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BSc (Hons) Biomedical Science

An Investigation into the Role of PPARγ and IL-6 in Exercise-Induced Alterations in Monocyte Gene Expression

February 2014

A thesis submitted in partial fulfilment of the requirements of Cardiff Metropolitan University for the degree of Doctor of Philosophy (PhD) by research in the discipline of Biomedical Science

Director of studies: Dr Andrew Thomas
Supervisors: Dr Richard Webb and Professor Keith Morris
(Cardiff School of Health Sciences)

The research contained within this thesis was undertaken under the auspices of Cardiff Metropolitan University Cardiff School of Health Sciences
Declaration

Statement 1

This work has not previously been accepted in substance for any other degree and is not currently submitted in candidature for any degree.

Signed ……………………………………………………………………… (Candidate)

Date ………………………

Statement 2

This thesis is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references.

Signed ……………………………………………………………………… (Candidate)

Date ………………………

Statement 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

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Date ………………………
Abstract

The pro-inflammatory M1 macrophage phenotype is linked to insulin resistance, T2D and associated cardiovascular co-morbidities, whereas M2 macrophages regulate the M1 inflammatory response and are, thus, believed to be preventative of these diseases. Exercise may induce markers of M2 polarisation and downregulate M1 markers in mononuclear cells but this has not yet been demonstrated in macrophage precursors, monocytes. Thus, the present research aimed to investigate the effects of exercise on M1/M2 marker expression in monocytes and to elucidate potential mechanisms for any observed changes.

Participation in exercise was found to beneficially alter T2D and CVD risk factors, including insulin sensitivity, in high-risk females. Exercise participation was also shown to induce markers of M2 polarisation and reduce expression of the M1 marker, MCP-1, in isolated monocytes. Additionally, a ‘key regulator’ of M2 polarisation, PPARγ was upregulated in monocytes and PPARγ activating properties were elevated in serum samples obtained immediately after exercise participation. Finally, IL-6 was also found to be upregulated immediately after participation in exercise. In THP-1 monocytes, PPARγ was shown to have no effect on M1/M2 marker expression. However, IL-6 was found to downregulate expression of MCP-1, in human monocytes co-cultured with lymphocytes.

In conclusion, PPARγ activation does not appear to play a role in the upregulation of M2 markers observed in monocytes following exercise participation. Instead, IL-6 generation may contribute to the impact of exercise on monocyte/macrophage phenotype. It is likely, however, that other exercise-associated factors may also contribute to the observed elevations in monocyte M2 marker expression following exercise participation but these are yet to be elucidated. Nonetheless, this research provides evidence that participation in an exercise intervention, such as the one administered in this study, may impact upon monocyte-macrophage polarisation towards the M2 phenotype, and that this may contribute to the improvements in insulin sensitivity and T2D/CVD risk which support prescription of physical activity in the prevention and management of T2D.
This PhD thesis is dedicated to Dad;

Thank you for being a great inspiration and thank you for reading it.

I’m glad I made you proud.
Acknowledgements

I would firstly like to acknowledge my supervisors, Dr Andrew Thomas, Dr Richard Webb and Professor Keith Morris – the support, guidance and knowledge that you each have given me has enabled me to progress and develop as a researcher. Also, the continual encouragement I have received has aided me throughout this PhD and for this, I am extremely grateful.

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Furthermore, my appreciation extends to Maninder Ahluwalia, Laura Watkeys, Hannah Donovan, Gareth Walters, Paul Jones, and the laboratory technicians who have supported me during my laboratory work. Additionally, I would like to thank Professor Marian Ludgate and Dr Lei Zhang (Cardiff University) for their guidance during our collaboration.

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I would like to thank my family and friends for your endless support and words of encouragement throughout the past few years. You have listened to me when I’ve needed you to, you have left me alone when necessary and you have brought joy to some very stressful periods. Thank you.

