) maximum respiratory capacity in BMDMs cultured in media (UT), or in the presence of FhHDM-1 (2.5µM) or a combination of FhHDM-1 and the glutamate inhibitor, BPTES (10µM), for 18h (n=6). (C, D) The differential expression of Acly and Fasn was quantified by qRT-PCR in lysates of cells (n=3) that were untreated, or treated with IL-4, FhHDM-1(2.5µM), or a combination of FhHDM-1 and the glutamate inhibitor, BPTES (10µM), for 6h. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test. Data is representative of three independent experiments and is presented as means ± SEMs.

2.5. Discussion

Mitochondrial metabolism, and specifically fuel preference, has a defining role in regulating the inflammatory responses of macrophages (147, 148). The data presented here shows that the parasite derived peptide, FhHDM-1, reprograms the metabolic activity of macrophages to utilise OXPHOS, which was fuelled by FAO and supported by elevated levels of citrate and α-KG produced by glutaminolysis. In contrast to IL-4, the treatment of macrophages with FhHDM-1 did not increase glycolytic activity and did not induce the expression of genes associated with an M2 phenotype.

It is now well established that macrophage lipid metabolism and inflammatory activation are linked (149, 150). The consensus has been that saturated fatty acids elicit pro-inflammatory responses, via activation of Toll-like receptor (TLR) signalling, in both human and murine macrophages (151, 152). The subsequent activation of c-Jun N-terminal kinase (JNK) and nuclear factor-κB (NF-κB) signalling pathways induces endoplasmic reticulum (ER) stress, which leads to inflammation (153, 154). Conversely, the catabolism of fatty acids through mechanisms including FAO, attenuates ER stress and inflammatory responses in mice and humans, and sustains an anti-inflammatory phenotype (155). Increased FAO is also correlated with efferocytosis, a process used by regulatory/anti-inflammatory macrophages to remove apoptotic cells to maintain tissue homeostasis (156). However, more recently this simple paradigm of inflammatory function has been challenged by several observations that, under certain conditions, FAO can also activate pro-inflammatory responses through the activation of the NLRP3 inflammasome and the synthesis of IL-1β (157). Although the exact mechanism is still not clear, increased FAO fuelled mitochondrial reactive oxygen species (ROS) production has been implicated (158-160).

These observations have led to a re-assessment of the biological activity for fatty acids and suggests that FAO can, depending on the context, support both pro- and anti-inflammatory functions. In pro-inflammatory macrophages, FAO may be used to produce ROS, which are needed to activate the NLRP3 inflammasome. Additionally, in a pro-inflammatory environment, FAO could fuel the TCA cycle to compensate for loss of metabolites, which are being extracted for synthesis of the macromolecules necessary to support the pro-inflammatory response (149). On the other hand, FAO contributes to the maintenance of anti-inflammatory functions, such as efferocytosis, by consuming FAs that would otherwise accumulate. Furthermore, oxidation of FAs from apoptotic cells enhances rates of IL-10 transcription and synthesis, thereby reinforcing an anti-inflammatory phenotype (161). In the context of the host

immune response to infection with *Fasciola hepatica*, it seems likely that FhHDM-1 is inducing a unique phenotype of macrophage, which, although not typically M2-like, utilises the metabolic pathways necessary to support an anti-inflammatory functional phenotype. However, this premise will need to be investigated further.

The data presented here indicated that the FAO in FhHDM-1 treated macrophages was reliant on the synthesis of endogenous fatty acids. While this conclusion would be strengthened by the inhibition of FAS and a measure of subsequent FAO, the biological activity of FhHDM-1 provides support for the hypothesis that FhHDM-1 is inducing *de novo* fatty acid synthesis. It has previously been shown that FhHDM-1 localizes to endolysosomes within macrophages where it inhibits vATPase (103, 111). This central role for the lysosome explains the difference in metabolic activity observed between FhHDM-1 and IL-4 treated macrophages, as the OXPHOS induced by IL-4 is primarily dependent on lysosomal lipolysis of exogenous fatty acids (130). As the lysosomes of FhHDM-1 treated macrophages would be less acidic (due to inhibition of vATPase activity), lipolysis would be inhibited, and consequently the macrophages would become more metabolically reliant on endogenous fatty acids synthesized from glutamine. This process would be dependent on increased expression levels of mitochondrial NADPH-dependent isocitrate dehydrogenase (IDH2), which would lead to increased amounts of citrate being exported from the mitochondrial matrix for FAS (162). However, it remains to be determined how the citrate observed in the FhHDM-1 treated macrophages was being produced. It is possible that citrate was generated via reductive decarboxylation, or carbon from glutamine being cycled around the TCA cycle, or anapleurosis (replenishing TCA cycle intermediates that have been extracted for biosynthesis). However, given the observation that glutamine increases cellular citrate, and increases OCR, and can be reversed by BPTES it is not dependent on the balance of those three known mechanisms.

The premise that FhHDM-1 induced vATPase inhibition is modulating macrophage metabolism is further corroborated by comparison to the biological activity of the vATPase inhibitor, bafilomycin A1 (BafA1). Like FhHDM-1, macrophages treated with BafA1 displayed a metabolic preference for OXPHOS, which was attributable to protons being rechannelled to the mitochondria due to impaired acidification of lysosomes (163). While the fuel source supporting this metabolic switch was not identified, Huh7 liver cells treated with the vATPase inhibitor, Archazolid-A, also displayed a metabolic shift to OXPHOS, which was shown to be glutamine dependent (164). Furthermore, increased cytosolic acidification in human fibroblasts was shown to induce the expression of glutaminase-1, and drive a switch

towards glutaminolysis (165). It has been proposed that this alteration in metabolic activity is required in response to the change in cellular pH, as the ammonia produced by glutaminolysis neutralised the cytosolic pH to prevent cell death (165). This association between lysosomal pH, intracellular movement of protons, and mitochondrial activity should be explored in FhHDM-1 treated cells to establish a definitive mechanistic link.

Glutaminolysis also leads to the production of α-KG, which, as shown here, was increased in FhHDM-1 treated macrophages. This metabolite functions as a checkpoint for the reprogramming of macrophage phenotype and functional activity (166-168). In IL-4 treated cells, α-KG is an essential cofactor for the histone demethylase, jumonji domain-containing protein 3 (JMJD3), which mediates the demethylation of the repressive trimethylated histone mark (H3K27me3) to promote the expression of its target genes, notably the characteristic M2 markers (166, 169). However, this outcome is subtly regulated by the concentration of α-KG (168), which might explain the lack of M2 gene expression in FhHDM-1 treated macrophages because these cells showed a lower abundance of α-KG than IL-4 induced M2 macrophages.

In addition to a role in the polarization of M2 macrophages, an increased abundance of α-KG has also been shown to reduce the expression levels of the DNA methyltransferase, DNMT3B, leading to a correlative reduction in the expression levels of dynamin-1-like protein (Drp1) (170). This is notable as Drp1 is an enzyme, which in macrophages catalyzes mitochondrial fission, a morphology that is associated with enhanced glycolysis (163). Thus, a switch in mitochondrial dynamics from fission to fusion promoted by an increase in α-KG, would support the FAO and OXPHOS observed in FhHDM-1 treated macrophages.

While some outstanding questions need to be resolved, the data presented here, combined with the current understanding of immune metabolism, and previous knowledge of FhHDM-1 biological activity, suggests an intriguing link between the macrophage lysosome, glutaminolysis, mitochondrial dynamics, and the regulation of macrophage inflammatory function. It also suggests that either blocking glutaminolysis or enhancing glycolysis in peritoneal macrophages during the early stages of infection with *F. hepatica*, may facilitate the development of new strategies for the control of infection with this parasite.

Chapter 3: FhHDM-1 reprogramming of metabolic activity supports an anti-inflammatory phenotype in macrophages

3.1. Publication statement

The experimental data presented in this chapter and in Chapter 2 contribute to a research article, which has published in *Frontiers in Immunology*:

Quinteros, S., von Krusenstiern, E., Snyder, NW., Tanaka A., O'Brien, B., & Donnelly, S. (2022). The helminth derived peptide FhHDM-1 redirects macrophage metabolism towards glutaminolysis to regulate the pro-inflammatory response. *Front Immunol*. 2023 Jan 25; 14:1018076.

The author contribution are described in the previous chapter, section 2.1. The introduction and discussion presented here differ from the published manuscript to better position the experimental work and data within the context of the PhD project and written thesis. In addition, numbering of sections, style of referencing, numbering of tables and figures have been edited to align with the formatting of the thesis.

3.2. Introduction

When pro-inflammatory cytokines are not required due to the regulation or resolution of an inflammatory response, or at the initiation of immune tolerance, the metabolic activity of macrophages is typically switched from glycolysis to predominantly OXPHOS (171, 172). Thus, given the observed redirection of metabolic activity in FhHDM-1 treated cells described previously (Chapter 2), it seemed feasible that FhHDM-1 was hijacking this mechanism of immune-regulation to prevent pro-inflammatory immune responses in macrophages.

The previous chapter established that the parasite secreted peptide, FhHDM-1, did not induce an M2 phenotype of macrophage, but rather switched the metabolic activity to a pathway that would support an anti-inflammatory functional phenotype. This activity aligns with the regulation of host immune responses that would be required as the parasite migrates from the intestine to the bile duct. As described in Chapter 1, after ingestion of the metacercariae, the NEJs excyst and perforate the intestinal epithelial as they move from the gut and through the peritoneal cavity to reach the liver capsule. Undoubtedly this activity releases host alarmins, due to damage to the epithelial layer, and causes the translocation of gut bacteria (64). Yet there is no evidence of a host protective pro-inflammatory response, the absence of which allows the parasite to complete its migratory path. Thus, the data presented in Chapter 2 suggested that FhHDM-1 directed a metabolic preference for OXPHOS, thereby reducing the ability of tissue alarmins and/or bacterial ligands to initiate the glycolytic pathways that would be necessary to generate an M1 pro-inflammatory response.

While a switch in macrophage phenotypes is critical to the outcome of infection with parasitic worms (Chapters 1 and 2), it can also change the progression of several human immune-mediated diseases. Compelling epidemiological evidence demonstrates a robust inverse correlation between the prevalence of endemic helminth infections and the incidence of immune-mediated disease globally (173, 174). Multiple animal-based experimental studies and human investigations have corroborated the potent ability of helminth infection, and/or treatment with their excretory-secretory products, to skew immune responses towards an anti-inflammatory/tolerogenic profile to prevent/reverse autoimmune/inflammatory disease (60, 175). Further, it has been shown that this protective effect can be simulated by the adoptive transfer of macrophages that have been exposed to helminth products and induced to undergo a functional modification to an anti-inflammatory/tolerogenic phenotype (176-179). Accordingly, understanding the mechanisms by which helminth molecules modulate the

functional phenotypes of macrophages opens new avenues to prevent and treat human immune-mediated disease.

The anti-inflammatory capacity of FhHDM-1 was previously discovered by assessing the protective efficacy of the peptide in animal models of immune-mediated T1D (non-obese diabetic [NOD] mice) and MS (Experimental Allergic Encephalitis; EAE). In these studies, the efficacy of recombinant/synthetic versions of the predominant proteins secreted by *F. hepatica*, proteomically identified as a cysteine protease (FhCL1), peroxiredoxin (FhPrx), and FhHDM-1 was tested. This comparison revealed that only FhHDM-1 prevented the development of disease in both models (102). Understanding that FhHDM-1 preferentially interacted with macrophages, these cells were tested for their inflammatory capacity *ex vivo*. Thus, peritoneal macrophages were harvested from mice that had previously received the therapeutic regime of FhHDM-1 (10μg given by i.p. injection delivered on alternate days for a total of 6 injections) and their ability to produce TNF in response to an *ex vivo* stimulation with bacterial LPS was measured. Macrophages isolated from mice that had been injected with FhHDM-1 produced significantly less TNF, as compared to animals that had received saline alone.

This data, combined with the observed prevention of inflammatory disease in mice injected with FhHDM-1, identified FhHDM-1 as a potent anti-inflammatory agent. However, an understanding of the mechanism(s) of action of FhHDM-1 and whether this was a direct or indirect effect of the peptide was yet to be elucidated. The possibility that an altered metabolic activity facilitated this anti-inflammatory effect (as supported by data in Chapter 2) was investigated in this chapter.

3.3. Materials and Methods

3.3.1. Methodology previously described

Several methods used in this chapter have been previously described in Chapter 2 (2.2.1-2.2.8). Additionally, an inactive mutant derivative [NHP;(111)], was synthesised to 95% purity and verified to be endotoxin free (GLBiochem, Shanghai, China). The peptide was solubilised in sterile, endotoxin-free water (Baxter), aliquoted, and stored at -80 ºC until use.

Table 3.1: qPCR primers specifically used in Chapter 3.

Name	Interrogated Sequence	Source
Stx5a (housekeeping)	NM_001167799.1	TaqMan® Mm00502335_m1
TNF	NM_001278601.1	TaqMan® Mm00443258_m1
HIF1α	NM_010431.2	TaqMan® Mm00468869_m1

3.3.2. Quantification of TNF and IL-6 Protein Secretion by Macrophages

The levels of TNF or IL-6 secreted by BMDMs in response to stimulation with LPS (20ng/mL) were quantified using cytokine specific ELISA kits (BD Bioscience), according to the manufacturer's instructions. Absorbance at 450nm was quantified using a Tecan plate reader. Absorbance readings were corrected for background absorbance, and then used to calculate cytokine concentrations by extrapolation from a standard curve.

3.3.3. Measurement of Hexokinase Activity

The hexokinase activity assay (Abcam-ab136957) was performed according to the manufacturer's instructions. BMDMs were treated with FhHDM-1 (2.5µM or 15µM) for 1h, supernatants were discarded, and cells were washed twice in RPMI 1640 medium (Gibco) containing 10% v/v heat-inactivated FCS (Gibco). Following this, cells were stimulated with LPS (20ng/mL) for 18h, and then collected and homogenized in 100µL of ice-cold assay buffer. To allow for the homogenization process to occur, cells were incubated for 10 min on ice before centrifugation (10,000xg for 5 min). The supernatant was collected and assayed using the

reaction mix, according to the manufacturer's instructions. The development of colour was then measured at 450 nm every 5 min for 30 min.

3.3.4. Statistical Analysis

In all cases, data are presented as means ± standard error of the means (SEMs) of several biological replicates (n). The comparison of data was determined by either an unpaired, two tailed Welch's t-test or a one-way ANOVA with Tukey's multiple comparison test using GraphPad Prism 7 software (GraphPad). Statistical significance was considered as a p-value <0.05.

3.4. Results

3.4.1. FhHDM-1 treatment of macrophages suppressed the pro-inflammatory response and reduced glycolysis, independently of a switch to OXPHOS.

FhHDM-1 has previously been shown to reduce the production of TNF by macrophages leading to its characterisation as an immune regulatory peptide (102). If the switch from glycolysis to OXPHOS induced by FhHDM-1 was indeed regulating the inflammatory response, it would be expected that the production of a several cytokines would be impacted. Therefore, the levels of pro-inflammatory cytokines secreted by macrophages treated with FhHDM-1 *in vitro* and subsequently stimulated with bacterial LPS were measured. This showed that FhHDM-1 also significantly reduced the secretion of IL-6, IL-1β, IL-12p70 and MCP-1 (Figure 3.1A). Further analysis of the suppression of TNF production in response to LPS stimulation demonstrated that this was a dose dependent effect, with increased concentrations of FhHDM-1 having a greater inhibitory effect, suggesting a direct effect of the peptide's biological activity (Figure 3.1B).

The anti-inflammatory cytokine, IL-10, has the capacity to regulate the expression of pro-inflammatory responses by inhibiting inflammatory induced glycolysis and promoting OXPHOS (40). However, FhHDM-1 treated macrophages showed no significant increase in the production of IL-10 in response to LPS stimulation, as compared to untreated cells (Figure 3.1A). This observation supported the premise that FhHDM-1 was specifically targeting the pro-inflammatory response in macrophages by altering their metabolism, rather than exerting indirect effects through the increased production of regulatory cytokines, such as IL-10.

It has been reported that the c-terminal amphipathic alpha helix of FhHDM-1 is critical to its biological activity, as disruption of the alpha-helix or alterations to the hydrophobicity prevents the inhibition of lysosomal vATPase (111). Macrophages treated with a mutant variant of FhHDM-1, which had 6 amino acid substitutions that abolished the hydrophobic face of the amphipathic region of the c-terminus (termed NHP), were not inhibited in their ability to produce TNF in response to subsequent stimulation with LPS (Figure 3.1B). This supports a direct role for FhHDM-1 and validates the hypothesis that the regulation of pro-inflammatory responses was mediated by changes to the lysosomal pH of macrophages following internalisation of FhHDM-1.

The activation of macrophages by pro-inflammatory ligands that signal through a wide range of TLRs (-2, -3, -4, and -9) results in the same shift in metabolic flux, switching

metabolism towards glycolysis to meet the high energetic and biosynthetic demand (180). Metabolites that accumulate due to glycolysis reinforce the pro-inflammatory response by activating intracellular signalling pathways, post-transcriptional and post-translational regulation of components of the inflammatory response, and mediating epigenetic changes (181). Two of the most important signalling pathways for M1 macrophage activation are NF-κB and Akt/mTOR. Both NF-κB and Akt pathways converge to stabilise hypoxia-inducible factor 1α (HIF-1α), which subsequently increases the expression levels of glucose transporters (182) and glycolytic enzymes to maintain the glycolytic flux (183). HIF-1α also promotes the expression of IL-1β by activating NF-κB (184) through direct binding with the IL-1β promoter (185), or by activating the inflammasome through the expression of the glycolytic enzymes, hexokinase-1 (186) and pyruvate kinase isozyme M2 (PKM2) (39).

The hypothesis that FhHDM-1 mediated an anti-inflammatory effect by altering metabolic preferences was tested. Accordingly, the metabolic flux and the abundance of elements of the glycolytic pathway in macrophages treated with FhHDM-1, and subsequently stimulated with bacterial LPS (a ligand for TLR-4), was examined. Corroborating this proposed mechanism of immune regulation, macrophages treated with FhHDM-1 showed significantly lower glycolytic activity in response to stimulation with LPS, as compared to untreated cells (Figures 3.2A, B). Furthermore, FhHDM-1 treated macrophages also displayed significantly lower levels of hexokinase-1 activity and HIF-1α expression levels (Figures 3.2C,D) after stimulation with LPS. These results validated the observed reduction in glycolysis. This outcome was dependent on the ability of FhHDM-1 to inhibit vATPase, as the inactive derivative of the FhHDM-1 peptide (NHP) exerted no effect on the metabolic activity or pro-inflammatory response of macrophages (Figures 3.2A, B).

Although FhHDM-1 induced an increase in OXPHOS metabolism in resting/unstimulated macrophages (Figure 3.3A, and chapter 2), a similar effect was not observed in FhHDM-1 treated macrophages that were also stimulated with LPS (Figures 3.3A, B). This outcome suggested that the inhibition of glycolysis, and the subsequent reduction in inflammatory cytokine production in response to LPS stimulation, was independent of OXPHOS metabolism induced by FhHDM-1.

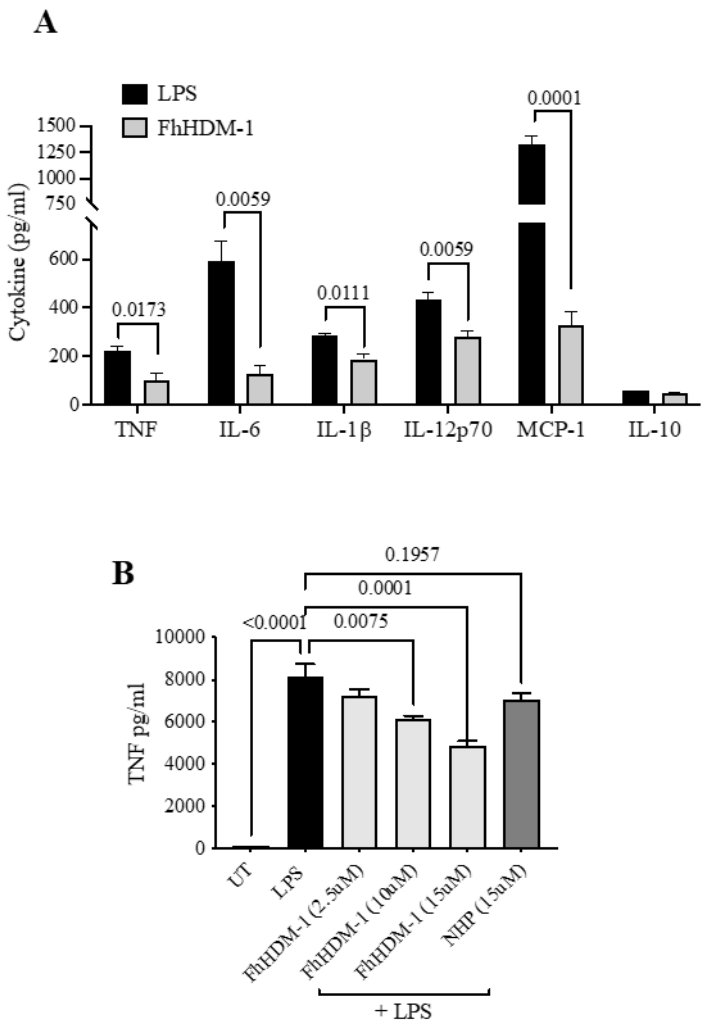

Figure 3.1: FhHDM-1 inhibited the secretion of pro-inflammatory cytokines in response to LPS stimulation via a mechanism that was dependent on the inhibition of lysosomal vATPase. (A) BMDMs were treated 20ng/mL LPS or a combination of FhHDM-1 (5µM) and LPS for 24h (n=4). The levels of cytokines secreted after an overnight incubation were quantified by Bioplex. (B) BMDMs were treated with 20ng/mL LPS or a combination of FhHDM-1 (2.5-15µM), or NHP (15µM) and LPS for 24h (n=4). The levels of TNF secreted after an overnight incubation were quantified by ELISA. Data is representative of three independent experiments and is presented as means ± SEMs. Statistical significance was determined by (A) an unpaired, two tailed Welch's t-test or (B) a one-way ANOVA with Tukey's multiple comparison test.