Finally, I would like to thank Dr Richard Metcalfe for showing up at the perfect time, for being a true support and for making it all worthwhile. If I could go back, would I choose to do it all again? 100% yes.
Publications, Posters and Oral Conference Contributions

PUBLICATIONS:


ORAL PRESENTATIONS:


Ruffino, J.S. (2013). *The Role of Exercise in Priming Human Monocytes into Anti-inflammatory M2 Macrophages*. Oral presentation (Invited Speaker) at: Wales Heart Research Institute, Cardiff University, February 25 2013, Cardiff, UK.

POSTERS:


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Abbreviations

#

13-oxoODE  13-OXO-9Z,11E-octadecadienoic acid
15d-PGJ2  15-deoxy-Δ12,14-prostaglandin J2
15-keto-PGE2  15-keto-prostaglandin E2
15-LOX  15-lipoxygenase
ΔRn  Delta Rn

\[\text{Abbreviations}\]

A

ABCG1  ATP-binding cassette transporter G1
ABIN3  A20-binding inhibitor of NF-κB activation 3
ANOVA  Analysis of variance
AP-1  Activator protein-1
ATM  Adipose tissue macrophages

B

BMI  Body mass index
BSA  Bovine serum albumin

C

C/EBP  CCAAT-enhancer binding proteins
cAMP  Cyclic adenosine monophosphate
CCL  CC chemokine ligand
CCR2  MCP-1 receptor
cdk  Cyclin dependent kinase
cDNA  Complimentary deoxyribonucleic acid
CREB  cAMP responsive element binding
CRP  C-reactive protein
CSF2Rα  Colony stimulating factor 2 receptor alpha
Ct  Cycle threshold
CVD  Cardiovascular disease
CX3CR1  Fractalkine receptor
CXCL  CXC chemokine ligand
CXCL10  C-X-C motif chemokine 10

D

DBD  DNA-binding domain
DMEM  Dulbecco’s Modified Eagle Medium
DMSO  Dimethyl Sulfoxide
dNTP  Deoxyribonucleotide
DPP  Diabetes Prevention Program
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>DPS</td>
<td>Diabetes Prevention Study</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DUSP1</td>
<td>Dual specificity phosphatase</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>F</td>
<td>Forward</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter-4</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen synthesis kinase-3</td>
</tr>
<tr>
<td>GUSB</td>
<td>Glucuronidase β</td>
</tr>
<tr>
<td>H3K27</td>
<td>Histone 3 Lys27</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HEK-293T</td>
<td>Human embryonic kidney cells</td>
</tr>
<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>HODE</td>
<td>Hydoxy-octadecadienoic acid</td>
</tr>
<tr>
<td>HOMA1</td>
<td>Homeostasis model of assessment</td>
</tr>
<tr>
<td>HOMA2</td>
<td>Updated homeostasis model assessment</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HRmax</td>
<td>Maximum heart rate</td>
</tr>
<tr>
<td>HTA</td>
<td>Human tissue act</td>
</tr>
<tr>
<td>I0</td>
<td>Fasting insulin</td>
</tr>
<tr>
<td>IC</td>
<td>Immune complex</td>
</tr>
<tr>
<td>IFN-β</td>
<td>Interferon-beta</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IKKβ</td>
<td>Inhibitor of kappa beta-kinase beta</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-4Rα</td>
<td>IL-4 receptor alpha-chain</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>iNOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>IPAQ</td>
<td>International physical activity questionnaire</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory transcription factor</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>ISI</td>
<td>Insulin sensitivity index</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of NF-κB</td>
</tr>
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**J**

<table>
<thead>
<tr>
<th>JAK</th>
<th>Janus kinase</th>
</tr>
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<tbody>
<tr>
<td>JMJD3</td>
<td>Jumonji domain containing-3</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
</tbody>
</table>

**K**

| KLF4   | Kruppel-like factor 4 |

**L**

<table>
<thead>
<tr>
<th>LAR II</th>
<th>Luciferase Assay Reagent II</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>LDS</td>
<td>Lithium dodecyl sulphate</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukocyte inhibitory factor</td>
</tr>
<tr>
<td>LNO₂</td>
<td>Nitrolinoleic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
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**M**

<table>
<thead>
<tr>
<th>MAPK</th>
<th>Mitogen-activated protein kinase</th>
</tr>
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<tbody>
<tr>
<td>MDM</td>
<td>Monocyte-derived macrophage</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>MET</td>
<td>Metabolic equivalent</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage inflammatory protein -1 alpha</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propane sulphonic acid</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene (88)</td>
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**N**

<table>
<thead>
<tr>
<th>NCBI</th>
<th>National Center for Biotechnology Information</th>
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<tbody>
<tr>
<td>N-CoR</td>
<td>Nuclear receptor co-repressor</td>
</tr>
</tbody>
</table>
**NF-κB** | Nuclear factor-kappa beta  
**NO** | Nitric oxide  
**NTC** | Non template control  

**O**

| OA-NO$_2$ | Nitrol-oleicacid  
| OD | Optical density  
| ONPG | o-Nitrophynyl-beta-D-galactopyranosidase  
| oxLDL | Oxidised low density lipoprotein  

**P**

| PBMC | Peripheral blood mononuclear cells  
| PBS | Phosphate buffered saline  
| PCR | Polymerase chain reaction  
| PGC-1 | PPARg co-activator  
| PI3K | Phosphoinositide 3-kinase  
| PMA | Phorbol 12-myristate 13-acetate  
| PPAR | Peroxisome proliferator-activated receptor  
| PPARγ1 | Peroxisome proliferator-activated receptor, isoform 1  
| PPARγ2 | Peroxisome proliferator-activated receptor, isoform 2  
| PPRE | Peroxisome proliferator response element  
| P-STAT-6 | Phospho -STAT-6  

**R**

| R | Reverse  
| Relm-α | Resistin like molecule-alpha  
| RIPA | Radio-Immunoprecipitation Assay  
| RLU | Relative light units  
| RNA | Ribonucleic acid  
| RNI | Reactive nitrogen intermediates  
| ROS | Reactive oxygen species  
| RPM | Revolutions per minute  
| RSG | Rosiglitazon  
| RT | Room temperature  
| RT-PCR | Reverse transcription polymerase chain reaction  
| RXR | Retinoid X receptor  

**S**

| S273 | Serine 273  
| SD | Standard deviation  
| SDS | Sodium dodecyl sulfate  
| SEM | Standard error of the mean


<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
</tr>
<tr>
<td>SR</td>
<td>Scavenger receptor</td>
</tr>
<tr>
<td>SR-1</td>
<td>Steroid receptor co-activator-1</td>
</tr>
<tr>
<td>SREC</td>
<td>School research ethics committee</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>sTNF-R</td>
<td>Soluble TNF-α receptor</td>
</tr>
<tr>
<td>SUMO-R</td>
<td>Small ubiquitin-like modifier</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 Diabetes</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumour associated macrophages</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline containing 0.1% Tween-20</td>
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<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TG</td>
<td>Fasting triglycerides</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
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<tr>
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<td>Transthyretinase 2</td>
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<td>Th</td>
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<td>THP-1 macrophages</td>
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<td>THP-1 mon</td>
<td>THP-1 monocytes</td>
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<td>Toll-like receptor</td>
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<td>T_m</td>
<td>Melting temperature</td>
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<td>Tumour necrosis factor alpha</td>
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<td>Regulatory T cells</td>
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<tr>
<td>TZD</td>
<td>Thiazolidinediones</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activation sequence</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VO_{2max}</td>
<td>Maximal oxygen consumption</td>
</tr>
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<td>xg</td>
<td>Times gravity</td>
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CHAPTER 1

General Introduction

1.1. An Introduction to Inflammation in the Pathology of Insulin Resistance

Inflammation is an integral defence process required for maintaining health and preventing disease. During an inflammatory response, cellular and molecular mediators work together to ensure that potential threats to the body, be they internal or external, are adequately neutralised and cleared in order to prevent damage and restore health (Dahlback, 2012). After an inflammatory response, to injury or an infection *per se*, inflammation normally returns to homeostatic levels (Roy *et al.*, 2012). However, in some circumstances chronic, low grade inflammation may persist, having implications in a range of pathological conditions such as insulin resistance, Type 2 diabetes (T2D), cardiovascular disease (CVD), cancer, arthritis and Alzheimer’s disease (Grivennikov *et al.*, 2010, Kiecolt-Glaser *et al.*, 2010).