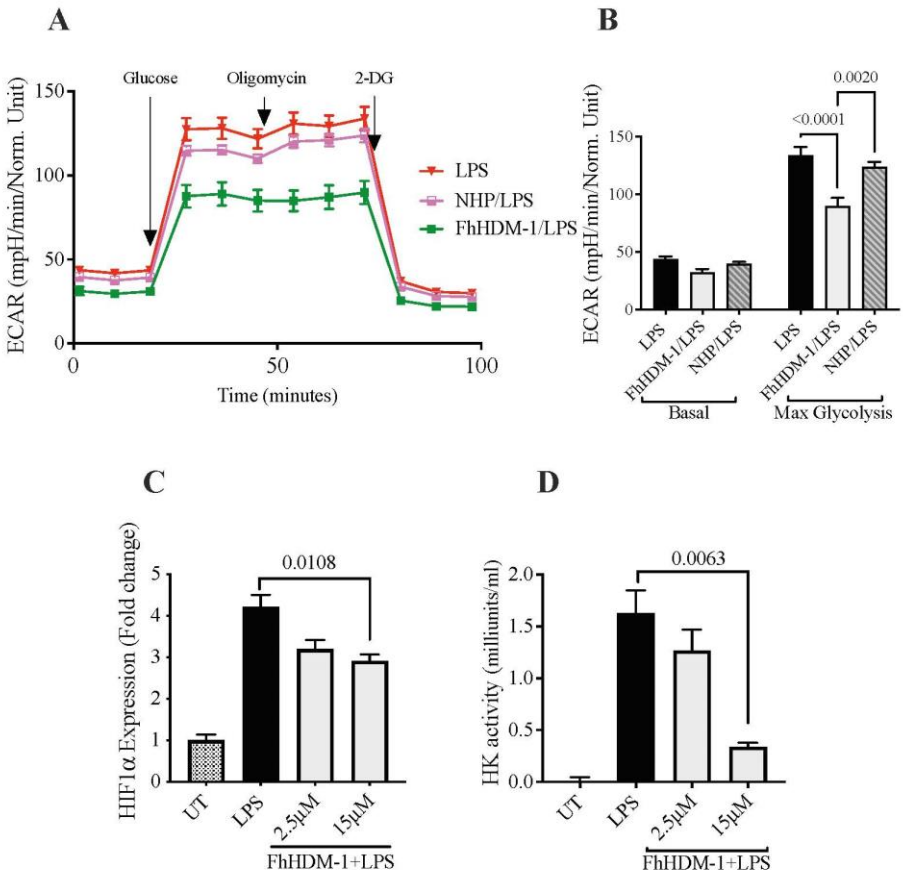

Figure 3.2: FhHDM-1 treatment of macrophages reduced LPS activation of the glycolytic pathway. (A) BMDMs were treated 20ng/mL LPS or a combination of FhHDM-1 (2.5μM) or NHP (2.5μM), and LPS for 24h (n=4). Then glycolytic activity was determined by measuring the extracellular acidification rate (ECAR) to determine (B) the maximum glycolytic activity as cells were treated with glucose, oligomycin and 2-deoxy-glucose (2-DG). (C, D) Macrophages derived from the bone marrow of C57BL/6 mice were either untreated (UT) or cultured for 1h with FhHDM-1 (2.5μM, and 15μM) and then stimulated with 20ng/ml LPS. (C) The levels of HIF1α gene expression at 6h after LPS treatment were determined by qRT-PCR (n=3). (D) Hexokinase activity was measured in BMDMs after an overnight incubation with 20ng/mL LPS (n=5). Data is representative of three independent experiments and is presented as means ± SEMs. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

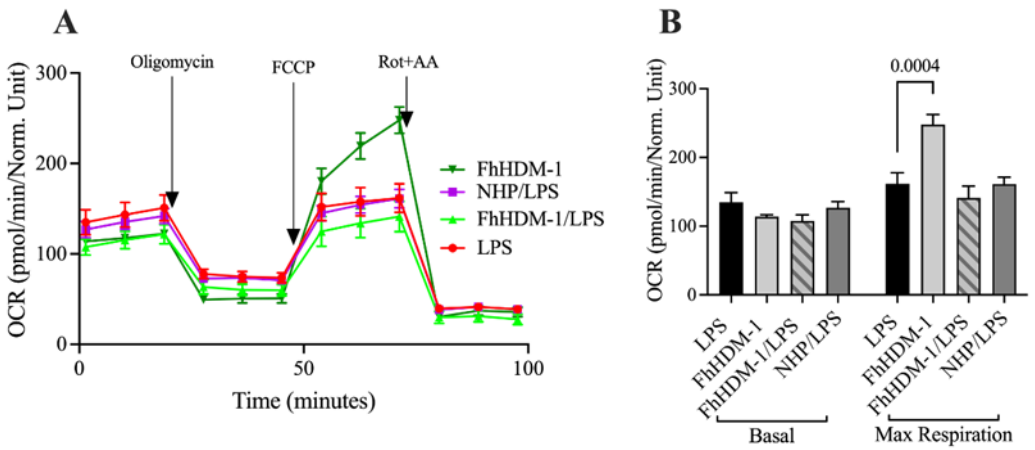

Figure 3.3: The reduction in glycolysis in FhHDM-1 treated, LPS stimulated macrophages was not associated with an increase in oxidative phosphorylation (OXPHOS). (A) BMDMs were either untreated (UT) or treated with LPS (20ng/ml), FhHDM-1 (2.5µM), FhHDM/LPS or NHP (2.5µM)/LPS for 18h (n=5). The oxygen consumption rate (OCR) was measured at basal levels and following sequential treatments with oligomycin, FCCP, and rotenone/antimycin A (Rot+AA) to determine (B) the maximum respiratory capacity of cells. Data is representative of three independent experiments and is presented as means ± SEMs. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

3.4.2. The regulation of macrophage inflammation by FhHDM-1 was dependent on the induction of glutaminolysis.

The data presented in Chapter 2 demonstrated that the OXPHOS in FhHDM-1 treated macrophages was dependent on glutaminolysis. There is evidence that the metabolites produced through this metabolic pathway can regulate inflammatory pathways. Therefore, a mechanistic link between the regulation of glycolysis and pro-inflammatory cytokine production and the metabolic conversion to glutaminolysis in FhHDM-1 treated macrophages was next investigated. Accordingly, both ECAR and cytokine secretion were measured in macrophages treated with FhHDM-1 and stimulated with bacterial LPS in the presence of the glutaminase inhibitor, BPTES.

The presence of BPTES modulated neither the induction of glycolysis (Figure 3.4A, B) nor the production of TNF (Figure 3.4C) and IL-6 (Figure 3.4D) in response to LPS stimulation. However, the addition of BPTES to FhHDM-1 treated macrophages completely reversed both the inhibition of glycolysis and the suppression of LPS-induced TNF and IL-6 production, with levels of both being restored to those observed in the LPS treated cells (Figure 3.4). These data support a conclusion that FhHDM-1 induced glutaminolysis was mediating the regulation of inflammatory responses in macrophages by inhibiting the metabolic utilisation of glucose.

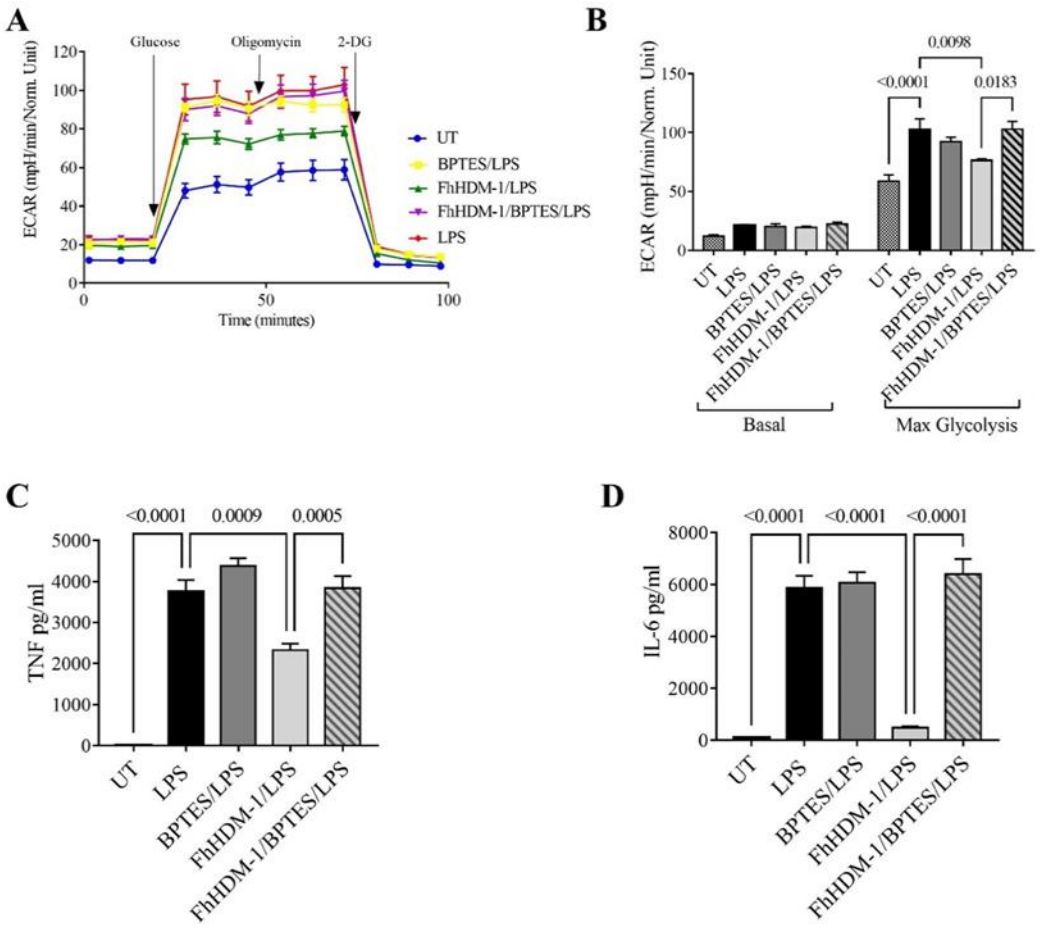

Figure 3.4: The reduction in glycolysis and pro-inflammatory cytokine production in FhHDM-1 treated macrophages was dependent on the induction of glutaminolysis. (A, B) BMDMs were either untreated (UT) or treated with LPS (20ng/mL), the glutamate inhibitor BPTES (10µM)/LPS, FhHDM-1 (2.5µM)/LPS or FhHDM-1/BPTES/LPS for 18h (n=4). (A) The glycolytic activity was determined by measuring the extracellular acidification rate (ECAR) and (B) the maximum glycolytic capacity of cells after subsequent treatment with glucose, oligomycin and 2-deoxy-glucose (2-DG). (C, D) BMDMs were cultured overnight with LPS (20ng/mL), BPTES (10µM)/LPS, FhHDM-1 (15µM)/LPS, or FhHDM-1/BPTES/LPS. (C) Secreted TNF (D) or IL-6 protein was quantified in the culture media by ELISA (n=3). Data is representative of three independent experiments and is presented as means ± SEMs. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

3.4.3. Increased glutaminolysis in FhHDM-1 treated macrophages was not associated with an increased abundance of succinate.

It was previously shown (Chapter 2) that the metabolism of glutamine by glutaminolysis resulted in the accumulation of α-KG. This metabolite then enters the TCA cycle where it can be converted to succinate. The resulting ratio of α-KG:succinate influences the inflammatory status of macrophages. Succinate acts to stimulate pro-inflammatory cytokine production in LPS-treated macrophages (185, 187). On the other hand, accumulation of α-KG inhibits LPS induced activation of the NF-κB pathway, via post-translational modification of IKKβ, resulting in reduced gene expression levels of several pro-inflammatory cytokines, including IL-1β, IL-6, IL-12, and TNF (168). In addition, an increase in α-KG levels mediates the induction of immune tolerance to LPS stimulation, suppressing the expression and production of pro-inflammatory cytokines. Importantly, these functions of α-KG appear to be independent of its conversion to succinate as the supplementation of α-KG-treated cells with succinate did not restore the inflammatory response to LPS (168).

Examining the intracellular abundance of metabolites showed that treatment of macrophages with FhHDM-1, either alone or in the presence of LPS, resulted in an increased ratio of α-KG:succinate (Figure 3.5A). Treatment of macrophages with IL-4 alters the abundance of both α-KG and succinate. However, the increased ratio of α-KG:succinate in FhHDM-1 treated macrophages was solely attributable to an increase in the levels of α-KG, with no significant change in the levels of succinate, in comparison to cells stimulated with LPS (Figure 3.5B, C). These observations demonstrate that the regulation of inflammatory responses induced by FhHDM-1 correlated to an increase in α-KG, thus corroborating the reliance on glutaminolysis.

Based on the described functions for α-KG, it was surprising to find that the expression levels of *tnf* mRNA remained unchanged, even at the highest concentration of FhHDM-1 used (Figure 3.5D). Although apparently paradoxical, this result upholds the hypothesis that FhHDM-1 treatment of macrophages is reducing glycolysis, as this metabolic pathway mediates the post-transcriptional regulation of TNF. When glycolytic flux is reduced, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme in the glycolytic pathway, remains bound to TNF mRNA, thereby repressing its translation (188), as observed in macrophages treated with FhHDM-1. However, this outcome also suggests that the increased abundance of α-KG in FhHDM-1 treated macrophages is mediating a different mechanism of

immune regulation, as opposed to the suppression of gene transcription or the induction of LPS tolerance.

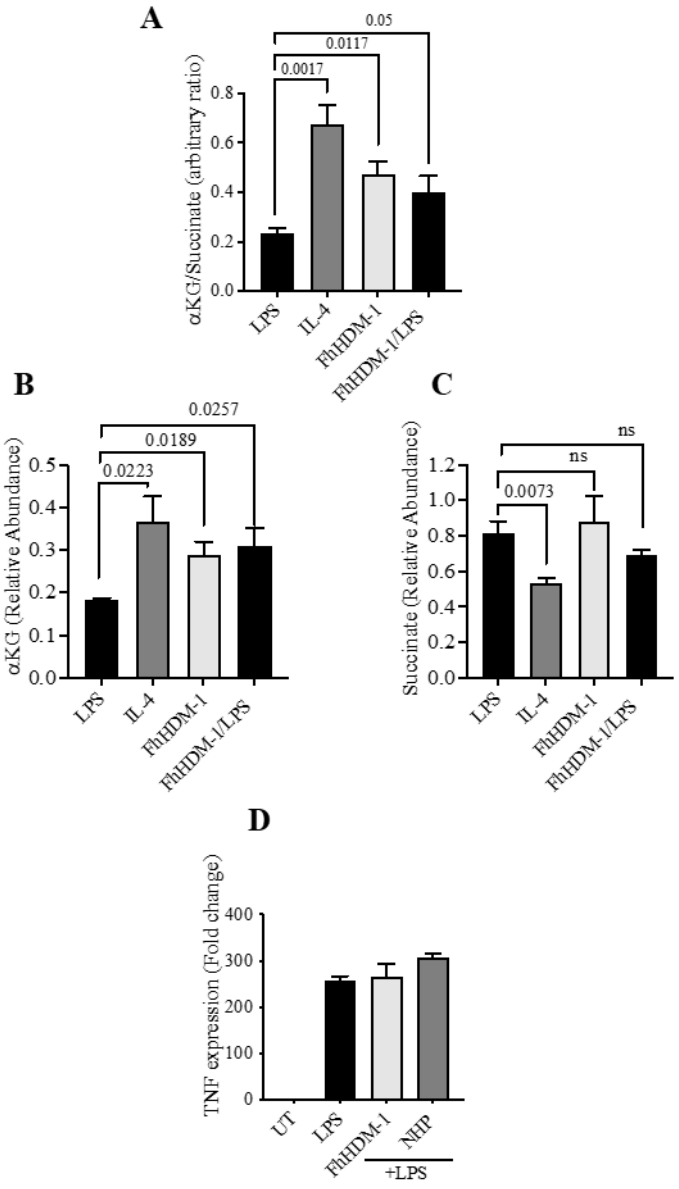

Figure 3.5: The increased ratio of KG to succinate in FhHDM-1 treated macrophages did not correlate with the reduced expression of pro-inflammatory cytokine genes. (A) The intracellular ratio of α-ketoglutarate(α-KG):succinate in BMDMs treated overnight with LPS (20ng/mL), IL-4 (20ng/mL), FhHDM-1 (15μM), or a combination of FhHDM-1 and LPS (n=6) was calculated from the abundance of the individual metabolites as quantified by mass spectroscopy. (B) Intracellular levels of α-KG and (C) succinate in BMDMs were measured by mass spectrometry after metabolite extraction. (D) The differential expression of TNF was measured by qRT-PCR in cell lysates of BMDMs that were either untreated (UT) or cultured with LPS (20ng/mL), FhHDM-1 (15μM), or a combination of FhHDM-1 and LPS or NHP (inactive peptide) and LPS for 6h (n=3). Data is representative of three independent experiments and is presented as means ± SEMs. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

3.4.4. The regulation of inflammation by FhHDM-1 was dependent on fatty acid oxidation.

While oxidative metabolism of glutamine supports the canonical TCA cycle, reductive carboxylation of glutamine-derived α-KG sustains FA synthesis. As described in Chapter 2, macrophages treated with FhHDM-1 had an increased abundance/expression of metabolites (citrate) and enzymes (*fasn* and *acly*) associated with this pathway. The subsequent oxidation of fatty acids mediated the observed increase in OXPHOS. Furthermore, although the mechanism is not fully elucidated, it is evident that in certain contexts, FAO mediates the regulation of pro-inflammatory responses.

While treatment with FhHDM-1 during LPS stimulation supressed the secretion of both TNF (Figure 4.4A) and IL-6 (Figure 4.4B) cytokines as expected, this effect was reversed by the inclusion of the fatty acid inhibitor, Etomoxir (ETO), used at 5μM to prevent off-target effects (189). This outcome suggests that the enhanced abundance of α-KG in FhHDM-1 treated cells was driving FAO, which was subsequently mediating the immune regulatory effect of the parasite peptide.

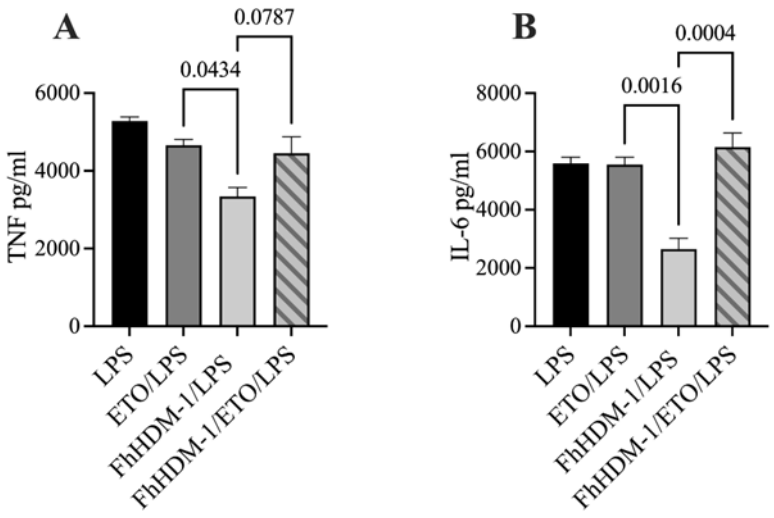

Figure 3.6: The regulation of pro-inflammatory cytokine production by FhHDM-1 was dependent on fatty acid oxidation. BMDMs were treated with LPS (20ng/mL), LPS and Etomoxir (ETO; 5µM), FhHDM-1 (2.5µM)/LPS or FhHDM-1/ETO/LPS for 18h (n=3). The quantity of (A) TNF, and (B) IL-6 secreted was quantified by ELISA. Data is representative of three independent experiments and is presented as means ± SEMs. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

3.5. Discussion

The data presented within this chapter, builds on the initial findings presented in Chapter 2, and supports the hypothesis that the reprogramming of macrophage metabolism mediates the regulation of pro-inflammatory responses. Specifically, this anti-inflammatory effect appears to be mediated by FAO, which is promoted by an increased abundance of α-KG produced through glutaminolysis. This functional activity was dependent on the inhibition of vATPase by FhHDM-1, providing further evidence for a link between the lysosome, metabolism, and the immune response in macrophages.

α-KG is a pleiotropic metabolite with key roles in multiple metabolic and cellular pathways (190). The mechanism by which α-KG induces immune tolerance has not been fully elucidated. However, it has been shown that as a co-factor for the histone demethylase, KDM5, α-KG mediates the demethylation of active histone marks (H3K4me1/3) to reduce the expression of pro-inflammatory cytokines in macrophages (167). Nonetheless, as FhHDM-1 treated cells did not show any change in the expression levels of TNF, this suggested an alternative function for α-KG in FhHDM-1 mediated anti-inflammatory effects. As described in Chapter 2, an increased abundance of α-KG reportedly reduces the expression of DNMT3B, which leads to a correlative reduction in the expression levels of Drp1 (191). Notably, Drp1 is enriched on mitochondria in LPS-activated macrophages, and is required for the induction of glycolysis, and the selective expression and/or secretion of pro-inflammatory cytokines. Corroborating the outcome of FhHDM-1 treatment, silencing Drp1 in macrophages resulted in the translational, but not transcriptional, regulation of TNF expression (192, 193). This pathway therefore adds to the evidence presented in Chapter 2 indicating that the increased abundance of α-KG in FhHDM-1 treated macrophages was altering mitochondrial dynamics (from fission to fusion), which, in turn, was supporting the switch from glycolysis to FAO, and this shift regulated the production of pro-inflammatory cytokines. As mentioned previously, the specific mechanism by which FAO is mediating this immune regulation remains to be elucidated. Nonetheless, it will be important to specifically investigate the changes in mitochondrial dynamics that are induced in macrophages treated with FhHDM-1 in a resting and inflammatory scenario and determine if this is indeed controlling the switch from glycolysis to FAO and OXPHOS.

This role for α-KG would also explain the discrepancy in the literature in which glutaminolysis has been found to support a pro-inflammatory response induced by several ligands (167, 185). However, in these scenarios, the induction of M1 macrophage functional

phenotypes required the increased abundance of metabolites that are downstream of α-KG in the TCA cycle, such as succinate and fumarate. Although not all cellular metabolites were measured in this study, it was of interest to note that the treatment of macrophages with FhHDM-1 did not affect the levels of succinate produced after stimulation with LPS, but did increase the accumulation of α-KG. This observation suggests that the α-KG induced by FhHDM-1 was being utilised to support both an increase in endogenous fatty acids for FAO and contributed to the TCA cycle and OXPHOS. In addition, the data shows that despite the reduction in pro-inflammatory cytokines in macrophages treated with FhHDM-1, this was a regulatory effect rather than a complete immune suppression. This conclusion was drawn because gene transcripts of pro-inflammatory cytokines and abundance of pro-inflammatory metabolites under conditions of LPS stimulation remained unchanged. This mechanism generates a novel strategy for the regulation of inflammatory responses by a parasite-derived peptide.

It is becoming widely acknowledged that metabolic reprogramming offers a unique strategy to fine-tune the functional activities of macrophages to limit inflammation, as opposed to globally suppressing inflammatory responses (126). This approach would allow for the targeted inhibition of pro-inflammatory pathologies, without compromising the ability of the patient to mount immune responses necessary for infection clearance and immunity after vaccination. Such a strategy represents a significant advantage over current immune therapeutic regimes, which are less targeted, and, accordingly, induce undesirable global immune suppression and detrimental off-target effects. The demonstration that FhHDM-1 preferentially targets macrophages to metabolically reprogram them towards immune regulation rather than complete immune suppression, makes this peptide an attractive therapeutic candidate for the multitude of conditions mediated by dysregulated or chronic inflammation.

Chapter 4: The helminth derived peptide, FhHDM-1, reverses the trained phenotype of NOD bone marrow derived macrophages to regulate pro-inflammatory responses

4.1. Introduction

The data presented in chapter 2 and 3 showed that FhHDM-1 reprogrammed the metabolic activity of macrophages to regulate pro-inflammatory responses, as evidenced by a shift in metabolism towards glutaminolysis. This, in turn, resulted in reduced production of pro-inflammatory effectors, suggesting the remarkable possibility that FhHDM-1 altered the 'trained' innate immune response to prevent autoimmune disease.

The concept of 'trained immunity' in the context of pro-inflammatory conditions, proposes that exposure to ligands of microbial pathogens imprints macrophages with a pro-inflammatory memory, which endows them with the capacity to respond faster and stronger to future stimuli (194). This creates a long-lasting effect which is mediated by modifications to histones by a process that is underpinned by a change in cellular metabolism towards glycolysis, and away from OXPHOS (195). During immune training, the addition of methyl groups to the lysine 4 on the histone H3 protein (H3K4me1/3) in particular, results in the persistent opening of chromatin (196). This allows enhanced access to the promoter regions of genes encoding the pro-inflammatory cytokines, IL-6, IL-1β and TNF. The production of these cytokines is typically amplified in trained macrophages in response to restimulation with inflammatory ligands, such as LPS (197). The activation of enzymes that control histone methylation (198) is regulated by the metabolic intermediates produced by the glycolytic pathway (199). Accordingly, the marking of histones is associated with increased aerobic glycolytic activity in trained macrophages, as compared to untrained cells.

Unlike the adaptive immune response, the trained innate response is not antigen specific. Accordingly, it has been proposed that this form of immune memory evolved to provide general protection against future infections of diverse origins (200). However, the induction of trained immunity is not limited to microbial ligands, and can also be induced by endogenous sterile stimuli, such as metabolites, fatty acids, glucose, and tissue damage (167, 201, 202). In contrast to the beneficial effects of trained immunity in the context of an infectious threat, the inappropriate activation of an enhanced inflammatory response by endogenous sterile stimuli is likely to be deleterious (203). Indeed, evidence is emerging that innate immune memory is involved in the rejection of heart and kidney transplants (204, 205), in the initiation of the autoimmune condition, systemic lupus erythematosus (206), and in the inflammatory diseases of diet-induced type 2 diabetes (T2D) and atherosclerosis (207, 208).

The inappropriate activation of pro-inflammatory responses is universally associated with a spectrum of seemingly disparate autoimmune/inflammatory pathologies that dominate present-day morbidity and mortality worldwide (209, 210). Therefore, it is conceivable that a trained innate immune response is the common initiating factor among these conditions. Furthermore, the inverse correlation of endemic helminth infection and autoimmune/inflammatory disease (as discussed in Chapter 3), combined with the metabolic shift away from glycolysis in response to parasite molecules (Chapters 2 and 3) suggests that parasitic worms may impact the activation of this innate immune memory to prevent immune-mediated diseases. Thus, due to the ubiquitous presence of helminth infections in our ancestors, the evolution of the trained immune response must have occurred in worm infected populations. The elimination of helminth infections from human populations, through increased sanitation and urbanisation, has removed the regulatory influence of helminth infection on the human immune response, consequently disturbing immune-metabolic homeostasis (211). As a result, the trained immune response is now unrestrained, and readily and robustly responds to endogenous sterile ligands, thereby incorrectly initiating inflammatory and metabolic pathways. This results in the increased prevalence of immune-mediated diseases, such as diabetes, MS, atherosclerosis, asthma, and Alzheimer's disease.

Previous observations that the administration of FhHDM-1 provided protection from several immune-mediated diseases, such as T1D (102), MS (102), and asthma (212) strongly suggests modulation of a common, broad biological process, such as trained immunity. Furthermore, the observation that only a short treatment regime (six i.p. injections on alternate days over 12 days) was sufficient to provide long-term (up to 30 weeks in NOD mice; experimental endpoint) protection from disease, suggests an influence on the epigenetic regulation of inflammatory responses. This possibility is supported by the discovery that BMDMs treated with FhHDM-1 *in vitro* had decreased glycolytic activity and a reduced inflammatory response to stimulation (Chapter 3), both hallmarks of a trained cell.

This chapter will test the hypothesis that FhHDM-1 regulates the trained immune response in macrophages *in vivo* to prevent the aberrant inflammatory responses, which initiate autoimmunity in T1D.

4.2. Materials and Methods

4.2.1. Previously Described Methodology

The methodology for the synthesis of the parasite-derived peptide; isolation and culture of BMDMs; RNA extraction, cDNA synthesis and qRT-PCR; analysis of metabolic pathways and cell normalization; and quantification of secreted TNF and IL-6 were performed as described in Chapter 2 (Sections 2.3.1, 2.3.2, 2.3.4, 2.3.5, and 2.3.6) and Chapter 3 (Section 3.3.2).

Table 4.1: qRT-PCR primers specifically used in Chapter 4.

Name	Interrogated Sequence	Source
Stx5a (housekeeping)	NM_001167799.1	TaqMan® Mm00502335_m1
TNF	NM_001278601.1	TaqMan® Mm00443258_m1
IL-6	NM_031168.1	TaqMan® Mm00446190_m1

4.3.2 Treatments of Mice with FhHDM-1

Female mice (C57BL/6, BALB/c and NOD/Lt) were purchased from the Australian Research Centre (ARC, Perth, Australia). NOD/Lt mice were chosen to study autoimmune diabetes. The NOD mouse is the most widely used and most relevant model in which to study the pathogenesis of TID. This is because there are striking similarities with the human disease. In both species, polymorphisms in MHC class II molecules confer the most disease risk, diabetes onset is preceded by the presence of circulating autoreactive T cells and autoantibodies that recognise many of the same islet antigens, and infiltration of destructive immune cells within the islets occurs in both humans and NOD mice (213). After acclimatisation, animals were randomly divided into groups (n=3-5) to remain untreated, or to receive FhHDM-1 (10 µg in 100 µl sterile saline), or sterile saline alone. The treatment regime consisted of six i.p. injections, delivered on alternate days, beginning at 6-7 weeks of age. Mice were then housed for a further three weeks with no additional intervention, with water and standard chow provided *ad libitum*. Ethical approval for these studies was granted by the University of Technology Sydney (UTS) Animal Care and Ethics Committee (Approval Numbers: ETH18-

2257, ETH21-5823, ETH22-7635), and experiments were conducted in accordance with the approved guidelines of 'The Australian Code for the Care and Use of Animals for Scientific Purposes'.

4.3.3 Western Blot Analyses

BMDMs (2 x 10^6) from NOD/Lt or BALB/c mice were cultured overnight in R10 media (untreated - media only) or in the presence of LPS (20ng/mL). After removal of the supernatant, cells were washed twice with saline and collected by pulse centrifugation at 16,000 g. The cell pellets were lysed by addition of 20µl Pierce RIPA buffer (Thermo Scientific) containing a 1x dilution of protease and phosphatase inhibitor cocktail (Thermo Scientific) with incubation on ice for 30 mins. Cell debris was then removed by centrifugation of the lysates for 15 mins at 14 000 rpm at 4 ºC. The protein contents of the resulting supernatants was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Each sample was normalised according to protein concentration by dilution in water and 20µl of each sample was then electrophoresed on 4-12% NuPAGE Bos-Tris gels (Invitrogen) in NuPAGE MES SDS running buffer. The Novex™ Sharp Pre-stained Protein Standard (Invitrogen) was used as a molecular weight marker. After complete separation of the samples, gels were transferred onto iBlot® 2 PVDF regular stacks using an iBlot 2 transfer device at P0 standard protocol. Membranes were blocked overnight in 1% BSA in TBS and then probed overnight with primary antibodies specific for H3K4me1 (Invitrogen, clone: 2H65L5) or H3K4me3 (Invitrogen, clone: G.532.8) at a concentration of 1 in 1000 in 1% BSA in TBS, followed by the addition of HRP-linked anti-rabbit IgG secondary antibody (Cell Signalling 7074) at 1 in 2000 in 1% BSA in TBS for 1h. To visualise protein loading of samples, blots were stripped by washing three times for 5 mins each in stripping buffer containing 1g/L SDS, 15g/L glycine and 1% tween 20 in milliQ water, blocked for 1h with 1% BSA in TBS, and re-probed for 1h with HRP-linked anti-mouse actin (mAbcam clone: 8226) at 1 in 5000 in 1% BSA in TBS. Blots were imaged using the Amersham Image Quant Imager 800.

4.3.4 Statistical Analysis

In all cases, data are presented as means ± standard error of the means (SEMs) of several biological replicates (n). The comparison of data was determined by either an unpaired, two tailed Welch's t-test or a one-way ANOVA with Tukey's multiple comparison test using

GraphPad Prism 7 software (GraphPad). Statistical significance was considered as a p-value <0.05.

4.4 Results

4.3.2 FhHDM-1 reprogrammed the metabolic and inflammatory activities of bone marrow derived macrophages *in vivo*.

The first stage of this investigation built on the findings of Chapters 2 and 3 and aimed to determine the ability of FhHDM-1 to alter the metabolic and inflammatory activities of macrophages progenitor cells *in vivo*. To replicate the previous investigation of the efficacy of FhHDM-1 to prevent disease, the same treatment regime was used here. Thus, C57BL6 mice (n=3-5) received six i.p. injections of FhHDM-1 (10μg in 100μl), or saline (100μl) delivered on alternate days. To determine the longevity of any effect, the bone marrow was harvested three weeks after the final injection, macrophages were derived, and these BMDMs were then stimulated with bacterial LPS. In addition, a cohort of mice were given the same treatment regime of the *F. hepatica* cathepsin L1 protease (FhCL1). As compared to FhHDM-1, FhCL1 exerted no protective therapeutic effect in any disease model studied (102). Therefore, FhCL1 served as a negative parasite-derived protein control in these experiments.

Corroborating the results from FhHDM-1 treatment of macrophages *in vitro* (Chapter 3), BMDMs of mice that received FhHDM-1 displayed a downwards trend in glycolytic activity in response to LPS stimulation, as compared to mice that received saline alone (Figure 4.1A, B). The administration of FhCL1 to mice exerted no effect on the levels of ECAR, with BMDMs from these mice displaying the same maximal glycolytic activity in response to LPS as mice that had been treated with saline alone (Figure 4.1A, B). Notably, in the BMDMs derived from mice that were treated with FhHDM-1, levels of glycolytic activity in cells treated with saline *ex vivo* were the same as those for BMDMs from mice given either FhCL-1 or saline (Figure 4.1A). This suggested that FhHDM-1 exerted a specific effect in regulating the response of macrophages to an inflammatory ligand, as opposed to inducing an alteration in the metabolic activity of resting, unstimulated cells. Consistent with this reduction in glycolytic flux, the secretion of TNF in response to LPS stimulation was significantly decreased in BMDMs of mice treated with FhHDM-1, but not in those from mice treated with FhCL1 or saline (Figure 4.2).

Combined, this data confirmed that FhHDM-1 altered both the metabolic and inflammatory pathways in macrophage precursors *in vivo*, which persisted up to 3 weeks after

FhHDM-1 treatment ceased. These observations strongly supported the premise that FhHDM-1 was positively regulating the trained immune response, which would have been capable of mediating the therapeutic effects previously observed in the animal models of immune-mediated disease.

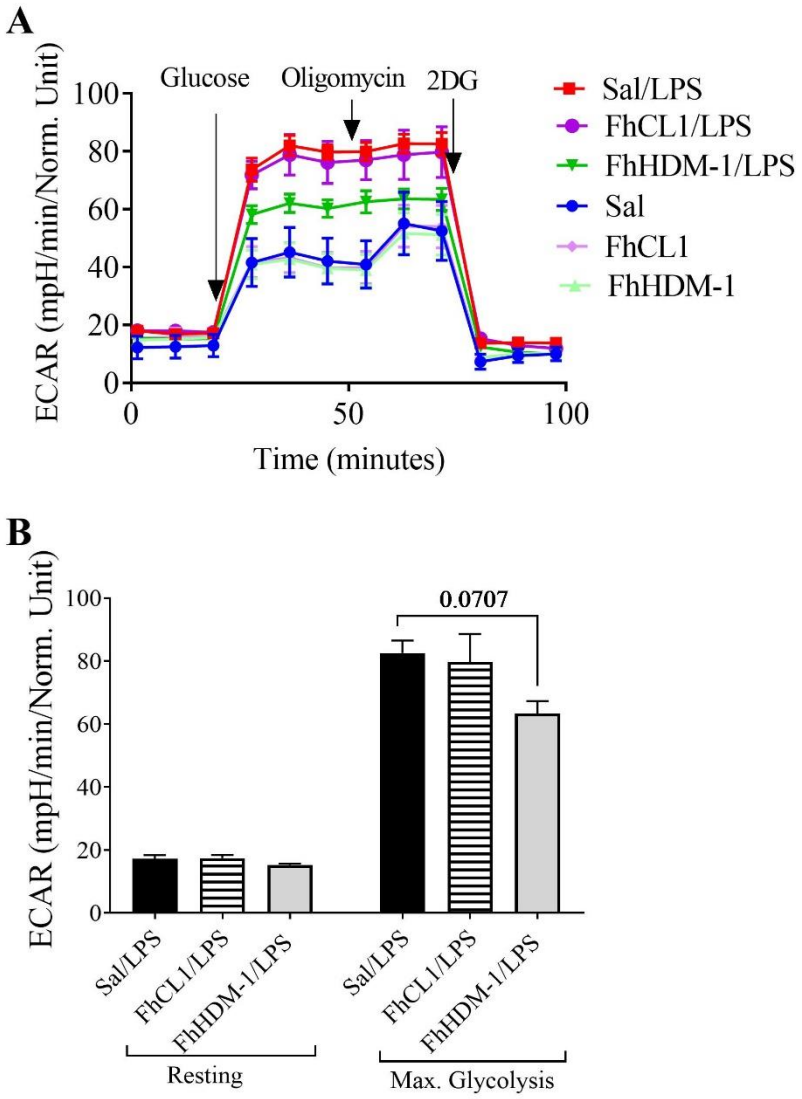

Figure 4.1: Treatment of mice with FhHDM-1 reduced glycolytic activity in bone marrow derived macrophages stimulated with LPS *ex vivo*. Macrophages were differentiated from bone marrow harvested 3 weeks after C57BL/6 mice had received 6 i.p. injections of 0.1mg/ml FhHDM-1 or FhCL1 or saline delivered on alternate days. Differentiated macrophages were then either untreated (UT) or treated with LPS for 24h *in vitro*. (A) The glycolytic activity was determined by measuring the extracellular acidification rate (ECAR) and (B) the resting and maximum glycolytic capacity of BMDMs after subsequent treatment with glucose, oligomycin and 2-deoxy-glucose (2-DG). Data is presented as means ± SEMs (n=3) and is representative of two independent experiments. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

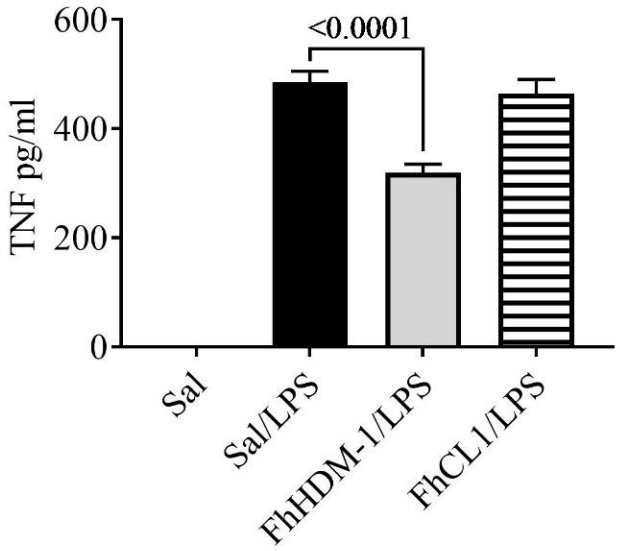

Figure 4.2: Treatment of mice with FhHDM-1 regulated the pro-inflammatory response of bone marrow derived macrophages to stimulation with LPS *ex vivo*. Bone marrow was harvested from C57BL/6 mice 3 weeks after they had been treated with 6 i.p. injections of 0.1mg/ml FhHDM-1 or FhCL1 or saline delivered on alternate days. Differentiated macrophages were then either treated with saline (Sal) or LPS overnight. Secreted TNF was quantified in the culture media by ELISA. Data is presented as means ± SEMs (n=5) and is representative of two independent experiments. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

4.3.3 Bone marrow derived macrophages from Non-Obese Diabetic (NOD) mice displayed the characteristics of a trained immune cell.

Before examining the ability of FhHDM-1 to specifically alter a trained immune response *in vivo*, an appropriate model was required. Given the previous work demonstrating that FhHDM-1 permanently reversed TID in NOD mice (102), this model was chosen. The NOD mouse is the most widely studied model used for preclinical/translational studies of T1D because disease pathogenesis mimics, in many respects, that of humans (214).

Although the initiating event for T1D has not been fully defined, viral and bacterial infections have long been implicated as a trigger for disease development (215), as they have been for other immune-mediated diseases (216, 217). Additionally, macrophages from both T1D patients and NOD mice display enhanced pro-inflammatory responses, and this phenotype is necessary for the initiation of autoimmunity (214, 218-221). Combining these observations with the new paradigm of trained immunity, it is plausible that in T1D patients, myeloid cells in the bone marrow are trained by a previous bacterial/viral infection, or an altered gut microbiota (215). The recruitment of these trained macrophages to the islets, induced by β-cell stress signals, produces an excessive/amplified pro-inflammatory response, triggering the initiation of autoimmunity (222) . The ongoing interactions between macrophages and β-cells perpetuates a cycle of inflammation, which culminates in the activation and clonal expansion of autoreactive T-cells (223) , which ultimately drive the accelerated and irreversible loss of β-cell mass leading to T1D. Based on the data here, and in previous chapters, it is conceivable that FhHDM-1 can prevent, or reverse, the centrally trained macrophage phenotype to mitigate the initiation of autoimmunity, and hence T1D.

To first test the hypothesis that macrophages from NOD mice were indeed trained, the hallmarks of innate immune cell training (197): (i) methylation of histones; (ii) increased glycolytic activity; and (iii) enhanced pro-inflammatory immune responses to stimulation were measured in BMDMs of untreated mice. These readouts were compared to those for BMDMs derived from diabetes-resistant, non-inflammatory BALB/c mice.

These data revealed, for the first time, that the histone H3K4 in macrophages differentiated from the bone marrow of untreated NOD mice (aged 11 weeks of age) was both mono (H3K4me1)- and tri (H3K4me3)-methylated (Figure 4.3). In comparison, the BMDMs

of age matched BALB/c mice showed no methylation of the same histone. This suggests that the immune progenitor cells in the bone marrow of NOD mice were 'trained', thus driving the inflammatory disease-promoting phenotype of macrophages. Notably, there was some variability in the extent of methylation across the entire cohort of NOD mice, as illustrated by the representative data in Figure 4.3. This likely reflects the asynchronous T1D onset and incomplete disease penetrance (80% among females) in the NOD mice (224).

Correlating with this increase in histone methylation, the glycolytic activity in NOD BMDMs that were unstimulated *ex vivo* was significantly higher than that of BMDMs from BALB/c mice. This observation was consistent regardless of whether the BMDMs were resting, stimulated with glucose, or functioning at maximal metabolic activity (Figure 4.4A, B). Furthermore, although the maximal glycolytic activity of BMDMs from NOD and BALB/c mice after LPS stimulation was the same (Figure 4.4A, B), there was a significant difference in the immediate response to glucose stimulation. Glycolytic activity in NOD BMDMs stimulated *ex vivo* with LPS increased to maximal levels within minutes of being exposed to glucose. These levels far exceeded the glycolytic activity in LPS treated BALB/c macrophages at the same timepoint (Figure 4.4A, B). These data indicate that the NOD BMDMs were metabolically primed for an immediate and enhanced response to stimulation with a secondary ligand.

Further verification that BMDMs from NOD mice were centrally trained came from observations of increased expression and secretion of pro-inflammatory cytokines. The production of both IL-6 and TNF in response to LPS stimulation was significantly higher in BMDMs from NOD mice, as compared to macrophages from BALB/c mice (Figure 4.5A-D).

Collectively, these data support the hypothesis that BMDMs in NOD mice are 'trained' by the methylation of histones, and therefore react with a quicker and heightened pro-inflammatory response to subsequent stimulation, as compared to untrained BMDMs from a non-inflammatory, diabetes-resistant strain of mouse.

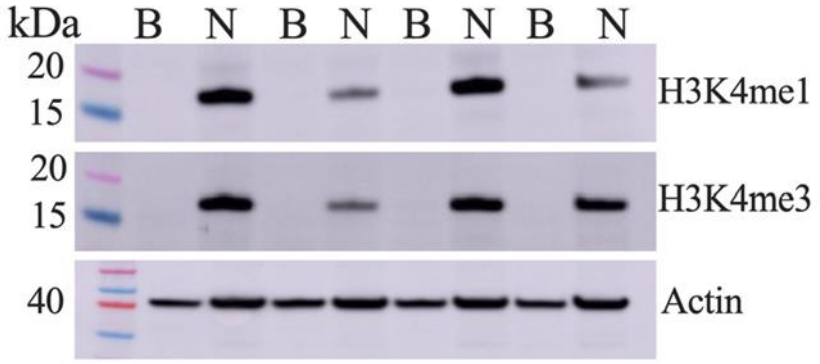

Figure 4.3: Lysine 4 on Histone 3 was mono- and tri- methylated in bone marrow derived macrophages from NOD mice. Macrophages were differentiated from the bone marrow of age-matched BALB/c (B) or NOD (N) mice at 11 weeks of age. Cell lysates were normalised by protein concentration, and the methylation status of H3K4 was analysed by western blot using primary antibodies specific for H3K4me1 and H3K4me3. The protein loading of each sample was determined by the detection of actin. The data shown is representative of n=5 BALB/c and NOD mice.

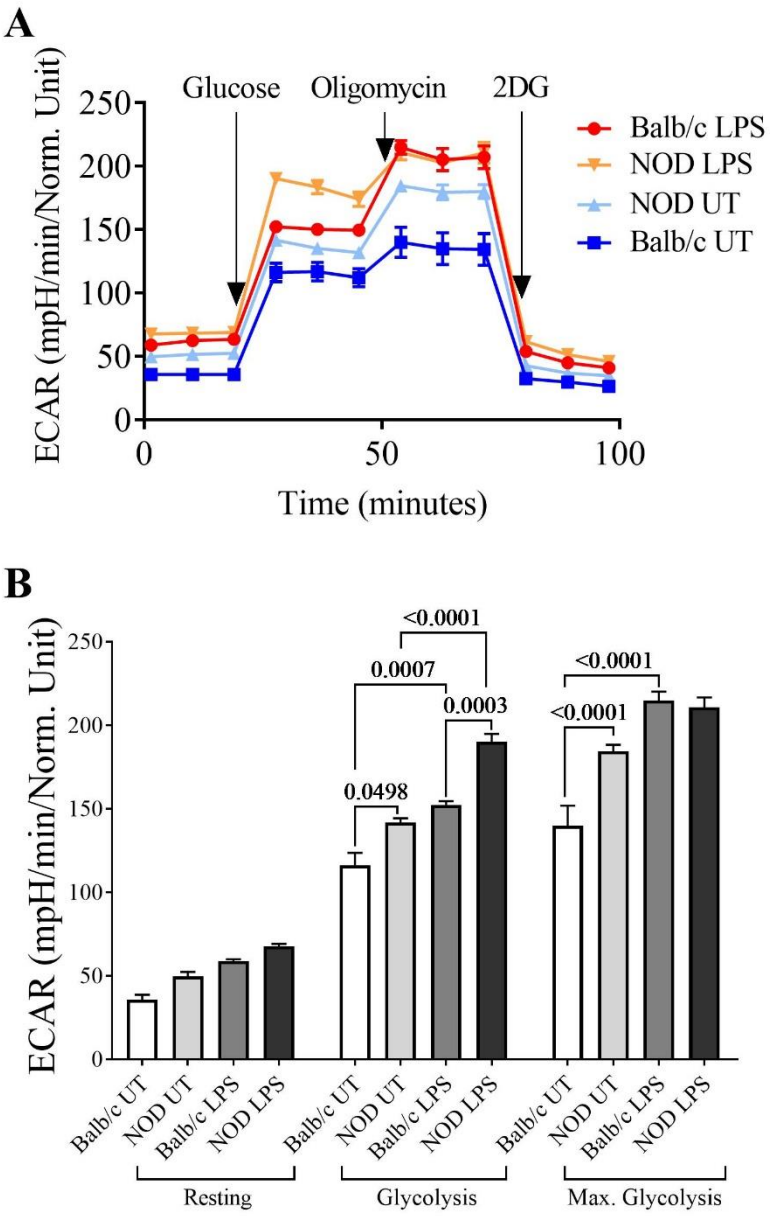

Figure 4.4: Bone marrow derived macrophages from NOD mice had higher glycolytic activity than macrophages from BALB/c mice. Macrophages were differentiated from the bone marrow of age-matched BALB/c or NOD mice at 11 weeks of age and then were either untreated (UT) or treated with LPS for 24h. (A) The glycolytic activity was determined by measuring the extracellular acidification rate (ECAR), and (B) the resting, immediate and maximum glycolytic capacity of macrophages after subsequent treatment with glucose, oligomycin and 2-deoxy-glucose (2-DG) Data is presented as means ± SEMs (n=5) and is representative of three independent experiments. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

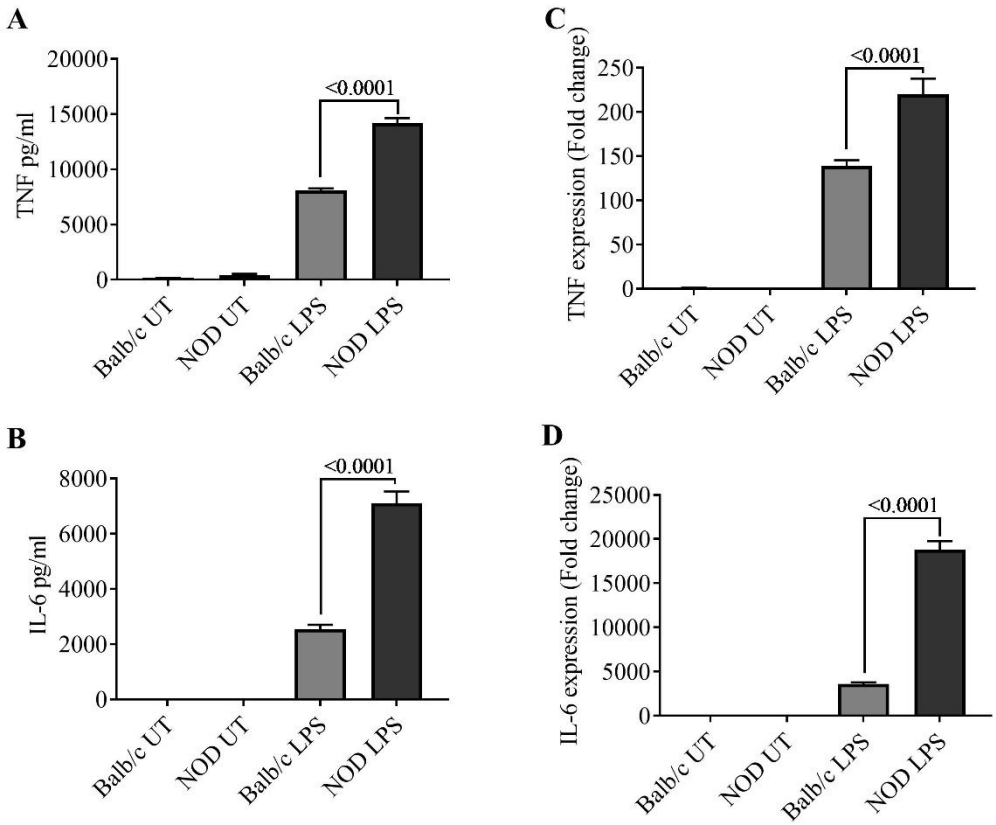

Figure 4.5: Bone marrow derived macrophages from NOD mice produce an increased pro-inflammatory response to LPS stimulation *ex vivo*, as compared to macrophages derived from the bone marrow of BALB/c mice. Macrophages were differentiated from the bone marrow of age-matched BALB/c or NOD mice at 11 weeks of age, and then were either untreated (UT) or treated with LPS overnight (A, B) or for 6h (C, D). Secreted TNF (A) and (B) IL-6 was quantified by ELISA. The differential expression of (C) TNF and (D) IL-6 was measured by qRT-PCR. Data is presented as means ± SEMs (n=5) and is representative of two independent experiments. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

4.3.4 FhHDM-1 downregulated the epigenetic markers to regulate, but not fully suppress, the pro-inflammatory immune response of NOD bone marrow derived macrophages.

Next, the hypothesis that FhHDM-1 regulated macrophage training in NOD mice to prevent T1D onset was tested. Once again, the previous treatment regime that prevented T1D was utilised. Thus, FhHDM-1 was delivered i.p. on alternate days, for a total of six injections, to NOD mice, beginning at 6 weeks of age. At 11 weeks of age, three weeks after the final injection of FhHDM-1, the bone marrow was harvested. Macrophages were differentiated *in vitro* after which the methylation of H3K4 was assessed. In addition, BMDMs from saline or FhHDM-1 treated NOD mice were stimulated with bacterial LPS, after which the glycolytic activity and cytokine production were measured.

Validating the hypothesis that FhHDM-1 was regulating the trained immune response, there was a notable reduction of mono-methylation, and a significant reduction of tri-methylation of histone H3K4 in macrophages differentiated from the bone marrow of NOD mice that had received FhHDM-1 (Figure 4.6). Furthermore, the glycolytic flux in response to LPS in the BMDMs of FhHDM-1 treated mice was also significantly reduced, as compared to those from NOD mice that had received saline (Figure 4.7A, B). Correlating with these changes, the production of both TNF (Figure 4.8A) and IL-6 (Figure 4.8B) in response to LPS was significantly suppressed in the FhHDM-1 treated NOD mice, as compared to those given saline alone. However, consistent with the previous *in vitro* analysis (chapter 3), this effect was not replicated at the gene expression level, with no change in the expression levels of IL-6 or TNF in response to LPS stimulation in macrophages from mice that had received either saline or FhHDM-1 (Figure 4.8C, D)

Notably, the administration of FhHDM-1 to NOD mice had no significant effect on the glycolytic activity of unstimulated BMDMs (Figure 4.7A), suggesting the specific regulation of only LPS activated, and not resting, macrophages. Furthermore, a direct comparison of LPS-induced cytokine levels in macrophages derived from the bone marrow of saline treated BALB/c mice, saline treated NOD mice, and FhHDM-1 treated NOD mice revealed that the pro-inflammatory response to bacterial LPS was not abolished in macrophages from FhHDM-1 treated NOD mice, but rather restored to the level observed in cells from immune competent

BALB/c mice (Figure 4.9). This finding implies a subtle fine-tuning of immune responses, rather than a switch to an anti-inflammatory phenotype, or complete immune suppression.

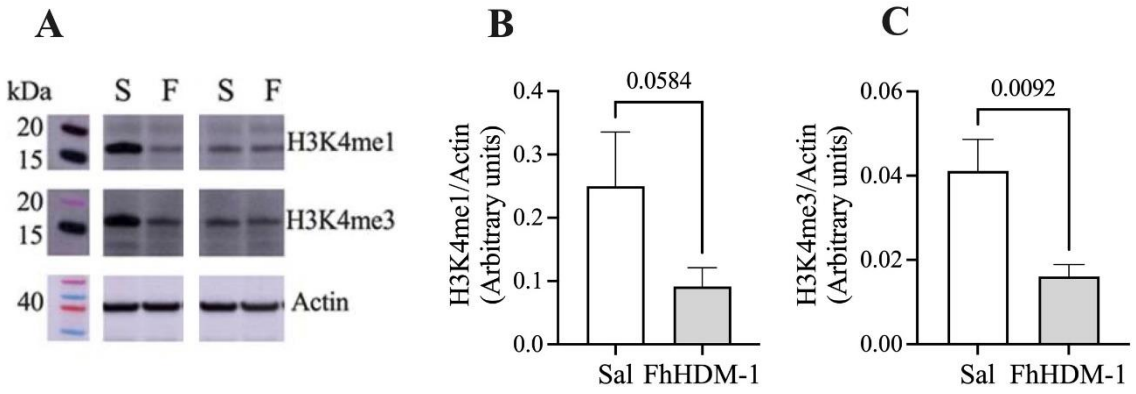

Figure 4.6: Methylation of H3K4 was reduced in bone marrow derived macrophages from NOD mice that had been treated with FhHDM-1. Macrophages were differentiated from the bone marrow of NOD mice 3 weeks after they had received 6 i.p. injections of 0.1mg/ml FhHDM-1 (F) or saline (S). (A) Cell lysates were normalised by protein concentration and the methylation status of H3K4 was analysed by western blot using primary antibodies specific for H3K4me1 and H3K4me3. The protein loading of each sample was determined by the detection of actin. The data shown is representative of n=10 NOD mice from each treatment. (B, C) Quantification of western blots were performed by the Amersham imager software in which each protein bands was normalised to its corresponding actin value generating an arbitrary unit. Statistical significance was determined by a paired t-test.

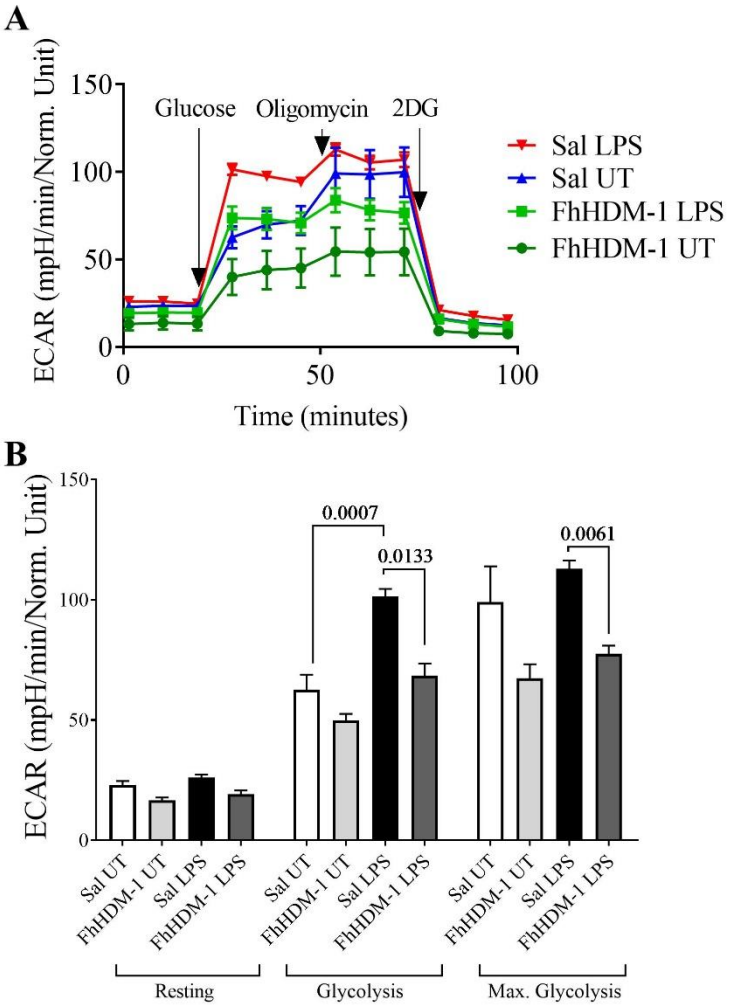

Figure 4.7: Treatment of NOD mice with FhHDM-1 significantly reduced the glycolytic activity induced by LPS stimulation of bone marrow derived macrophages *ex vivo*. Macrophages were differentiated from the bone marrow of NOD mice harvested 3 weeks after treatment with 6 i.p. injections of 0.1mg/ml FhHDM-1 or saline, and then stimulated *in vitro* with LPS for 24h. (A) The glycolytic activity was determined by measuring the extracellular acidification rate (ECAR), and (B) the maximum glycolytic capacity of cells after subsequent treatment with glucose, oligomycin and 2-deoxy-glucose (2-DG). Data is presented as means ± SEMs (n=5) and is representative of two independent experiments. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

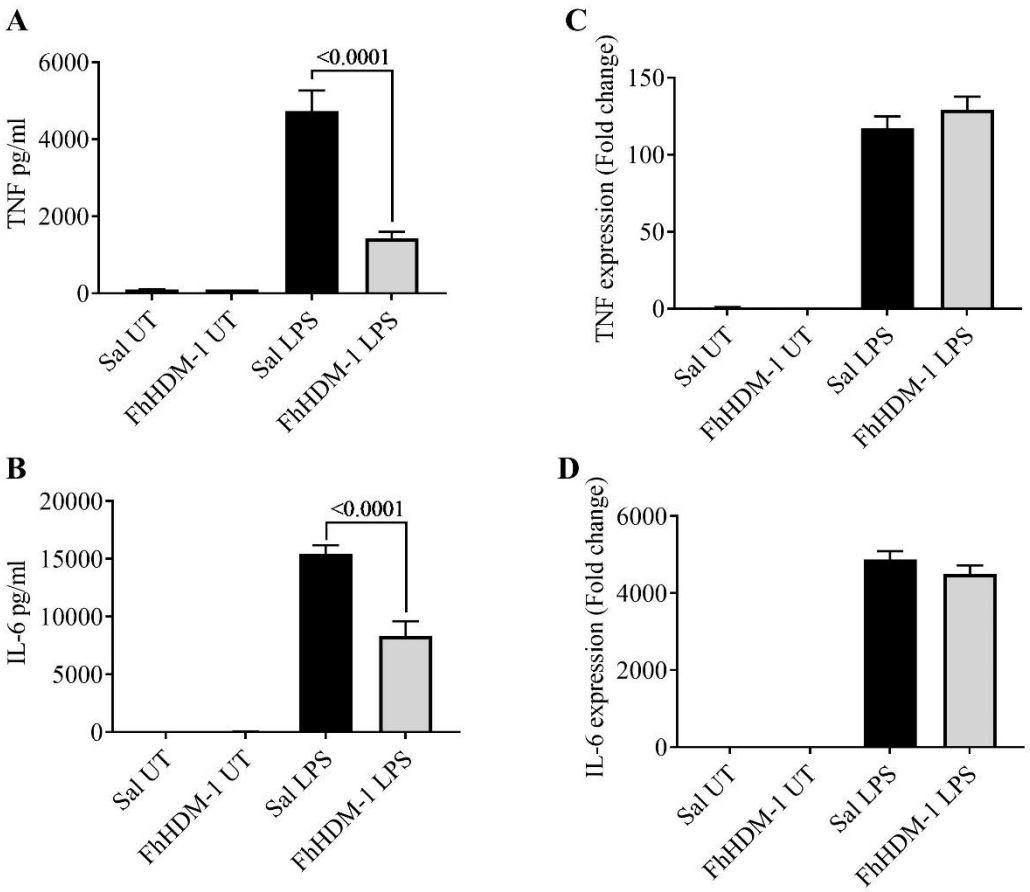

Figure 4.8: Treatment of NOD mice with FhHDM-1 reduced the production, but not gene expression levels of TNF and IL-6, in response to LPS stimulation of bone marrow derived macrophages *in vitro*. Macrophages were differentiated from the bone marrow of NOD mice 3 weeks after treatment with 6 i.p. injections of 0.1mg/ml FhHDM-1 or saline, and then stimulated *in vitro* with LPS either overnight (A, B) or for 6h (C, D). Secreted (A) TNF and (B) IL-6 was quantified by ELISA. The differential expression of (C) TNF and (D) IL-6 was measured by qRT-PCR. Data is presented as means ± SEMs (n=5) and is representative of two independent experiments. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

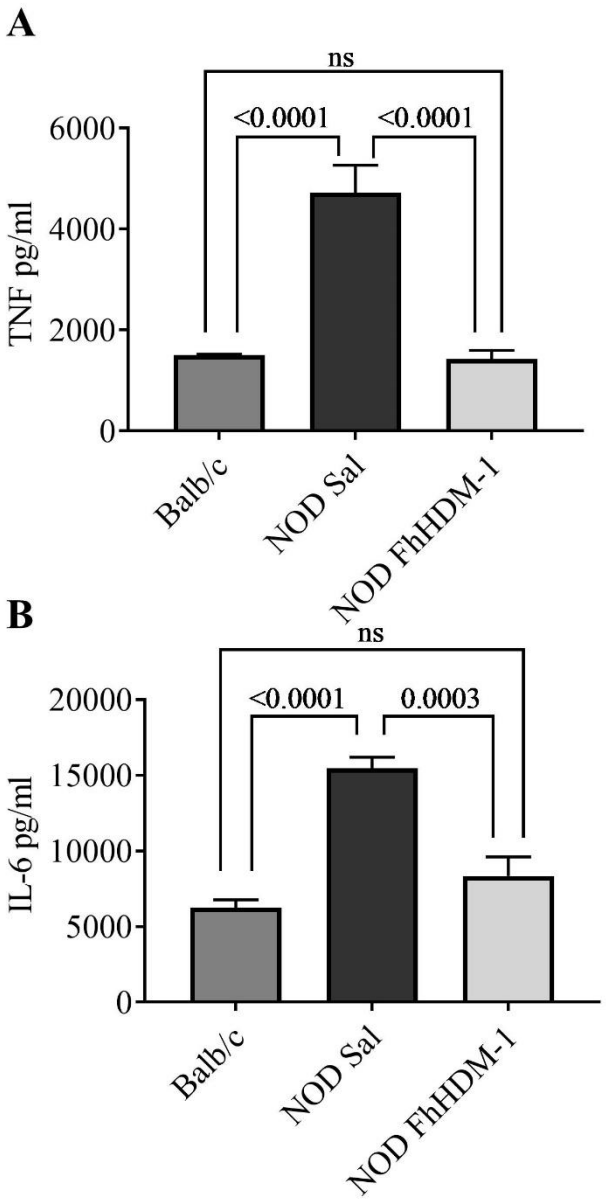

Figure 4.9: Treatment of NOD mice with FhHDM-1 prevented the enhanced (trained) pro-inflammatory response of macrophages to LPS. The production of (A) TNF and (B) IL-6 was measured in the supernatants collected from LPS stimulated macrophages derived from the bone marrow of BALB/c mice (as shown in Figure 4.5A, B), NOD mice treated with saline, and NOD mice treated with FhHDM-1 (as shown in Figure 4.9A, B). For direct comparison, these samples were re-analysed in the same ELISA. Data is presented as means ± SEMs (n=5) and is representative of two independent experiments. Statistical significance was determined by a one-way ANOVA with Tukey's multiple comparison test.

4.4 Discussion

This chapter presents the first demonstration of a trained innate immune response in T1D and provides the first evidence that a single helminth derived peptide regulates this phenomenon. Bone marrow derived macrophages from untreated NOD mice, but not from T1D resistant BALB/c mice, displayed increased mono- and tri- methylation of H3K4, enhanced glycolytic activity, and elevated secretion of TNF and IL-6, in response to stimulation with bacterial LPS. All hallmarks of a centrally trained immune response. The administration of FhHDM-1 to NOD mice, using the treatment regime that prevented the onset of T1D, significantly reduced the methylation of H3K4 in BMDMs. The variability seen in the methylation levels from NOD mice within the saline group was attributed to the asynchronous nature and incomplete disease penetrance in this colony of mice. Nonetheless, methylation ofH3K4 was reduced in every mouse treated with FhHDM-1 suggesting that in the instances where macrophages from NOD mice were methylated, FhHDM-1 significantly reduced this. Remarkably, this was associated with the regulation, but not suppression, of the response to inflammatory ligands, with levels of pro-inflammatory cytokines produced reflecting the response of macrophages from immune competent, non-diabetic mice. Collectively, these data reveal that NOD macrophages are trained, and that FhHDM-1 can reverse this central training to re-establish immune-metabolism homeostasis and prevent T1D.

It has long been recognised that macrophages from diabetic patients (and mouse models of disease) display enhanced inflammatory responses, an immune profile that is central to the initiation of autoimmunity and disease development (207, 208). Testament to this, elimination of these macrophage populations halts diabetes development (225). The data presented here provides support for the premise that this state of heightened reactivity is attributable to an aberrant trained innate immune response, characterised by increased glycolytic activity and methylation of H3K4 in BMDMs. This discovery also provides a putative mechanistic framework to explain the long-term association of T1D with previous viral infections (226-228), most recently the SARS-CoV-2 virus (229). Whether viral proteins are directly training the macrophages, or if the resulting imbalance in the gut microbiome and associated bacterial translocation, provides the activating signal remains to be elucidated (215). However, as the NOD mice used in this study were housed in specific pathogen free conditions, alterations to gut microbiota, rather than exposure to an infectious agent, may have been initiating the training of macrophages reported here. Indeed, it has been reported that NOD mice develop disease with an increased incidence in specific pathogen free, as compared to germ free,

conditions suggesting a protective role for gut commensals, which has also been demonstrated for humans and autoimmune disease (230). Furthermore, in NOD mice there is a higher penetrance of disease in the females, as compared to the male mice (224, 231), which has been attributed, in part, to differing gut microbiota (232, 233). Subsequent analysis of the microbiota between newborn NOD female mice and non-autoimmune strains of mice (BALB/c and C57BL6) confirmed an association between gut dysbiosis, pro-inflammatory responses, and the diabetogenic immune response (232).

An association with an altered gut microbiota and aberrant inflammatory responses is now becoming universally associated with a spectrum of seemingly disparate pathologies that dominate present-day morbidity and mortality worldwide (234, 235). However, exactly how the immune system and the microbiota interact to promote and maintain disease remains unknown. Despite variation in dysbiosis across autoimmune diseases, there is evidence to suggest that specific bacteria can promote or inhibit immune responses, collectively suggesting that there may be a significant polymicrobial impact on inflammatory states (236-238). Of relevance to this study, patients with T1D display changes in the ratios of the main phyla within the gut microbiome. Patients with T1D exhibit decreased Bacteroidetes and lower bacterial diversity, as compared to that of age-matched, genotype-matched, non-autoimmune individuals (239). Additional evidence shows that T1D incidence is associated with an increased abundance of *Clostridium* and decreased *Bifidobacterium* and *Lactobacillus* microbes, as compared to healthy subjects (240). Further, a study in children showed that a low abundance of bacteria responsible for lactate and butyrate production was associated with β-cell autoimmunity (241). Based on the evidence presented here, it is conceivable that a trained innate immune response initiated by ligands from altered populations of gut microbiota may be a common event initiating disease development.

However, in the same way that the immune system has co-evolved with the gut microbiota, it has also co-evolved in the presence of helminth parasites. Therefore, it is highly likely that helminths and gut microbiota must also have co-evolved. Accordingly, there is growing evidence that infection with helminth parasites can alter the composition of the gut microbiota (242-245), which, in turn, mediates a beneficial effect in preventing immune-mediated disease (246, 247). While the focus of most studies has been on a correlation between helminth infection and altered populations of gut bacteria, the evidence that in germ-free mice helminth infection can still regulate the immune response to viral infection (248), indicates that

the beneficial effects of parasitic worms can also occur independently of any change in the resident commensal microbiota.

The data presented within this chapter proposes a likely mechanism to explain this regulation of immune responses, which, although not directly altering the population of commensal bacteria, may be preventing the aberrant induction of a trained immune response induced by an altered microbiota. The discovery of reduced histone methylation, a switch to glycolysis, and reduced pro-inflammatory responses, adds support to the emerging hypothesis that helminth infections, and their secreted proteins, are imprinting a long-lasting regulation of macrophage activation. This would, in turn, mediate the beneficial effects of parasitic worms in preventing/reversing immune-mediated disease. Bone marrow derived myeloid cells from *S. mansoni* infected Apoe$^{-/-}$ mice (a model for cardiovascular disease and atherosclerosis) displayed increased OXPHOS, and other metabolic signatures (133) associated with reduced inflammatory signalling. Furthermore, this reprogrammed phenotype was adoptively transferable to uninfected mice, which were subsequently protected from obesity and glucose intolerance, suggesting a long-lasting memory of metabolic reprogramming and immune regulation (133). Although not explored, these findings suggest that *S. mansoni* infection is metabolically reprogramming the myeloid lineage to regulate the training of macrophages by glucose and lipids, thereby preventing the aberrant activation of the pro-inflammatory responses that drive immune-mediated metabolic disease. Similarly, the administration of either total extracts, or the excretory/secretory products, of *F. hepatica* to mice altered the metabolic profile, and reduced pro-inflammatory responses in BMDMs (179, 249). This effect was sufficiently potent to provide protection from experimental allergic encephalomyelitis (EAE) after adoptive transfer (179). However, in contrast to the effects of FhHDM-1 observed here, in all the aforementioned studies BMDMs displayed characteristics of an M2 phenotype and a general anti-inflammatory profile, characterised by increased secretion of cytokines, such as IL-10 and TGFβ. While not tested, this may result in the more generalised immune suppression associated with helminth infections, rather than the subtle regulation of training and the restoration of immune homeostasis observed after administration of FhHDM-1 to NOD mice. This difference likely reflects the broad, heterogenous mix of molecules (both host and parasite) present during a helminth infection, or within the excretions and secretions of parasites. In contrast, the administration of a single isolated peptide is able to produces a more subtle regulation of inflammatory responses. It is now important to validate this apparent rebalancing of immunity induced by FhHDM-1, and to demonstrate that the beneficial effects

of FhHDM-1 treatment in the prevention of autoimmunity do not prevent the protective immune response to infection or vaccination. Some support for this outcome has been previously reported. There was no change in T-cell proliferative responses or cytokine production observed in the lymph nodes of mice treated with FhHDM-1, as compared to controls. In addition, the antibody response to multiple injections of keyhole limpet haemocyanin (KLH) was unaffected in FhHDM-1 treated mice (102), suggesting an intact B-cell and T-cell adaptive immune responses. To build on these observations, FhHDM-1 treated NOD mice could be challenged with a viral or bacterial infection, or a vaccination, and the subsequent stimulation of protective pathogen specific immune responses measured.

This fine-tuning of macrophage pro-inflammatory responses by FhHDM-1 can be explained by the observation that the peptide regulates immune responses post-transcriptionally, as illustrated by the significant reduction in TNF and IL-6 protein production, without any change to the gene transcription levels of these cytokines. This is contradictory to the reported impact of methylation of H3K4 which has been correlated to enhanced transcription levels of TNF and IL-6 and removal of these methylation marks reduces access to the promoters for these cytokines, thus decrease their levels of transcription (250, 251). Although H3K4me1/3 has been the focus of most previous studies of the epigenetic regulation of the trained innate immune response, several other histone modifications are associated with the transcriptional regulation of TNF and IL-6 (252-255). In addition, there is evidence of differential methylation and acetylation of histones in macrophages from different mouse strains, which results in distinct transcriptional profiles in response to stimulation (256). Therefore, it will be important to next expand on the data presented here and perform a global analysis of histone modifications, and associated open chromatin regions, in macrophages from NOD mice with and without FhHDM-1 treatment. This could be achieved using chromatin immunoprecipitation and assay of transposase-accessible chromatin using sequencing (ATAC-seq) following stimulation of macrophages with LPS, and comparing results to untreated cells. These studies would provide a definitive functional association between specific methylation or acetylation marks on relevant histones and altered transcription of specific cytokines in NOD mice and determine if this is modulated by FhHDM-1.

Independent of a role in the regulation of gene transcription, the methylation of H3K4 also controls mitochondrial dynamics (257), with the absence of methylation associated with the reduced expression of Drp1 (258), an enzyme that is necessary for mitochondrial fission. As previously discussed in Chapters 2 and 3, like the effect of FhHDM-1, silencing Drp1 in

macrophages results in the post-transcriptional regulation of TNF (192, 193). Thus, the discovery here that FhHDM-1 acts to demethylase H3K4 corroborates the previous *in vitro* data presented in Chapters 2 and 3. The data also further supports a mechanism in which FhHDM-1 controls the mitochondrial dynamics of macrophages to reprogram metabolic activity, and consequently regulate pro-inflammatory responses. Further support for the association between mitochondrial dynamics and regulation of cytokine transcription is provided by reports that shifts in mitochondrial activity, and the utilisation of metabolic pathways, promotes the phosphorylation of eukaryotic translation initiation factor 2 (eIF2), which, in turn, represses the translation of several cellular and viral mRNAs (259). Additionally, elF4E is a similar cap-binding protein also regulated by mitochondrial dynamics (260), and has been shown to regulate innate immunity and promote M2 macrophages (261, 262). The elF4E protein has more specifically been shown to regulate the post-transcriptional regulation of TNF and IL-6 expression in macrophages in response to LPS stimulation (263). To test this hypothesis, the dynamics of macrophage mitochondria following stimulation with LPS (to induce fission), in the presence and absence of FhHDM-1, could be visualised and quantified using confocal microscopy to follow the uptake of MitoTracker™. These observations could then be correlated to a switch in mitochondrial activity, and the translational regulation of cytokine production.

Collectively, the results described in this chapter suggest the feasibility of a new treatment paradigm involving the therapeutic targeting of the trained immune response to specifically modulate the pro-inflammatory responses of macrophages to prevent autoimmunity, without the induction of immune suppress *per se*. It has previously been reported that FhHDM-1 prevents T1D, and MS, in murine models (102). This protective effect was attributed to the preferential interaction of FhHDM-1 with macrophages, thereby offering a targeted peptide treatment (102). The current study has established that macrophages from NOD mice are centrally trained. Importantly, FhHDM-1 can remove the epigenetic markers and reprogram metabolism in NOD macrophages. Furthermore, FhHDM-1 does not impact the metabolic activity of resting cells, instead specifically regulating, but not fully suppressing, the response to stimulation with an inflammatory ligand. These findings suggest that FhHDM-1 can recreate the ancestral microenvironment in which innate immune training evolved, and thus restore the balance between activation and regulation of inflammation. While a causal link to disease outcomes, and a deeper understanding of the molecular mechanisms that mediate these effects *in vivo* will be required, the current study offers a potential, and previously

unexplored, therapeutic strategy for T1D, and likely other autoimmune/inflammatory disorders.

Chapter 5: General Discussion

The innate immune system evolved to provide protection from infection and tissue damage. However, in recent decades, excessive and inappropriate inflammatory responses have caused an increased incidence of inflammatory diseases, such as diabetes mellitus, non-alcoholic fatty liver disease, neurodegenerative conditions, and various autoimmune disorders (264). Immune-mediated disease is now the most predominant cause of death globally, with more than 50% of all deaths being attributable to these disorders, and currently there are no cures for these diseases. The existing state-of-the-art in therapeutics target key components of the inflammatory pathways. While these strategies do suppress pathophysiology and symptoms in some patients, this is achieved against a burden of toxicity and global immune-suppression, and typically full remission from disease does not occur or is transient (265). Thus, alternative treatments for the vast array of immune-mediated diseases, which share the aetiology of aberrant macrophage activation, are urgently required.

Previously, a single peptide, FhHDM-1, secreted by the liver fluke, *Fasciola hepatica*, was shown to prevent multiple disparate immune-mediated diseases: T1D, MS and asthma in murine models (102, 212). This PhD project has determined a significant mechanism of action by which FhHDM-1 exerts this efficacy. Namely, FhHDM-1 can restore the balance between the activation and regulation of inflammatory responses in mammalian macrophages. Specifically, by reprogramming the metabolic activity of macrophages, FhHDM-1 altered histone methylation, and post-transcriptional regulation of pro-inflammatory cytokine (TNF and IL-6) production. Importantly, this effect was observed in BMDMs of NOD mice (which develop immune-mediated diabetes). Only a short time course of FhHDM-1 treatment (previously shown to prevent T1D in NOD mice) reduced pro-inflammatory cytokine production to the levels observed in immune-competent, nondiabetic-prone mice. Furthermore, the changes to macrophage metabolism and cytokine production were only observed after stimulation with the inflammatory ligand, LPS, and not in resting cells. As such, the data presented in this project indicates that FhHDM-1 does not induce an anti-inflammatory phenotype or cause immunosuppression, rather it re-establishes metabolic/immune homeostasis. These data authenticate FhHDM-1 as a novel peptide for the treatment of immune-mediated diseases caused by excessive and pro-inflammatory responses of macrophages.

Inflammatory pathways evolved in an ancestral microenvironment in which parasitic worms were ubiquitous (173, 242). This is not the case today. The traditionally endemic prevalence of helminths in human populations has been broadly attributed to their ability to

regulate host immune responses, such that chronic infections are established, which ensure longevity and reproductive success [(266); and reviewed in Chapter 1]. The bystander effect of immune regulation by helminths is the prevention of diseases that are mediated by chronic inflammation or autoimmunity. Indeed, clinical and experimental evidence has established an inverse correlation between helminth infection and immune mediated diseases globally (173). Accordingly, understanding the immune-regulatory mechanisms utilised by helminths will inform the development of strategies for the prevention/treatment of immune mediated diseases and parasite infections.

Macrophages are central to the immediate immune response to infection with all helminths (reviewed in Chapter 1), and in the initiation of most immune-mediated diseases (267). Accordingly, targeting macrophages is a novel immune therapy for both parasite infections (61, 268) and human inflammatory diseases (269). As described in Chapter 1, macrophages have been phenotypically classified and exist along a continuum of functional activities, with pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages at each pole of this continuum. This polarisation of macrophage phenotype/function ensures an efficient response to challenges within dynamic microenvironments. During the initial stages of infection or injury, macrophages differentiate into a pro-inflammatory M1 functional state to facilitate efficient microbial killing. In contrast, M2 macrophages predominate during the resolution of inflammation. This sequential dominance of different macrophage phenotypes ensures the appropriate induction and then termination of inflammatory responses, and the induction of tissue repair mechanisms. During helminth infections, there is a deficit of M1 macrophages, and instead M2 cells dominate. This alteration to the highly evolved mammalian response to infection is partly mediated by the molecules excreted/secreted by the parasites (described in Chapter 1), which regulate the activation of an M1 phenotype and/or induce the differentiation of M2 macrophages. Therefore, understanding how these immune-modulatory parasite molecules achieve these outcomes would provide a potential strategy for effective anti-helminth treatments. Furthermore, as M1 macrophages are most typically associated with the initiation of autoimmunity and chronic inflammation in humans, exploiting the mechanisms by which helminths regulate macrophage responses, would provide a novel therapeutic strategy for immune mediated diseases.

The peptide, FhHDM-1, which was the focus of this project, had previously been shown to reduce pro-inflammatory responses in macrophages, and to prevent immune-mediated diabetes, autoimmune demyelination, and allergy induced asthma in murine models (102, 212,

270). Although it was also known that FhHDM-1 was internalised by macrophages, after which it inhibited vATPase, thereby preventing lysosome acidification (103), it was unknown how FhHDM-1 impacted the regulation of macrophage immune responses. The initial investigation in this project (Chapter 2) was inspired by the emerging association between lysosomal pH and mitochondrial metabolism (271), and the functional link between macrophage metabolism and immune functionality (272). Examining the metabolic activity of macrophages treated *in vitro* with FhHDM-1 revealed a switch from glycolysis to OXPHOS, which was supported by endogenous FAO with a dependency on glutaminolysis. Although this metabolic signature has been typically associated with an M2 phenotype (induced by IL-4), FhHDM-1 treated macrophages did not alter the expression levels of the characteristic M2 markers. However, a predominant switch to OXPHOS and utilisation of FAO is also associated with the resolution of inflammation, which suggested that FhHDM-1 was mimicking mammalian immune regulation to prevent pro-inflammatory immune responses, which would be detrimental to the invading Fasciola parasites. This hypothesis was examined by stimulating FhHDM-1 treated macrophages with the M1 inducing ligand, LPS (Chapter 3). The data confirmed that the switch to glutaminolysis induced by FhHDM-1 reduced the glycolytic activity of LPS stimulated macrophages, which consequently reduced the secretion of TNF and IL-6. These outcomes were dependent on the inhibition of lysosomal acidification, which established an explicit link between macrophage lysosomes, mitochondria, and inflammation.

Importantly, this ability of FhHDM-1 to modulate the metabolic activity of macrophages suggested a broad therapeutic effect because glycolysis is central to the induction of a trained immune response (273). While it was proposed that trained immunity evolved as a mechanism to protect against infection, it is now acknowledged that the training of macrophages can lead to the induction of inappropriate and amplified pro-inflammatory responses, which mediate human disease (274). To test the possibility that FhHDM-1 could indeed influence the immune response of a trained macrophage, it was first established that macrophages from NOD mice, which spontaneously develop immune-mediated diabetes, displayed the characteristics of a trained immune cell (Chapter 4). Remarkably, the administration of FhHDM-1 to NOD mice (using the same treatment regime that prevented disease) removed the epigenetic marks on the BMDMs, and regulated, but did not fully suppress, the secretion of pro-inflammatory cytokines in response to LPS stimulation. Surprisingly, given the demethylation of H3K4, the gene expression levels of TNF and IL-6 were unaltered by FhHDM-1 treatment despite an observed reduction in TNF and IL-6

production. These findings suggest a fine-tuning of macrophage pro-inflammatory responses by FhHDM-1, rather than a complete suppression.

Collectively, this study has provided new insights into the mechanisms by which FhHDM-1 modulates the response of macrophages to inflammatory stimulation, and explains the protective effect previously reported in murine models of immune mediated disease (Figure 5.1). By changing the pH of the macrophage lysosome, FhHDM-1 is initiating a cascade of events mediated primarily by the production of α-ketoglutarate (α-KG) through the induction of glutaminolysis. The increased abundance of α-KG, controls histone methylation and mitochondrial dynamics, the combined effect of which supports the metabolic switch to OXPHOS and away from glycolysis, and the post-transcriptional regulation of TNF and IL-6. As a result, the pro-inflammatory response of macrophages to stimulation is delicately balanced to ensure a response that is not destructive in nature.

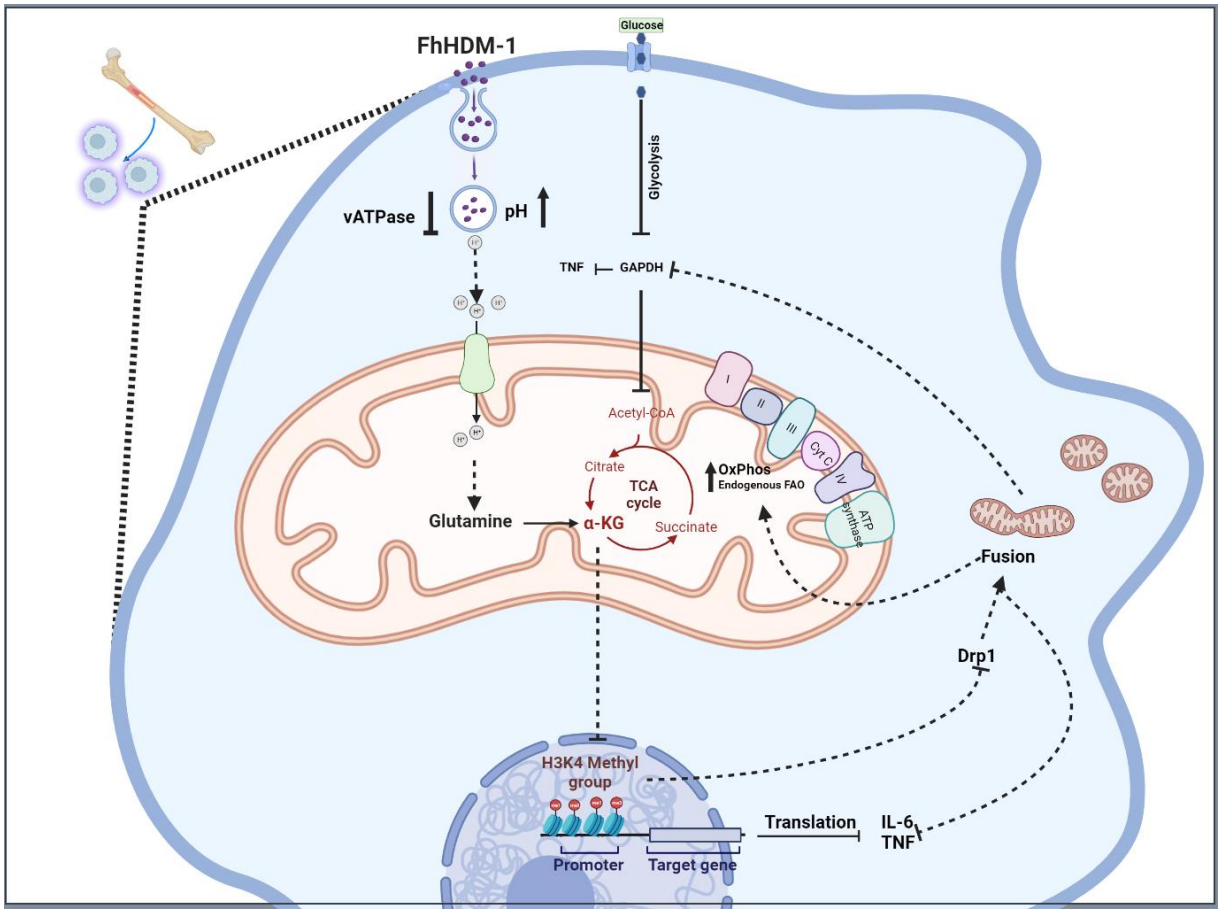

Figure 5.1: Proposed mechanism of action of FhHDM-1 within macrophages. After internalisation of FhHDM-1 by macrophages it localises to the endolysosome and inhibits vATPase, resulting in increased lysosomal pH. The resultant release of protons from the lysosome drives the induction of glutaminolysis, which, in turn, leads to the production of α-ketoglutarate (α-KG). The lack of H3K4 methylation associated with FhHDM-1 treatment of macrophages is likely mediated by α-KG because it is a co-factor for multiple demethylases. Additionally, in FhHDM-1 treated macrophages, α-KG is converted to citrate, which facilitates the synthesis of fatty acids required to fuel fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS). The absence of methylation on H3K4 and the increased abundance of α-KG promotes mitochondrial fusion. Fusion is a physical state that supports OXPHOS, rather than glycolysis, and regulates the post-transcriptional expression of pro-inflammatory cytokines. Because of these changes, FhHDM-1 can mediate a balanced macrophage response to inflammation, thereby preventing immune-mediated disease while maintaining the protective immune responses to microbial infection or tissue injury.

Some steps in this mechanistic cascade are hypothesised (dotted lines, Figure 5.1), based on comprehensive analyses of the existing literature in relation to the experimental findings. Accordingly, the observed changes to metabolic activity (increased glutaminolysis; switch to OXPHOS), and the resultant demethylation of H3K4 and the post-transcriptional regulation of pro-inflammatory cytokine expression, can be explained by: [1] a release of protons from the lysosome; [2] α-KG-mediated demethylation of histones; and [3] promotion of mitochondrial fusion. It will now be important to validate these steps experimentally by [1] tracking the movement of protons between the lysosome and mitochondria, [2] inhibiting α-KG and assessing the methylation state of H3K4, and finally [3] looking at mitochondrial morphological changes in response to FhHDM-1 treatment to fully demonstrate the intracellular events induced by FhHDM-1.

The previous demonstration that FhHDM-1 treatment of macrophages prevented the induction of the NLRP3 inflammasome (103), lends support to the possibility that the alteration in mitochondrial dynamics is central to the immune regulation of macrophages by FhHDM-1. It was deduced that the altered pH of macrophage lysosomes observed after treatment with FhHDM-1 was reducing the activation of lysosomal cathepsin B, which, in turn, was preventing the oligomerisation and activation of cytoplasmic NLRP3 (103). However, more recently a role for mitochondrial dynamics in the regulation of inflammasome signalling has emerged (275). Furthermore, the glycolytic potential under stress conditions is regulated by the interaction between the outer mitochondrial membrane protein and the NLRP3 inflammasome (276). This relationship is not clear-cut, given that both mitochondrial fission (277) and mitochondrial fusion (278) have been reported to promote the activation of NLRP3 in macrophages. Nonetheless, this association provides credence to the mechanistic hypothesis proposed (Figure 5.1).

The mechanism of action of FhHDM-1 presented here is consistent with an emerging body of evidence that the methylation profiles of cells are altered following helminth infection (279) or exposure to helminth extracts (280) or antigens (281). Like the effect of FhHDM-1, these changes correlated to a regulation of inflammation in the target cell (hepatocyte, dendritic cell, synovial fibroblast, respectively), although no functional, link was established. Thus, the findings here expand on this association, building a mechanism by which the helminth mediated demethylation of cellular histones regulates the pro-inflammatory response. Furthermore, the discovery that FhHDM-1 induced these changes to the bone marrow progenitor cells to stably imprint the regulation of inflammation provides a mechanism to

explain the absence of any effective protective (pro-inflammatory) host immune responses to helminth parasites. This would support the parasites' ability to retain a constant endemic presence in human populations. Indeed, the remodelled DNA-methylation signatures and defective pro-inflammatory immune responses in children infected with *Schistosoma haematobium* were maintained for at least 6 months following successful deworming (282). Furthermore, the offspring of mothers who were chronically infected with helminths during pregnancy displayed epigenetically rewired regulation of their pro-inflammatory immune responses (283, 284). FhHDM-1 is a single parasite-derived peptide that alters the demethylation of histones, and consequently regulates the macrophage inflammatory response. Therefore, this peptide holds significant promise to not only combat helminth infection, but also in the development of therapeutic strategies to counter the rise in prevalence of chronic inflammatory diseases, increasingly associated with the eradication of helminth infections.

Accordingly, it will be of interest to determine if the demethylation of histones observed in the bone marrow derived from FhHDM-1 treated NOD mice (Chapter 4), is recapitulated in other models of immune-mediated disease, and in monocytes/macrophages derived from patients. As mentioned, in addition to a beneficial effect against the development of T1D in NOD mice, FhHDM-1 has also been shown to prevent the progression of experimental allergic encephalomyelitis (EAE; a model of multiple sclerosis), and allergy induced asthma, in mouse models (102, 212). This may seem somewhat surprising given the different immune profiles associated with these conditions, with T1D and EAE (MS) typically associated to a Th1 type immune response, while asthma can be mediated by either a Th2, or mixed Th1/Th2 immune response (285-287). However, given the significant contribution that macrophage metabolism makes in determining disease outcomes, it is now apparent that increased glycolytic activity, a profile characterising immune training, may be the common underlying mechanism driving the progression of immune mediated diseases (127, 288-290). Expanding the analysis of the epigenetic, metabolic, and immune status of BMDMs from FhHDM-1 treated mice in other disease models would explore this premise, and putatively elevate FhHDM-1 to the status of master regulator of trained immunity.

However, to establish FhHDM-1 as an effective therapeutic it will be important to demonstrate the specificity of this effect. The adoptive transfer of BMDMs from FhHDM-1 treated mice to recipient mice would prove that the regulation of inflammation is stably imprinted to the myeloid progenitor cells and is transferable. In addition, the quantification of the immune response in FhHDM-1 treated mice, or macrophage recipients, to an infectious

challenge or vaccination would validate the proposition that FhHDM-1 is fine-tuning, rather than suppressing, the immune response.

Finally, the localisation of FhHDM-1 to the bone marrow would suggest that it is exerting a direct and specific immune-regulatory effect. It has been previously established that FhHDM-1 preferentially interacts with macrophages *in vivo*, with macrophages being the only peritoneal immune cell staining positive for the peptide 20 mins after i.p. injection (102). In addition, when incubated with human PBMCs *in vitro*, FhHDM-1 selectively bound to the monocyte population (Donnelly, personal communication). However, it is currently unknown whether FhHDM-1 administered by i.p. injection, traffics to the bone marrow to have a direct interaction with the myeloid progenitor cells. Given the cell specificity previously reported, and the remarkable similarity in effects between the *in vitro* and *in vivo* macrophages seen here, it seems likely. Furthermore, it has been reported that the addition of an anionic amphiphile (the HDM peptides are amphipathic) to the surface of liposomal vesicles directed them to bone marrow macrophages (291). This could be explored using labelled peptide and *ex vivo* imaging of the bone marrow cells.

The strategy of regulating trained immunity to rebalance the inflammatory response represents a significant advantage over current immune therapeutic regimes, which are less specific, and accordingly induce undesirable global immune suppression and detrimental off-target effects. Given the pivotal role for lysosomes in coordinating cell metabolism, and consequently the epigenetic landscape, there is precedence in targeting the functions of this organelle (197). Proof of principle for this approach exists in the efficacy of chloroquine (CQ)/hydroxychloroquine (HCQ), weak bases that passively diffuse to the lysosome, and the vATPase inhibitor, Balfilomycin1 (Baf1). Using an *in vitro* model of trained immunity in which human monocytes were cultured for 24 h with heat killed *Candida albicans,* rested for 5 days, and then stimulated with an inflammatory ligand, the production of TNF and IL-6 by the second stimuli was abrogated when either CQ, HCQ or Baf1 were included in the first 24 h incubation (292). In addition, the tri-methylation of H3K4 was prevented suggesting that these compounds are potent inhibitors of trained immunity. However, in contrast to FhHDM-1, these compounds also reduced the transcription levels of pro-inflammatory cytokines, which indicates a much broader suppressive effect. Furthermore, unlike FhHDM-1 (293) these compounds have high cytotoxicity and cause a range of adverse events (294, 295). By establishing that macrophages from NOD mice are centrally trained and demonstrating that a short course treatment with FhHDM-1 can remove the epigenetic markers and reprogram

metabolism in NOD macrophages, this study provides the first ever evidence that altering the lysosomal pH can regulate trained immunity *in vivo*. Furthermore, the analysis here showed that FhHDM-1 does not impact the metabolic activity of resting cells, instead specifically regulating, but not fully suppressing, the response to inflammatory stimulation.

It will be of interest to determine if the biological activity uncovered in this project is common to all the members of the HDM peptides. As all HDM peptides display a similar structure (alpha helix and an amphipathic C-terminal) which is required for the immune-modulatory activity mediated by FhHDM-1 (111), it is likely that there is a close association between structure and function. Indeed, it has been shown that the Sm16-(34-117)-HDM peptides also interacts with macrophages and like FhHDM-1 localises to the endo-lysosome and decreases the pro-inflammatory response to subsequent stimulation with LPS. However, in contrast (as described in chapter 1) the recombinant version of HDM from *C. sinensis* induces a pro-inflammatory response in macrophages. It will be important to determine if these differences are associated with different methods of peptide synthesis (chemical versus recombinant) and how the biological outcomes relate to the HDMs specific to each parasite to fully establish how the HDMs are functionally linked and how the trematode worms utilise this peptide family to modulate host immune responses.

Nonetheless, the collective from this PhD suggest that FhHDM-1, a peptide that is naturally secreted by a parasitic worm, can recreate the natural ancestral microenvironment in which innate immune training evolved. In doing so, FhHDM-1 subtly restores the balance between activation and regulation of inflammation to prevent autoimmune/inflammatory disease. In addition, the data indicates that blocking the activity of FhHDM-1 during infection with *Fasciola hepatica*, may prevent the parasite mediated suppression of host responses, allowing mammalian hosts to actively expel invading parasites and induce an immune memory to protect against future infection. Therefore, FhHDM-1 is a highly promising therapeutic that fine tunes metabolic activity regulating pro-inflammatory responses restoring immune homeostasis.

Bibliography

1. Haswell-Elkins MR, Mairiang E, Mairiang P, Chaiyakum J, Chamadol N, Loapaiboon V, et al. Cross-sectional study of Opisthorchis viverrini infection and cholangiocarcinoma in communities within a high-risk area in northeast Thailand. International journal of cancer. 1994;59(4):505-9.

2. Kaplan RM. Fasciola hepatica: a review of the economic impact in cattle and considerations for control. Vet Ther. 2001;2(1):40-50.

3. Valero M, Girones N, Garcia-Bodelon M, Periago M, Chico-Calero I, Khoubbane M, et al. Anaemia in advanced chronic fasciolosis. Acta tropica. 2008;108(1):35-43.

4. Kim J-H, Choi M-H, Bae YM, Oh J-K, Lim MK, Hong S-T. Correlation between discharged worms and fecal egg counts in human clonorchiasis. PLoS neglected tropical diseases. 2011;5(10):e1339.

5. Fried B, Reddy A, Mayer D. Helminths in human carcinogenesis. Cancer letters. 2011;305(2):239-49.

6. Lim JH. Liver flukes: the malady neglected. Korean journal of radiology. 2011;12(3):269-79.

7. De Martel C, Ferlay J, Franceschi S, Vignat J, Bray F, Forman D, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. The lancet oncology. 2012;13(6):607-15.

8. Kaya M, Beştaş R, Çetin S. Clinical presentation and management of Fasciola hepatica infection: single-center experience. World journal of gastroenterology: WJG. 2011;17(44):4899.

9. Machicado C, Machicado JD, Maco V, Terashima A, Marcos LA. Association of Fasciola hepatica infection with liver fibrosis, cirrhosis, and cancer: a systematic review. PLoS neglected tropical diseases. 2016;10(9):e0004962.

10. Machicado C, Bertani S, Herrera-Velit P, Espinoza J, Ruiz E, Marcos L. Negative serology of Fasciola hepatica infection in patients with liver cancer in Peru: a preliminary report. Revista da Sociedade Brasileira de Medicina Tropical. 2018;51:231-3.

11. Lu X-T, Gu Q-Y, Limpanont Y, Song L-G, Wu Z-D, Okanurak K, et al. Snail-borne parasitic diseases: an update on global epidemiological distribution, transmission interruption and control methods. Infectious diseases of poverty. 2018;7(1):1-16.

12. Sripa B, Kaewkes S, Intapan PM, Maleewong W, Brindley PJ. Food-borne trematodiases in Southeast Asia: epidemiology, pathology, clinical manifestation and control. Advances in parasitology. 2010;72:305-50.

13. Mas-Coma S, Valero MA, Bargues MD. Fasciola, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Advances in parasitology. 2009;69:41-146.

14. Fedorova OS, Fedotova MM, Zvonareva OI, Mazeina SV, Kovshirina YV, Sokolova TS, et al. Opisthorchis felineus infection, risks, and morbidity in rural Western Siberia, Russian Federation. PLoS neglected tropical diseases. 2020;14(6):e0008421.

15. Steele JA, Richter CH, Echaubard P, Saenna P, Stout V, Sithithaworn P, et al. Thinking beyond Opisthorchis viverrini for risk of cholangiocarcinoma in the lower Mekong region: a systematic review and meta-analysis. Infectious diseases of poverty. 2018;7(1):1-13.

16. Andrews SJ. The life cycle of Fasciola hepatica. Fasciolosis. 1999;3:1-29.

17. Sithithaworn P, Sripa B, Kaewkes S, Nawa Y, Haswell MR. Food-borne trematodes. Manson's Tropical Infectious Diseases: Twenty-Third Edition[23rd ed]. 2014:726-36.

18. Cwiklinski K, Dalton JP. Advances in Fasciola hepatica research using 'omics' technologies. International journal for parasitology. 2018;48(5):321-31.

19. Lalor R, Cwiklinski K, Calvani NED, Dorey A, Hamon S, Corrales JL, et al. Pathogenicity and virulence of the liver flukes Fasciola hepatica and Fasciola Gigantica that cause the zoonosis Fasciolosis. Virulence. 2021;12(1):2839-67.

20. Attwood H, Chou S. The longevity of Clonorchis sinensis. Pathology. 1978;10(2):153-6.

21. Ramsay RJ, Sithithaworn P, Prociv P, Moorhouse DE, Methaphat C. Density-dependent fecundity of Opisthorchis viverrini in humans, based on faecal recovery of flukes. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1989;83(2):241-2.

22. Mas-Coma S, Valero MA, Bargues MD. Fascioliasis. Digenetic trematodes. 2014:77-114.

23. Durbin C. Longevity of the liver fluke, Fasciola sp. in sheep. Proceedings of the Helminthological Society of Washington. 1952;19(2).

24. Radostits OM, Gay C, Hinchcliff KW, Constable PD. A textbook of the diseases of cattle, horses, sheep, pigs and goats. Veterinary medicine. 2007;10:2045-50.

25. Cox N, Pokrovskii M, Vicario R, Geissmann F. Origins, biology, and diseases of tissue macrophages. Annual Review of Immunology. 2021;39:313-44.

26. Nobs SP, Kopf M. Tissue-resident macrophages: Guardians of organ homeostasis. Trends in immunology. 2021;42(6):495-507.

27. Wen Y, Lambrecht J, Ju C, Tacke F. Hepatic macrophages in liver homeostasis and diseases-diversity, plasticity and therapeutic opportunities. Cellular & molecular immunology. 2021;18(1):45-56.

28. Chen S, Yang J, Wei Y, Wei X. Epigenetic regulation of macrophages: from homeostasis maintenance to host defense. Cellular & molecular immunology. 2020;17(1):36-49.

29. Ghisletti S, Barozzi I, Mietton F, Polletti S, De Santa F, Venturini E, et al. Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages. Immunity. 2010;32(3):317-28.

30. Netea MG, Domínguez-Andrés J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, et al. Defining trained immunity and its role in health and disease. Nature Reviews Immunology. 2020;20(6):375-88.

31. Divangahi M, Aaby P, Khader SA, Barreiro LB, Bekkering S, Chavakis T, et al. Trained immunity, tolerance, priming and differentiation: distinct immunological processes. Nature immunology. 2021;22(1):2-6.

32. O'Neill LA, Pearce EJ. Immunometabolism governs dendritic cell and macrophage function. Journal of Experimental Medicine. 2015:jem. 20151570.

33. Murray PJ. Macrophage Polarization. Annual Review of Physiology. 2017;79(1):541-66.

34. Liu J, Geng X, Hou J, Wu G. New insights into M1/M2 macrophages: key modulators in cancer progression. Cancer Cell International. 2021;21(1):1-7.

35. Varga T, Mounier R, Horvath A, Cuvellier S, Dumont F, Poliska S, et al. Highly dynamic transcriptional signature of distinct macrophage subsets during sterile inflammation, resolution, and tissue repair. The Journal of Immunology. 2016;196(11):4771-82.

36. Kieler M, Hofmann M, Schabbauer G. More than just protein building blocks: How amino acids and related metabolic pathways fuel macrophage polarization. The FEBS journal. 2021;288(12):3694-714.

37. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity. 2014;41(1):14-20.

38. Palsson-McDermott EM, Curtis AM, Goel G, Lauterbach MA, Sheedy FJ, Gleeson LE, et al. Pyruvate kinase M2 regulates Hif-1α activity and IL-1β induction and is a critical determinant of the warburg effect in LPS-activated macrophages. Cell metabolism. 2015;21(1):65-80.

39. Xie M, Yu Y, Kang R, Zhu S, Yang L, Zeng L, et al. PKM2-dependent glycolysis promotes NLRP3 and AIM2 inflammasome activation. Nature communications. 2016;7:13280.

40. Ip WE, Hoshi N, Shouval DS, Snapper S, Medzhitov R. Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages. Science. 2017;356(6337):513-9.

41. Tao J, Zhang J, Ling Y, McCall CE, Liu TF. Mitochondrial Sirtuin 4 Resolves Immune Tolerance in Monocytes by Rebalancing Glycolysis and Glucose Oxidation Homeostasis. Front Immunol. 2018;9:419.

42. Wang F, Zhang S, Vuckovic I, Jeon R, Lerman A, Folmes CD, et al. Glycolytic stimulation is not a requirement for M2 macrophage differentiation. Cell metabolism. 2018;28(3):463-75. e4.

43. Sanin DE, Ge Y, Marinkovic E, Kabat AM, Castoldi A, Caputa G, et al. Predictive framework of macrophage activation. bioRxiv. 2021.

44. Colegio OR, Chu N-Q, Szabo AL, Chu T, Rhebergen AM, Jairam V, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. Nature. 2014;513(7519):559.

45. Parisi L, Gini E, Baci D, Tremolati M, Fanuli M, Bassani B, et al. Macrophage Polarization in Chronic Inflammatory Diseases: Killers or Builders? Journal of immunology research. 2018;2018.

46. Helm O, Held-Feindt J, Grage-Griebenow E, Reiling N, Ungefroren H, Vogel I, et al. Tumor-associated macrophages exhibit pro-and anti-inflammatory properties by which they impact on pancreatic tumorigenesis. International journal of cancer. 2014;135(4):843-61.

47. Ginhoux F, Schultze JL, Murray PJ, Ochando J, Biswas SK. New insights into the multidimensional concept of macrophage ontogeny, activation and function. Nature immunology. 2016;17(1):34.

48. Chen X, Liu Y, Gao Y, Shou S, Chai Y. The roles of macrophage polarization in the host immune response to sepsis. International Immunopharmacology. 2021;96:107791.

49. Zhou M, Wang C, Lu S, Xu Y, Li Z, Jiang H, et al. Tumor-associated macrophages in cholangiocarcinoma: complex interplay and potential therapeutic target. EBioMedicine. 2021;67:103375.

50. Andrejeva G, Rathmell JC. Similarities and distinctions of cancer and immune metabolism in inflammation and tumors. Cell metabolism. 2017;26(1):49-70.

51. Mazzone M, Menga A, Castegna A. Metabolism and TAM functions—it takes two to tango. The FEBS Journal. 2018;285(4):700-16.

52. Biswas SK, Gangi L, Paul S, Schioppa T, Saccani A, Sironi M, et al. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-κB and enhanced IRF-3/STAT1 activation). Blood. 2006;107(5):2112-22.

53. Lavin Y, Kobayashi S, Leader A, Amir E-aD, Elefant N, Bigenwald C, et al. Innate immune landscape in early lung adenocarcinoma by paired single-cell analyses. Cell. 2017;169(4):750-65. e17.

54. Su P, Wang Q, Bi E, Ma X, Liu L, Yang M, et al. Enhanced Lipid Accumulation and Metabolism Are Required for the Differentiation and Activation of Tumor-Associated MacrophagesEnhanced Lipid Metabolism Promotes TAM Generation. Cancer research. 2020;80(7):1438-50.

55. Chanmee T, Ontong P, Konno K, Itano N. Tumor-associated macrophages as major players in the tumor microenvironment. Cancers. 2014;6(3):1670-90.

56. Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. Immunity. 2014;41(1):49-61.

57. Salmaninejad A, Valilou SF, Soltani A, Ahmadi S, Abarghan YJ, Rosengren RJ, et al. Tumor-associated macrophages: role in cancer development and therapeutic implications. Cellular oncology. 2019;42(5):591-608.

58. Duan Z, Luo Y. Targeting macrophages in cancer immunotherapy. Signal transduction and targeted therapy. 2021;6(1):1-21.

59. Cortes-Selva D, Fairfax K. Schistosome and intestinal helminth modulation of macrophage immunometabolism. Immunology. 2021;162(2):123-34.

60. Lechner A, Bohnacker S, Esser-von Bieren J, editors. Macrophage regulation & function in helminth infection. Seminars in immunology; 2021: Elsevier.

61. Coakley G, Harris NL. Interactions between macrophages and helminths. Parasite immunology. 2020;42(7):e12717.

62. Walsh KP, Brady MT, Finlay CM, Boon L, Mills KH. Infection with a helminth parasite attenuates autoimmunity through TGF-β-mediated suppression of Th17 and Th1 responses. The journal of immunology. 2009;183(3):1577-86.

63. Pérez-Caballero R, Javier Martínez-Moreno F, Zafra R, Molina-Hernández V, Pacheco IL, Teresa Ruiz-Campillo M, et al. Comparative dynamics of peritoneal cell immunophenotypes in sheep during the early and late stages of the infection with Fasciola hepatica by flow cytometric analysis. Parasites & vectors. 2018;11(1):1-9.

64. Valero M, Navarro M, Garcia-Bodelon M, Marcilla A, Morales M, Hernandez J, et al. High risk of bacterobilia in advanced experimental chronic fasciolosis. Acta Tropica. 2006;100(1-2):17-23.

65. Ruiz-Campillo MT, Molina Hernandez V, Escamilla A, Stevenson M, Perez J, Martinez-Moreno A, et al. Immune signatures of pathogenesis in the peritoneal compartment during early infection of sheep with Fasciola hepatica. Scientific reports. 2017;7(1):1-14.

66. Piedrafita D, Parsons JC, Sandeman RM, Wood P, Estuningsih S, Partoutomo S, et al. Antibody-dependent cell-mediated cytotoxicity to newly excysted juvenile Fasciola hepatica in vitro is mediated by reactive nitrogen intermediates. Parasite immunology. 2001;23(9):473-82.

67. Golden O, Flynn RJ, Read C, Sekiya M, Donnelly S, Stack C, et al. Protection of cattle against a natural infection of Fasciola hepatica by vaccination with recombinant cathepsin L1 (rFhCL1). Vaccine. 2010;28(34):5551-7.

68. Molina-Hernández V, Mulcahy G, Pérez J, Martínez-Moreno Á, Donnelly S, O'Neill SM, et al. Fasciola hepatica vaccine: we may not be there yet but we're on the right road. Veterinary parasitology. 2015;208(1-2):101-11.

69. Donnelly S, O'Neill SM, Sekiya M, Mulcahy G, Dalton JP. Thioredoxin peroxidase secreted by Fasciola hepatica induces the alternative activation of macrophages. Infection and immunity. 2005;73(1):166-73.

70. Stempin CC, Motrán CC, Aoki MP, Falcón CR, Cerbán FM, Cervi L. PD-L2 negatively regulates Th1-mediated immunopathology during Fasciola hepatica infection. Oncotarget. 2016;7(47):77721.

71. Alvarez Rojas CA, Ansell BR, Hall RS, Gasser RB, Young ND, Jex AR, et al. Transcriptional analysis identifies key genes involved in metabolism, fibrosis/tissue repair and

the immune response against Fasciola hepatica in sheep liver. Parasites & vectors. 2015;8(1):1-14.

72. Lund ME, O'Brien BA, Hutchinson AT, Robinson MW, Simpson AM, Dalton JP, et al. Secreted proteins from the helminth Fasciola hepatica inhibit the initiation of autoreactive T cell responses and prevent diabetes in the NOD mouse. PloS one. 2014;9(1):e86289.

73. Guasconi L, Chiapello LS, Masih DT. Fasciola hepatica excretory-secretory products induce CD4+ T cell anergy via selective up-regulation of PD-L2 expression on macrophages in a Dectin-1 dependent way. Immunobiology. 2015;220(7):934-9.

74. Sripa B, Jumnainsong A, Tangkawattana S, Haswell MR. Immune response to Opisthorchis viverrini infection and its role in pathology. Advances in parasitology. 2018;102:73-95.

75. Wang N, Bai X, Jin X, Tang B, Yang Y, Sun Q, et al. The dynamics of select cellular responses and cytokine expression profiles in mice infected with juvenile Clonorchis sinensis. Acta Tropica. 2021;217:105852.

76. Bhamarapravati N, Thammavit W, Vajrasthira S. Liver changes in hamsters infected with a liver fluke of man, Opisthorchis viverrini. The American Journal of Tropical Medicine and Hygiene. 1978;27(4):787-94.

77. Kim E-M, Kwak YS, Yi M-H, Kim JY, Sohn W-M, Yong T-S. Clonorchis sinensis antigens alter hepatic macrophage polarization in vitro and in vivo. PLoS neglected tropical diseases. 2017;11(5):e0005614.

78. Bility MT, Sripa B. Chronic Opisthorchis viverrini infection and associated hepatobiliary disease is associated with iron loaded M2-like macrophages. The Korean journal of parasitology. 2014;52(6):695.

79. Thanee M, Loilome W, Techasen A, Namwat N, Boonmars T, Pairojkul C, et al. Quantitative changes in tumor-associated M2 macrophages characterize cholangiocarcinoma and their association with metastasis. Asian Pacific Journal of Cancer Prevention. 2015;16(7):3043-50.

80. Yan C, Wu J, Xu N, Li J, Zhou Q-Y, Yang H-M, et al. TLR4 deficiency exacerbates biliary injuries and peribiliary fibrosis caused by clonorchis sinensis in a resistant mouse strain. Frontiers in Cellular and Infection Microbiology. 2021;10:526997.

81. Pinlaor S, Ma N, Hiraku Y, Yongvanit P, Semba R, Oikawa S, et al. Repeated infection with Opisthorchis viverrini induces accumulation of 8-nitroguanine and 8-oxo-7, 8-dihydro-2′-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase. Carcinogenesis. 2004;25(8):1535-42.

82. Group IW. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans: Biological agents. Volume 100 B. A review of human carcinogens. IARC Monogr Eval Carcinog Risks Hum. 2012;100(pt B):1.

83. Sripa B, Kaewkes S. Localisation of parasite antigens and inflammatory responses in experimental opisthorchiasis. International journal for parasitology. 2000;30(6):735-40.

84. Tliba O, Sibille P, Boulard C, Chauvin A. Local hepatic immune response in rats during primary infection with Fasciola hepatica. Parasite. 2000;7(1):9-18.

85. Ravidà A, Cwiklinski K, Aldridge AM, Clarke P, Thompson R, Gerlach JQ, et al. Fasciola hepatica surface tegument: glycoproteins at the interface of parasite and host. Molecular & Cellular Proteomics. 2016;15(10):3139-53.

86. Suttiprapa S, Sotillo J, Smout M, Suyapoh W, Chaiyadet S, Tripathi T, et al. Opisthorchis viverrini proteome and host–parasite interactions. Advances in parasitology. 2018;102:45-72.

87. Shi Y, Yu K, Liang A, Huang Y, Ou F, Wei H, et al. Identification and analysis of the tegument protein and excretory-secretory products of the carcinogenic liver fluke Clonorchis Sinensis. Frontiers in Microbiology. 2020;11:555730.

88. Mulvenna J, Sripa B, Brindley PJ, Gorman J, Jones MK, Colgrave ML, et al. The secreted and surface proteomes of the adult stage of the carcinogenic human liver fluke Opisthorchis viverrini. Proteomics. 2010;10(5):1063-78.

89. Kaewpitoon N, Laha T, Kaewkes S, Yongvanit P, Brindley PJ, Loukas A, et al. Characterization of cysteine proteases from the carcinogenic liver fluke, Opisthorchis viverrini. Parasitology research. 2008;102(4):757-64.

90. Pinlaor P, Kaewpitoon N, Laha T, Sripa B, Kaewkes S, Morales ME, et al. Cathepsin F cysteine protease of the human liver fluke, Opisthorchis viverrini. PLoS neglected tropical diseases. 2009;3(3):e398.

91. Kang J-M, Bahk Y-Y, Cho P-Y, Hong S-J, Kim T-S, Sohn W-M, et al. A family of cathepsin F cysteine proteases of Clonorchis sinensis is the major secreted proteins that are expressed in the intestine of the parasite. Molecular and Biochemical Parasitology. 2010;170(1):7-16.

92. Cwiklinski K, Dalton JP, Dufresne PJ, La Course J, Williams DJ, Hodgkinson J, et al. The Fasciola hepatica genome: gene duplication and polymorphism reveals adaptation to the host environment and the capacity for rapid evolution. Genome biology. 2015;16(1):1-13.

93. Donnelly S, O'Neill SM, Stack CM, Robinson MW, Turnbull L, Whitchurch C, et al. Helminth cysteine proteases inhibit TRIF-dependent activation of macrophages via degradation of TLR3. Journal of Biological Chemistry. 2010;285(5):3383-92.

94. Perrigoue JG, Marshall FA, Artis D. On the hunt for helminths: innate immune cells in the recognition and response to helminth parasites. Cellular microbiology. 2008;10(9):1757-64.

95. Cliffe LJ, Humphreys NE, Lane TE, Potten CS, Booth C, Grencis RK. Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. Science. 2005;308(5727):1463-5.

96. Zhao A, Urban Jr JF, Anthony RM, Sun R, Stiltz J, Van Rooijen N, et al. Th2 cytokine-induced alterations in intestinal smooth muscle function depend on alternatively activated macrophages. Gastroenterology. 2008;135(1):217-25. e1.

97. Cortés A, Muñoz-Antoli C, Martín-Grau C, Esteban JG, Grencis RK, Toledo R. Differential alterations in the small intestine epithelial cell turnover during acute and chronic infection with Echinostoma caproni (Trematoda). Parasites & vectors. 2015;8(1):1-9.

98. Wynn TA, Cheever AW, Jankovic D, Poindexter RW, Caspar P, Lewis FA, et al. An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection. Nature. 1995;376(6541):594-6.

99. Hesse M, Modolell M, La Flamme AC, Schito M, Fuentes JM, Cheever AW, et al. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. The Journal of Immunology. 2001;167(11):6533-44.

100. Barron L, Wynn TA. Macrophage activation governs schistosomiasis-induced inflammation and fibrosis. European journal of immunology. 2011;41(9):2509-14.

101. Robinson MW, Donnelly S, Hutchinson AT, To J, Taylor NL, Norton RS, et al. A family of helminth molecules that modulate innate cell responses via molecular mimicry of host antimicrobial peptides. PLoS Pathog. 2011;7(5):e1002042.

102. Lund ME, Greer J, Dixit A, Alvarado R, McCauley-Winter P, To J, et al. A parasite-derived 68-mer peptide ameliorates autoimmune disease in murine models of Type 1 diabetes and multiple sclerosis. Sci Rep. 2016;6:37789.

103. Alvarado R, To J, Lund ME, Pinar A, Mansell A, Robinson MW, et al. The immune modulatory peptide FhHDM-1 secreted by the helminth Fasciola hepatica prevents NLRP3 inflammasome activation by inhibiting endolysosomal acidification in macrophages. FASEB J. 2017;31(1):85-95.

104. Kang J-M, Yoo WG, Lê HG, Lee J, Sohn W-M, Na B-K. Clonorchis sinensis MF6p/HDM (CsMF6p/HDM) induces pro-inflammatory immune response in RAW 264.7 macrophage cells via NF-κB-dependent MAPK pathways. Parasites & vectors. 2020;13(1):1-13.

105. Shiels J, Cwiklinski K, Alvarado R, Thivierge K, Cotton S, Santana BG, et al. Schistosoma mansoni immunomodulatory molecule Sm16/SPO-1/SmSLP is a member of the trematode-specific helminth defence molecules (HDMs). PLoS neglected tropical diseases. 2020;14(7):e0008470.

106. Aguayo V, Valdés Fernandez BN, Rodríguez-Valentín M, Ruiz-Jiménez C, Ramos-Benítez MJ, Méndez LB, et al. Fasciola hepatica GST downregulates NF-κB pathway effectors

and inflammatory cytokines while promoting survival in a mouse septic shock model. Scientific reports. 2019;9(1):1-16.

107. Martin I, Cabán-Hernández K, Figueroa-Santiago O, Espino AM. Fasciola hepatica fatty acid binding protein inhibits TLR4 activation and suppresses the inflammatory cytokines induced by lipopolysaccharide in vitro and in vivo. The Journal of Immunology. 2015;194(8):3924-36.

108. Figueroa-Santiago O, Espino AM. Fasciola hepatica fatty acid binding protein induces the alternative activation of human macrophages. Infection and immunity. 2014;82(12):5005-12.

109. Ramos-Benítez MJ, Ruiz-Jiménez C, Aguayo V, Espino AM. Recombinant Fasciola hepatica fatty acid binding protein suppresses toll-like receptor stimulation in response to multiple bacterial ligands. Scientific reports. 2017;7(1):1-14.

110. Sulaiman AA, Zolnierczyk K, Japa O, Owen JP, Maddison BC, Emes RD, et al. A trematode parasite derived growth factor binds and exerts influences on host immune functions via host cytokine receptor complexes. PLoS pathogens. 2016;12(11):e1005991.

111. Robinson MW, Alvarado R, To J, Hutchinson AT, Dowdell SN, Lund M, et al. A helminth cathelicidin-like protein suppresses antigen processing and presentation in macrophages via inhibition of lysosomal vATPase. FASEB J. 2012;26(11):4614-27.

112. Zhou L, Shi M, Zhao L, Lin Z, Tang Z, Sun H, et al. Clonorchis sinensis lysophospholipase A upregulates IL-25 expression in macrophages as a potential pathway to liver fibrosis. Parasites & vectors. 2017;10(1):1-10.

113. Pinlaor S, Tada-Oikawa S, Hiraku Y, Pinlaor P, Ma N, Sithithaworn P, et al. Opisthorchis viverrini antigen induces the expression of Toll-like receptor 2 in macrophage RAW cell line. International journal for parasitology. 2005;35(6):591-6.

114. Techasen A, Loilome W, Namwat N, Duenngai K, Cha'on U, Thanan R, et al. Opisthorchis viverrini-antigen induces expression of MARCKS during inflammation-associated cholangiocarcinogenesis. Parasitology international. 2012;61(1):140-4.

115. Hongsrichan N, Intuyod K, Pinlaor P, Khoontawad J, Yongvanit P, Wongkham C, et al. Cytokine/chemokine secretion and proteomic identification of upregulated annexin A1 from peripheral blood mononuclear cells cocultured with the liver fluke Opisthorchis viverrini. Infection and immunity. 2014;82(5):2135-47.

116. Mostafa MH, Sheweita S, O'Connor P. Relationship between schistosomiasis and bladder cancer. Clinical microbiology reviews. 1999;12(1):97-111.

117. Ray D, Nelson TA, Fu C-L, Patel S, Gong DN, Odegaard JI, et al. Transcriptional profiling of the bladder in urogenital schistosomiasis reveals pathways of inflammatory fibrosis and urothelial compromise. PLoS neglected tropical diseases. 2012;6(11):e1912.

118. Wu J, Hayes BW, Phoenix C, Macias GS, Miao Y, Choi HW, et al. A highly polarized TH2 bladder response to infection promotes epithelial repair at the expense of preventing new infections. Nature immunology. 2020;21(6):671-83.

119. Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, Allen JE. Helminth parasites–masters of regulation. Immunological reviews. 2004;201(1):89-116.

120. Motran CC, Silvane L, Chiapello LS, Theumer MG, Ambrosio LF, Volpini X, et al. Helminth infections: recognition and modulation of the immune response by innate immune cells. Frontiers in immunology. 2018;9:664.

121. Johnston CJ, McSorley HJ, Anderton SM, Wigmore SJ, Maizels RM. Helminths and immunological tolerance. Transplantation. 2014;97(2):127.

122. Behnke JM, Barnard CJ, Wakelin D. Understanding chronic nematode infections: evolutionary considerations, current hypotheses and the way forward. International journal for parasitology. 1992;22(7):861-907.

123. Bossaert K, Farnir F, Leclipteux T, Protz M, Lonneux J-F, Losson B. Humoral immune response in calves to single-dose, trickle and challenge infections with Fasciola hepatica. Veterinary parasitology. 2000;87(2-3):103-23.

124. Mazeri S, Rydevik G, Handel I, Bronsvoort BM, Sargison N. Estimation of the impact of Fasciola hepatica infection on time taken for UK beef cattle to reach slaughter weight. Scientific reports. 2017;7(1):1-15.

125. Tran N, Ricafrente A, To J, Lund M, Marques TM, Gama-Carvalho M, et al. Fasciola hepatica hijacks host macrophage miRNA machinery to modulate early innate immune responses. Scientific reports. 2021;11(1):1-11.

126. Pålsson-McDermott EM, O'Neill LA. Targeting immunometabolism as an anti-inflammatory strategy. Cell research. 2020;30(4):300-14.

127. Kaushik DK, Bhattacharya A, Mirzaei R, Rawji KS, Ahn Y, Rho JM, et al. Enhanced glycolytic metabolism supports transmigration of brain-infiltrating macrophages in multiple sclerosis. J Clin Invest. 2019;129(8):3277-92.

128. Zahoor I, Suhail H, Datta I, Ahmed ME, Poisson LM, Waters J, et al. Blood-based untargeted metabolomics in relapsing-remitting multiple sclerosis revealed the testable therapeutic target. Proc Natl Acad Sci U S A. 2022;119(25):e2123265119.

129. Davanso MR, Crisma AR, Braga TT, Masi LN, do Amaral CL, Leal VNC, et al. Macrophage inflammatory state in Type 1 diabetes: triggered by NLRP3/iNOS pathway and attenuated by docosahexaenoic acid. Clin Sci (Lond). 2021;135(1):19-34.

130. Huang SC, Everts B, Ivanova Y, O'Sullivan D, Nascimento M, Smith AM, et al. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. Nat Immunol. 2014;15(9):846-55.

131. Ip WKE, Hoshi N, Shouval DS, Snapper S, Medzhitov R. Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages. Science. 2017;356(6337):513-9.

132. He W, Heinz A, Jahn D, Hiller K. Complexity of macrophage metabolism in infection. Curr Opin Biotechnol. 2021;68:231-9.

133. Cortes-Selva D, Gibbs L, Maschek JA, Nascimento M, Van Ry T, Cox JE, et al. Metabolic reprogramming of the myeloid lineage by Schistosoma mansoni infection persists independently of antigen exposure. PLoS Pathog. 2021;17(1):e1009198.

134. Dalton JP, Robinson MW, Mulcahy G, O'Neill SM, Donnelly S. Immunomodulatory molecules of Fasciola hepatica: candidates for both vaccine and immunotherapeutic development. Veterinary parasitology. 2013;195(3-4):272-85.

135. Tanaka A. Characterisation of an immune-modulating peptide secreted by a helminth worm; PhD thesis, University of Technology Sydney, 2018.

136. Haneklaus M, O'Neill LA. NLRP3 at the interface of metabolism and inflammation. Immunological reviews. 2015;265(1):53-62.

137. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3(7):RESEARCH0034.

138. Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004;64(15):5245-50.

139. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnol Lett. 2004;26(6):509-15.

140. Tanaka A, To J, O'Brien B, Donnelly S, Lund M. Selection of reliable reference genes for the normalisation of gene expression levels following time course LPS stimulation of murine bone marrow derived macrophages. BMC Immunology. 2017;18:43.

141. Frey AJ, Feldman DR, Trefely S, Worth AJ, Basu SS, Snyder NW. LC-quadrupole/Orbitrap high-resolution mass spectrometry enables stable isotope-resolved simultaneous quantification and 13C-isotopic labeling of acyl-coenzyme A thioesters. Analytical and bioanalytical chemistry. 2016;408(13):3651-8.

142. Wise DR, Ward PS, Shay JE, Cross JR, Gruber JJ, Sachdeva UM, et al. Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability. Proc Natl Acad Sci U S A. 2011;108(49):19611-6.

143. Trefely S, Ashwell P, Snyder NW. FluxFix: automatic isotopologue normalization for metabolic tracer analysis. BMC bioinformatics. 2016;17(1):1-8.

144. Guo L, Worth AJ, Mesaros C, Snyder NW, Glickson JD, Blair IA. Diisopropylethylamine/hexafluoroisopropanol-mediated ion-pairing ultra-high-performance

liquid chromatography/mass spectrometry for phosphate and carboxylate metabolite analysis: utility for studying cellular metabolism. Rapid Commun Mass Spectrom. 2016;30(16):1835-45.

145. Jha AK, Huang SC-C, Sergushichev A, Lampropoulou V, Ivanova Y, Loginicheva E, et al. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. Immunity. 2015;42(3):419-30.

146. Metallo CM, Gameiro PA, Bell EL, Mattaini KR, Yang J, Hiller K, et al. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. Nature. 2012;481(7381):380-4.

147. Dominguez-Andres J, Netea MG. Long-term reprogramming of the innate immune system. J Leukoc Biol. 2019;105(2):329-38.

148. Wang Y, Li N, Zhang X, Horng T. Mitochondrial metabolism regulates macrophage biology. J Biol Chem. 2021;297(1):100904.

149. Almeida L, Everts B. Fa (c) t checking: How fatty acids shape metabolism and function of macrophages and dendritic cells. European Journal of Immunology. 2021;51(7):1628-40.

150. Batista-Gonzalez A, Vidal R, Criollo A, Carreño LJ. New insights on the role of lipid metabolism in the metabolic reprogramming of macrophages. Frontiers in immunology. 2020;10:2993.

151. Milanski M, Degasperi G, Coope A, Morari J, Denis R, Cintra DE, et al. Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. Journal of Neuroscience. 2009;29(2):359-70.

152. Rocha D, Caldas A, Oliveira L, Bressan J, Hermsdorff H. Saturated fatty acids trigger TLR4-mediated inflammatory response. Atherosclerosis. 2016;244:211-5.

153. Hu P, Han Z, Couvillon AD, Kaufman RJ, Exton JH. Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1α-mediated NF-κB activation and down-regulation of TRAF2 expression. Molecular and cellular biology. 2006;26(8):3071-84.

154. Pathinayake PS, Hsu AC-Y, Waters DW, Hansbro PM, Wood LG, Wark PA. Understanding the unfolded protein response in the pathogenesis of asthma. Frontiers in immunology. 2018;9.

155. Moutinho M, Nunes MJ, Rodrigues E. Cholesterol 24-hydroxylase: Brain cholesterol metabolism and beyond. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2016;1861(12):1911-20.

156. Park D, Han CZ, Elliott MR, Kinchen JM, Trampont PC, Das S, et al. Continued clearance of apoptotic cells critically depends on the phagocyte Ucp2 protein. Nature. 2011;477(7363):220-4.

157. Wen H, Gris D, Lei Y, Jha S, Zhang L, Huang MT-H, et al. Fatty acid–induced NLRP3-ASC inflammasome activation interferes with insulin signaling. Nature immunology. 2011;12(5):408-15.

158. Hall CJ, Boyle RH, Astin JW, Flores MV, Oehlers SH, Sanderson LE, et al. Immunoresponsive gene 1 augments bactericidal activity of macrophage-lineage cells by regulating β-oxidation-dependent mitochondrial ROS production. Cell metabolism. 2013;18(2):265-78.

159. Moon J-S, Nakahira K, Chung K-P, DeNicola GM, Koo MJ, Pabón MA, et al. NOX4-dependent fatty acid oxidation promotes NLRP3 inflammasome activation in macrophages. Nature medicine. 2016;22(9):1002-12.

160. Martinon F. Signaling by ROS drives inflammasome activation. European journal of immunology. 2010;40(3):616-9.

161. Zhang S, Weinberg S, DeBerge M, Gainullina A, Schipma M, Kinchen JM, et al. Efferocytosis fuels requirements of fatty acid oxidation and the electron transport chain to polarize macrophages for tissue repair. Cell metabolism. 2019;29(2):443-56. e5.

162. Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang X-Y, Pfeiffer HK, et al. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. Proceedings of the National Academy of Sciences. 2008;105(48):18782-7.

163. Wang X, Lin L, Lan B, Wang Y, Du L, Chen X, et al. IGF2R-initiated proton rechanneling dictates an anti-inflammatory property in macrophages. Sci Adv. 2020;6(48):eabb7389.

164. Bartel K, Pein H, Popper B, Schmitt S, Janaki-Raman S, Schulze A, et al. Connecting lysosomes and mitochondria - a novel role for lipid metabolism in cancer cell death. Cell Commun Signal. 2019;17(1):87.

165. Johmura Y, Yamanaka T, Omori S, Wang T-W, Sugiura Y, Matsumoto M, et al. Senolysis by glutaminolysis inhibition ameliorates various age-associated disorders. Science. 2021;371(6526):265-70.

166. Lee KM-C, Achuthan AA, De Souza DP, Lupancu TJ, Binger KJ, Lee MK, et al. Type I interferon antagonism of the JMJD3-IRF4 pathway modulates macrophage activation and polarization. Cell Reports. 2022;39(3):110719.

167. Arts RJ, Novakovic B, Ter Horst R, Carvalho A, Bekkering S, Lachmandas E, et al. Glutaminolysis and Fumarate Accumulation Integrate Immunometabolic and Epigenetic Programs in Trained Immunity. Cell Metab. 2016;24(6):807-19.

168. Liu P-S, Wang H, Li X, Chao T, Teav T, Christen S, et al. α-ketoglutarate orchestrates macrophage activation through metabolic and epigenetic reprogramming. Nature immunology. 2017;18(9):985.

169. Takeuch O, Akira S. Epigenetic control of macrophage polarization. European journal of immunology. 2011;41(9):2490-3.

170. Cieslar-Pobuda A, Ahrens TD, Caglayan S, Behringer S, Hannibal L, Staerk J. DNMT3B deficiency alters mitochondrial biogenesis and α-ketoglutarate levels in human embryonic stem cells. Stem Cells. 2020;38(11):1409-22.

171. Vats D, Mukundan L, Odegaard JI, Zhang L, Smith KL, Morel CR, et al. Oxidative metabolism and PGC-1beta attenuate macrophage-mediated inflammation. Cell Metab. 2006;4(1):13-24.

172. Griffiths HR, Gao D, Pararasa C. Redox regulation in metabolic programming and inflammation. Redox biology. 2017;12:50-7.

173. Versini M, Jeandel PY, Bashi T, Bizzaro G, Blank M, Shoenfeld Y. Unraveling the Hygiene Hypothesis of helminthes and autoimmunity: origins, pathophysiology, and clinical applications. BMC Med. 2015;13(1):81.

174. Rook GA. 99th Dahlem conference on infection, inflammation and chronic inflammatory disorders: Darwinian medicine and the 'hygiene'or 'old friends' hypothesis. Clinical & Experimental Immunology. 2010;160(1):70-9.

175. White MPJ, McManus CM, Maizels RM. Regulatory T-cells in helminth infection: induction, function and therapeutic potential. Immunology. 2020;160(3):248-60.

176. Ziegler T, Rausch S, Steinfelder S, Klotz C, Hepworth MR, Kühl AA, et al. A novel regulatory macrophage induced by a helminth molecule instructs IL-10 in CD4+ T cells and protects against mucosal inflammation. The Journal of Immunology. 2015:1401217.

177. Kang S, Park M-K, Park SK, Choi JH, Lee DI, Song SM, et al. Adoptive transfer of Trichinella spiralis-activated macrophages can ameliorate both Th1-and Th2-activated inflammation in murine models. Scientific reports. 2019;9(1):1-17.

178. Terrazas C, de Dios Ruiz-Rosado J, Amici SA, Jablonski KA, Martinez-Saucedo D, Webb LM, et al. Helminth-induced Ly6C(hi) monocyte-derived alternatively activated macrophages suppress experimental autoimmune encephalomyelitis. Sci Rep. 2017;7(1):40814.

179. Cunningham KT, Finlay CM, Mills KH. Helminth imprinting of hematopoietic stem cells sustains anti-inflammatory trained innate immunity that attenuates autoimmune disease. The Journal of Immunology. 2021;206(7):1618-30.

180. Rodríguez-Prados J-C, Través PG, Cuenca J, Rico D, Aragonés J, Martín-Sanz P, et al. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. The Journal of Immunology. 2010;185(1):605-14.

181. Soto-Heredero G, de las Heras MMG, Gabandé-Rodríguez E, Oller J, Mittelbrunn M. Glycolysis: a key player in the inflammatory response. The FEBS Journal. 2020.

182. Wang T, Liu H, Lian G, Zhang S-Y, Wang X, Jiang C. HIF1α-induced glycolysis metabolism is essential to the activation of inflammatory macrophages. Mediators of inflammation. 2017;2017.

183. Kim J-w, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell metabolism. 2006;3(3):177-85.

184. Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, et al. NF-κB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1α. Nature. 2008;453(7196):807-11.

185. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate is an inflammatory signal that induces IL-1[bgr] through HIF-1[agr]. Nature. 2013;496(7444):238-42.

186. Moon J-S, Hisata S, Park M-A, DeNicola GM, Ryter SW, Nakahira K, et al. mTORC1-induced HK1-dependent glycolysis regulates NLRP3 inflammasome activation. Cell reports. 2015;12(1):102-15.

187. Lampropoulou V, Sergushichev A, Bambouskova M, Nair S, Vincent EE, Loginicheva E, et al. Itaconate links inhibition of succinate dehydrogenase with macrophage metabolic remodeling and regulation of inflammation. Cell metabolism. 2016;24(1):158-66.

188. Millet P, Vachharajani V, McPhail L, Yoza B, McCall CE. GAPDH binding to TNF-α mRNA contributes to posttranscriptional repression in monocytes: a novel mechanism of communication between inflammation and metabolism. The Journal of Immunology. 2016;196(6):2541-51.

189. O'Connor RS, Guo L, Ghassemi S, Snyder NW, Worth AJ, Weng L, et al. The CPT1a inhibitor, etomoxir induces severe oxidative stress at commonly used concentrations. Sci Rep. 2018;8(1):6289.

190. Zdzisińska B, Żurek A, Kandefer-Szerszeń M. Alpha-ketoglutarate as a molecule with pleiotropic activity: well-known and novel possibilities of therapeutic use. Archivum immunologiae et therapiae experimentalis. 2017;65(1):21-36.

191. Cieslar-Pobuda A, Ahrens TD, Caglayan S, Behringer S, Hannibal L, Staerk J. DNMT3B deficiency alters mitochondrial biogenesis and alpha-ketoglutarate levels in human embryonic stem cells. Stem Cells. 2020;38(11):1409-22.

192. Kapetanovic R, Afroz SF, Ramnath D, Lawrence GM, Okada T, Curson JE, et al. Lipopolysaccharide promotes Drp1-dependent mitochondrial fission and associated inflammatory responses in macrophages. Immunol Cell Biol. 2020;98(7):528-39.

193. Gao F, Reynolds MB, Passalacqua KD, Sexton JZ, Abuaita BH, O'Riordan MXD. The Mitochondrial Fission Regulator DRP1 Controls Post-Transcriptional Regulation of TNF-alpha. Front Cell Infect Microbiol. 2020;10:593805.

194. Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirefah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. science. 2014;345(6204):1251086.

195. Riksen NP, Netea MG. Immunometabolic control of trained immunity. Molecular aspects of medicine. 2021;77:100897.

196. Pekowska A, Benoukraf T, Zacarias-Cabeza J, Belhocine M, Koch F, Holota H, et al. H3K4 tri-methylation provides an epigenetic signature of active enhancers. The EMBO journal. 2011;30(20):4198-210.

197. Ochando J, Mulder WJ, Madsen JC, Netea MG, Duivenvoorden R. Trained immunity—basic concepts and contributions to immunopathology. Nature Reviews Nephrology. 2023;19(1):23-37.

198. Ferrara AL, Liotti A, Pezone A, De Rosa V. Therapeutic opportunities to modulate immune tolerance through the metabolism-chromatin axis. Trends in Endocrinology & Metabolism. 2022.

199. Nieborak A, Schneider R. Metabolic intermediates – Cellular messengers talking to chromatin modifiers. Molecular Metabolism. 2018;14:39-52.

200. Netea MG, van der Meer JW. Trained immunity: an ancient way of remembering. Cell host & microbe. 2017;21(3):297-300.

201. van der Heijden CD, Keating ST, Groh L, Joosten LA, Netea MG, Riksen NP. Aldosterone induces trained immunity: the role of fatty acid synthesis. Cardiovascular Research. 2020;116(2):317-28.

202. Keating ST, Groh L, Thiem K, Bekkering S, Li Y, Matzaraki V, et al. Rewiring of glucose metabolism defines trained immunity induced by oxidized low-density lipoprotein. Journal of Molecular Medicine. 2020;98:819-31.

203. Bekkering S, Domínguez-Andrés J, Joosten LA, Riksen NP, Netea MG. Trained immunity: reprogramming innate immunity in health and disease. Annual review of immunology. 2021;39:667-93.

204. Amores-Iniesta J, Barberà-Cremades M, Martínez CM, Pons JA, Revilla-Nuin B, Martínez-Alarcón L, et al. Extracellular ATP activates the NLRP3 inflammasome and is an early danger signal of skin allograft rejection. Cell reports. 2017;21(12):3414-26.

205. Zhao H, Alam A, Soo AP, George AJ, Ma D. Ischemia-reperfusion injury reduces long term renal graft survival: mechanism and beyond. EBioMedicine. 2018;28:31-42.

206. Jing C, Castro-Dopico T, Richoz N, Tuong ZK, Ferdinand JR, Lok LS, et al. Macrophage metabolic reprogramming presents a therapeutic target in lupus nephritis. Proceedings of the National Academy of Sciences. 2020;117(26):15160-71.

207. Noz MP, Bekkering S, Groh L, Nielen TM, Lamfers EJ, Schlitzer A, et al. Reprogramming of bone marrow myeloid progenitor cells in patients with severe coronary artery disease. Elife. 2020;9:e60939.

208. Edgar L, Akbar N, Braithwaite AT, Krausgruber T, Gallart-Ayala H, Bailey J, et al. Hyperglycemia induces trained immunity in macrophages and their precursors and promotes atherosclerosis. Circulation. 2021;144(12):961-82.

209. Furman D, Campisi J, Verdin E, Carrera-Bastos P, Targ S, Franceschi C, et al. Chronic inflammation in the etiology of disease across the life span. Nature medicine. 2019;25(12):1822-32.

210. Hunter P. The inflammation theory of disease: The growing realization that chronic inflammation is crucial in many diseases opens new avenues for treatment. EMBO reports. 2012;13(11):968-70.

211. Doolan R, Putananickal N, Tritten L, Bouchery T. How to train your myeloid cells: a way forward for helminth vaccines? Frontiers in Immunology. 2023;14.

212. Tanaka A, Allam V, Simpson J, Tiberti N, Shiels J, To J, et al. The parasitic 68-mer peptide FhHDM-1 inhibits mixed granulocytic inflammation and airway hyperreactivity in experimental asthma. J Allergy Clin Immunol. 2018;141(6):2316-9.

213. Chen D, Thayer TC, Wen L, Wong FS. Mouse Models of Autoimmune Diabetes: The Nonobese Diabetic (NOD) Mouse. Methods Mol Biol. 2020;2128:87-92.

214. Sen P, Bhattacharyya S, Wallet M, Wong CP, Poligone B, Sen M, et al. NF-κB hyperactivation has differential effects on the APC function of nonobese diabetic mouse macrophages. The Journal of Immunology. 2003;170(4):1770-80.

215. Morse ZJ, Horwitz MS. Virus infection is an instigator of intestinal dysbiosis leading to type 1 diabetes. Frontiers in Immunology. 2021;12:4312.

216. Delogu LG, Deidda S, Delitala G, Manetti R. Infectious diseases and autoimmunity. The Journal of Infection in Developing Countries. 2011;5(10):679-87.

217. Lavine N, Ohayon A, Mahroum N. Renal autoimmunity: The role of bacterial and viral infections, an extensive review. Autoimmunity Reviews. 2022;21(5):103073.

218. Uno S, Imagawa A, Okita K, Sayama K, Moriwaki M, Iwahashi H, et al. Macrophages and dendritic cells infiltrating islets with or without beta cells produce tumour necrosis factor-α in patients with recent-onset type 1 diabetes. Diabetologia. 2007;50:596-601.

219. Jun H-S, Yoon C-S, Zbytnuik L, van Rooijen N, Yoon J-W. The Role of Macrophages in T Cell–mediated Autoimmune Diabetes in Nonobese Diabetic Mice. The Journal of Experimental Medicine. 1999;189(2):347.

220. Devaraj S, Glaser N, Griffen S, Wang-Polagruto J, Miguelino E, Jialal I. Increased monocytic activity and biomarkers of inflammation in patients with type 1 diabetes. Diabetes. 2006;55(3):774-9.

221. Bradshaw EM, Raddassi K, Elyaman W, Orban T, Gottlieb PA, Kent SC, et al. Monocytes from patients with type 1 diabetes spontaneously secrete proinflammatory cytokines inducing Th17 cells. The Journal of Immunology. 2009;183(7):4432-9.

222. Roep BO, Thomaidou S, van Tienhoven R, Zaldumbide A. Type 1 diabetes mellitus as a disease of the β-cell (do not blame the immune system?). Nature Reviews Endocrinology. 2021;17(3):150-61.

223. Cosentino C, Regazzi R. Crosstalk between macrophages and pancreatic β-cells in islet development, homeostasis and disease. International Journal of Molecular Sciences. 2021;22(4):1765.

224. Mullen Y. Development of the nonobese diabetic mouse and contribution of animal models for understanding type 1 diabetes. Pancreas. 2017;46(4):455.

225. Padgett LE, Burg AR, Lei W, Tse HM. Loss of NADPH oxidase–derived superoxide skews macrophage phenotypes to delay type 1 diabetes. Diabetes. 2015;64(3):937-46.

226. Filippi CM, von Herrath MG. Viral trigger for type 1 diabetes: pros and cons. Diabetes. 2008;57(11):2863.

227. Coppieters KT, Boettler T, von Herrath M. Virus infections in type 1 diabetes. Cold Spring Harbor perspectives in medicine. 2012;2(1):a007682.

228. Wu R, Mumtaz M, Maxwell AJ, Isaacs SR, Laiho JE, Rawlinson WD, et al. Respiratory infections and type 1 diabetes: Potential roles in pathogenesis. Reviews in Medical Virology. 2023;33(2):e2429.

229. Kendall EK, Olaker VR, Kaelber DC, Xu R, Davis PB. Association of SARS-CoV-2 infection with new-onset type 1 diabetes among pediatric patients from 2020 to 2021. JAMA Network Open. 2022;5(9):e2233014-e.

230. King C, Sarvetnick N. The incidence of type-1 diabetes in NOD mice is modulated by restricted flora not germ-free conditions. PloS one. 2011;6(2):e17049.

231. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, et al. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science. 2013;339(6123):1084-8.

232. Liang W, Enée E, Andre-Vallee C, Falcone M, Sun J, Diana J. Intestinal cathelicidin antimicrobial peptide shapes a protective neonatal gut microbiota against pancreatic autoimmunity. Gastroenterology. 2022;162(4):1288-302. e16.

233. Yurkovetskiy L, Burrows M, Khan AA, Graham L, Volchkov P, Becker L, et al. Gender bias in autoimmunity is influenced by microbiota. Immunity. 2013;39(2):400-12.

234. Vijay A, Valdes AM. Role of the gut microbiome in chronic diseases: A narrative review. European Journal of Clinical Nutrition. 2022;76(4):489-501.

235. Gomez A, Luckey D, Taneja V. The gut microbiome in autoimmunity: Sex matters. Clinical Immunology. 2015;159(2):154-62.

236. Yamamoto EA, Jørgensen TN. Relationships between vitamin D, gut microbiome, and systemic autoimmunity. Frontiers in immunology. 2020;10:3141.

237. Dao MC, Everard A, Aron-Wisnewsky J, Sokolovska N, Prifti E, Verger EO, et al. Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. Gut. 2016;65(3):426-36.

238. Chelakkot C, Ghim J, Ryu SH. Mechanisms regulating intestinal barrier integrity and its pathological implications. Experimental & Molecular Medicine. 2018;50(8):1-9.

239. Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, et al. Toward defining the autoimmune microbiome for type 1 diabetes. The ISME journal. 2011;5(1):82-91.

240. Murri M, Leiva I, Gomez-Zumaquero JM, Tinahones FJ, Cardona F, Soriguer F, et al. Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. BMC medicine. 2013;11:1-12.

241. De Goffau MC, Luopajärvi K, Knip M, Ilonen J, Ruohtula T, Härkönen T, et al. Fecal microbiota composition differs between children with β-cell autoimmunity and those without. Diabetes. 2013;62(4):1238-44.

242. Loke P, Lim Y. Helminths and the microbiota: parts of the hygiene hypothesis. Parasite immunology. 2015;37(6):314-23.

243. Llinás-Caballero K, Caraballo L. Helminths and bacterial microbiota: the interactions of two of humans'"old friends". International Journal of Molecular Sciences. 2022;23(21):13358.

244. Rausch S, Midha A, Kuhring M, Affinass N, Radonic A, Kühl AA, et al. Parasitic nematodes exert antimicrobial activity and benefit from microbiota-driven support for host immune regulation. Frontiers in immunology. 2018;9:2282.

245. Pillai A, Ueno S, Zhang H, Lee JM, Kato Y. Cecropin P1 and novel nematode cecropins: a bacteria-inducible antimicrobial peptide family in the nematode Ascaris suum. Biochemical Journal. 2005;390(1):207-14.

246. Jenkins TP, Pritchard DI, Tanasescu R, Telford G, Papaiakovou M, Scotti R, et al. Experimental infection with the hookworm, Necator americanus, is associated with stable gut microbial diversity in human volunteers with relapsing multiple sclerosis. BMC Biol. 2021;19(1):74.

247. Shute A, Callejas BE, Li S, Wang A, Jayme TS, Ohland C, et al. Cooperation between host immunity and the gut bacteria is essential for helminth-evoked suppression of colitis. Microbiome. 2021;9(1):186.

248. Osborne LC, Monticelli LA, Nice TJ, Sutherland TE, Siracusa MC, Hepworth MR, et al. Virus-helminth coinfection reveals a microbiota-independent mechanism of immunomodulation. Science. 2014;345(6196):578-82.

249. Quinn SM, Cunningham K, Raverdeau M, Walsh RJ, Curham L, Malara A, et al. Anti-inflammatory trained immunity mediated by helminth products attenuates the induction of T cell-mediated autoimmune disease. Frontiers in Immunology. 2019;10:1109.

250. Quintin J, Saeed S, Martens JH, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. Cell host & microbe. 2012;12(2):223-32.

251. Berglind D, Müller P, Willmer M, Sinha I, Tynelius P, Näslund E, et al. Differential methylation in inflammation and type 2 diabetes genes in siblings born before and after maternal bariatric surgery. Obesity. 2016;24(1):250-61.

252. Nakano K, Whitaker JW, Boyle DL, Wang W, Firestein GS. DNA methylome signature in rheumatoid arthritis. Annals of the rheumatic diseases. 2013;72(1):110-7.

253. Foster SL, Hargreaves DC, Medzhitov R. Gene-specific control of inflammation by TLR-induced chromatin modifications. Nature. 2007;447(7147):972-8.

254. Barski A, Cuddapah S, Cui K, Roh T-Y, Schones DE, Wang Z, et al. High-resolution profiling of histone methylations in the human genome. Cell. 2007;129(4):823-37.

255. Kapellos TS, Iqbal AJ. Epigenetic control of macrophage polarisation and soluble mediator gene expression during inflammation. Mediators of inflammation. 2016;2016.

256. Hoeksema MA, Shen Z, Holtman IR, Zheng A, Spann NJ, Cobo I, et al. Mechanisms underlying divergent responses of genetically distinct macrophages to IL-4. Science advances. 2021;7(25):eabf9808.

257. Afroz SF, Raven KD, Lawrence GM, Kapetanovic R, Schroder K, Sweet MJ. Mitochondrial dynamics in macrophages: divide to conquer or unite to survive? Biochemical Society Transactions. 2023;51(1):41-56.

258. Mei Nh, Guo Sm, Zhou Q, Zhang Yr, Liu Xz, Yin Y, et al. H3K4 Methylation Promotes Expression of Mitochondrial Dynamics Regulators to Ensure Oocyte Quality in Mice. Advanced Science. 2023;10(12):2204794.

259. Donnelly N, Gorman AM, Gupta S, Samali A. The eIF2α kinases: their structures and functions. Cellular and molecular life sciences. 2013;70:3493-511.

260. Chatterjee S, Chakrabarty Y, Banerjee S, Ghosh S, Bhattacharyya SN. Mitochondria control mTORC1 activity-linked compartmentalization of eIF4E to regulate extracellular export of microRNAs. J Cell Sci. 2020;133(24).

261. Li F, Sun H, Li Y, Bai X, Dong X, Zhao N, et al. High expression of eIF4E regulates immune cell infiltration and leads to poor prognosis in breast cancer. 2021.

262. Bao Y, Wu X, Chen J, Hu X, Zeng F, Cheng J, et al. Brd4 modulates the innate immune response through Mnk2–eIF4E pathway-dependent translational control of IκBα. Proceedings of the National Academy of Sciences. 2017;114(20):E3993-E4001.

263. Wan Y, Xiao H, Affolter J, Kim TW, Bulek K, Chaudhuri S, et al. Interleukin-1 receptor-associated kinase 2 is critical for lipopolysaccharide-mediated post-transcriptional control. Journal of Biological Chemistry. 2009;284(16):10367-75.

264. Roth GA, Abate D, Abate KH, Abay SM, Abbafati C, Abbasi N, et al. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017. The Lancet. 2018;392(10159):1736-88.

265. McInnes IB, Gravallese EM. Immune-mediated inflammatory disease therapeutics: past, present and future. Nature Reviews Immunology. 2021;21(10):680-6.

266. Babu S, Nutman TB. Immune responses to helminth infection. Clinical Immunology: Elsevier; 2019. p. 437-47. e1.

267. Ardura JA, Rackov G, Izquierdo E, Alonso V, Gortazar AR, Escribese MM. Targeting Macrophages: Friends or Foes in Disease? Front Pharmacol. 2019;10:1255.

268. Chen F, El-Naccache DW, Ponessa JJ, Lemenze A, Espinosa V, Wu W, et al. Helminth resistance is mediated by differential activation of recruited monocyte-derived alveolar macrophages and arginine depletion. Cell reports. 2022;38(2).

269. Yousaf H, Khan MIU, Ali I, Munir MU, Lee KY. Emerging role of macrophages in non-infectious diseases: An update. Biomedicine & Pharmacotherapy. 2023;161:114426.

270. Alvarado R, O'Brien B, Tanaka A, Dalton JP, Donnelly S. A parasitic helminth-derived peptide that targets the macrophage lysosome is a novel therapeutic option for autoimmune disease. Immunobiology. 2015;220(2):262-9.

271. Deus CM, Yambire KF, Oliveira PJ, Raimundo N. Mitochondria–lysosome crosstalk: from physiology to neurodegeneration. Trends in molecular medicine. 2020;26(1):71-88.

272. Van den Bossche J, O'Neill LA, Menon D. Macrophage Immunometabolism: Where Are We (Going)? Trends Immunol. 2017;38(6):395-406.

273. Cheng S-C, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, et al. mTOR- and HIF-1α–mediated aerobic glycolysis as metabolic basis for trained immunity. Science. 2014;345(6204):1250684.

274. Funes SC, Rios M, Fernández-Fierro A, Di Genaro MS, Kalergis AM. Trained immunity contribution to autoimmune and inflammatory disorders. Frontiers in Immunology. 2022;13:868343.

275. Mishra SR, Mahapatra KK, Behera BP, Patra S, Bhol CS, Panigrahi DP, et al. Mitochondrial dysfunction as a driver of NLRP3 inflammasome activation and its modulation through mitophagy for potential therapeutics. The International Journal of Biochemistry & Cell Biology. 2021;136:106013.

276. Liu P-S, Ho P-C. Mitochondria: A master regulator in macrophage and T cell immunity. Mitochondrion. 2018;41:45-50.

277. Wang X, Jiang W, Yan Y, Gong T, Han J, Tian Z, et al. RNA viruses promote activation of the NLRP3 inflammasome through a RIP1-RIP3-DRP1 signaling pathway. Nature immunology. 2014;15(12):1126-33.

278. Ichinohe T, Yamazaki T, Koshiba T, Yanagi Y. Mitochondrial protein mitofusin 2 is required for NLRP3 inflammasome activation after RNA virus infection. Proceedings of the National Academy of Sciences. 2013;110(44):17963-8.

279. Mota EA, Patrocínio ABd, Rodrigues V, da Silva JS, Pereira VC, Guerra-Sa R. Epigenetic and parasitological parameters are modulated in EBi3-/-mice infected with Schistosoma mansoni. PLoS Neglected Tropical Diseases. 2020;14(2):e0008080.

280. Mei X-F, Shi W, Zhang Y-Y, Zhu B, Wang Y-R, Hou L-J, et al. DNA methylation and hydroxymethylation profiles reveal possible role of highly methylated TLR signaling on Fasciola gigantica excretory/secretory products (FgESPs) modulation of buffalo dendritic cells. Parasites & vectors. 2019;12:1-13.

281. Corbet M, Pineda MA, Yang K, Tarafdar A, McGrath S, Nakagawa R, et al. Suppression of inflammatory arthritis by the parasitic worm product ES-62 is associated with epigenetic changes in synovial fibroblasts. PLoS Pathogens. 2021;17(11):e1010069.

282. DiNardo AR, Nishiguchi T, Mace EM, Rajapakshe K, Mtetwa G, Kay A, et al. Schistosomiasis induces persistent DNA methylation and tuberculosis-specific immune changes. The Journal of Immunology. 2018;201(1):124-33.

283. Klar K, Perchermeier S, Bhattacharjee S, Harb H, Adler T, Istvanffy R, et al. Chronic schistosomiasis during pregnancy epigenetically reprograms T-cell differentiation in offspring of infected mothers. European journal of immunology. 2017;47(5):841-7.

284. Dewals BG, Layland LE, Prazeres da Costa C, Horsnell WG. Maternal helminth infections and the shaping of offspring immunity. Parasite Immunology. 2019;41(3):e12599.

285. Jensen DM, Hendricks KV, Mason AT, Tessem JS. Good cop, bad cop: the opposing effects of macrophage activation state on maintaining or damaging functional β-cell mass. Metabolites. 2020;10(12):485.

286. Yang S, Zhao M, Jia S. Macrophage: Key player in the pathogenesis of autoimmune diseases. Frontiers in Immunology. 2023;14:1080310.

287. Girodet P-O, Nguyen D, Mancini JD, Hundal M, Zhou X, Israel E, et al. Alternative macrophage activation is increased in asthma. American journal of respiratory cell and molecular biology. 2016;55(4):467-75.

288. Goretzki A, Zimmermann J, Rainer H, Lin Y-J, Schülke S. Immune metabolism in TH2 responses: New opportunities to improve allergy treatment—Disease-Specific Findings (Part 1). Current Allergy and Asthma Reports. 2023;23(1):29-40.

289. Lechner A, Henkel FD, Hartung F, Bohnacker S, Alessandrini F, Gubernatorova EO, et al. Macrophages acquire a TNF-dependent inflammatory memory in allergic asthma. Journal of Allergy and Clinical Immunology. 2022;149(6):2078-90.

290. Fransson J, Bachelin C, Deknuydt F, Ichou F, Guillot-Noël L, Ponnaiah M, et al. Dysregulated functional and metabolic response in multiple sclerosis patient macrophages correlate with a more inflammatory state, reminiscent of trained immunity. bioRxiv. 2021:2021.01. 13.426327.

291. Sou K, Goins B, Takeoka S, Tsuchida E, Phillips WT. Selective uptake of surface-modified phospholipid vesicles by bone marrow macrophages in vivo. Biomaterials. 2007;28(16):2655-66.

292. Rother N, Yanginlar C, Lindeboom RG, Bekkering S, van Leent MM, Buijsers B, et al. Hydroxychloroquine inhibits the trained innate immune response to interferons. Cell Reports Medicine. 2020;1(9).

293. Thivierge K, Cotton S, Schaefer DA, Riggs MW, To J, Lund ME, et al. Cathelicidin-like helminth defence molecules (HDMs): absence of cytotoxic, anti-microbial and anti-protozoan activities imply a specific adaptation to immune modulation. PLoS neglected tropical diseases. 2013;7(7):e2307.

294. Was H, Barszcz K, Czarnecka J, Kowalczyk A, Bernas T, Uzarowska E, et al. Bafilomycin A1 triggers proliferative potential of senescent cancer cells in vitro and in NOD/SCID mice. Oncotarget. 2017;8(6):9303.

295. Jamshidzadeh A, Niknahad H, Kashafi H. Cytotoxicity of chloroquine in isolated rat hepatocytes. Journal of Applied Toxicology: An International Journal. 2007;27(4):322-6.