Of particular importance to this thesis is the link between inflammation and insulin resistance and how physical activity may influence this interaction. This introduction will review the role of inflammation in the development of insulin resistance and associated T2D and its cardiovascular complications. A particular emphasis will be placed on the importance of monocytes and macrophages in this process; identifying which of these cells’ subsets are involved in insulin resistance and discussing potential interventions which may beneficially alter monocyte/macrophage phenotype, so as to prevent insulin resistance and T2D. Of these interventions, a focus will be placed on the health benefits of physical activity, specifically highlighting the impact of physical activity on monocyte/macrophage-derived inflammation and on the development of insulin resistance and T2D.
1.2. The Link between Inflammation and Insulin Resistance

Since insulin enables glucose uptake into cells, insulin resistance ultimately perturbs glucose metabolism and often predates the onset of diabetes (Olefsky and Glass, 2010, Donath and Shoelson, 2011). Diabetes occurs when the body is unable to produce or react to insulin sufficiently, resulting in hyperglycaemia. It is the fourth most common noncommunicable cause of death worldwide; mortality often resulting from diabetic complications, particularly CVD (Morrish et al., 2001, American Diabetes, 2013, Hunter and Reddy, 2013).

The link between inflammation and diabetes was first suggested in 1876, when Ebstein demonstrated that salicylates, a group of anti-inflammatory drugs, were able to eliminate glycosuria in patients with diabetes mellitus. Recent studies have also found that anti-inflammatory agents, such as aspirin and salsalates, promote metabolic improvements in diabetic subjects (Hundal et al., 2002, Chai et al., 2011, Goldfine et al., 2013). Hence, it is now evident that impairments in insulin secretion and/or sensitivity are associated with a heightened pro-inflammatory status (reviewed in Goldfine et al., 2013). In fact, some have found that chronically elevated levels of inflammatory mediators (interleukin- (IL-) 1β, IL-6 and C-reactive protein (CRP)) may be useful in predicting T2D risk (Pradhan et al., 2001, Spranger et al., 2003, Donath and Shoelson, 2011). Concordantly, many antidiabetic interventions have been shown to exert some of their beneficial effects by advantageously altering inflammatory status. These interventions include therapeutic agents, such as metformin and thiazolidinediones (TZDs), and lifestyle changes, such as weight loss and increased physical activity (Goldfine et al., 2013).
1.3. Inflammation in the Disruption of Insulin Signalling

The insulin receptor belongs to a subfamily of receptor tyrosine kinases. It is composed of α and β subunits. In health, insulin binds to the α subunit, activating the receptor’s intrinsic tyrosine kinase (IRK), allowing autophosphorylation of the receptor by the β subunit (Boura-Halfon and Zick, 2009, Rains and Jain, 2011). Subsequently, the activated insulin receptor directly phosphorylates tyrosine residues on the insulin receptor substrates (IRSs), of which there are four (IRS-1-4) and of which two (IRS-1 and IRS-2) participate in glucose transport (Rains and Jain, 2011). IRS-associated kinases (PI3-K, PDK-1, PKB/Akt and PKC) drive insulin’s metabolic actions, such as translocation of the glucose transporter 4 (GLUT4) to skeletal muscle cell membrane, glycogen synthesis via phosphorylation of glycogen synthesis kinase 3 (GSK-3) and increased lipogenesis due to up-regulation of the fatty acid synthase gene (Rains and Jain, 2011, Dishman et al., 2013).

Certain inflammatory mediators, such as free fatty acids (FFAs), glycerol, hormones and pro-inflammatory cytokines, are thought to disrupt the normal signalling of insulin (Gustafson, 2010, Boden, 2011). Many pro-inflammatory cytokines, including IL-1β, IL-6 and tumour necrosis factor α (TNFα), are known to activate two key inflammatory pathways; the inhibitor of κB kinase-β (IKKβ) pathway and the Jun N-terminal kinase (JNK) pathway. These kinases interfere with insulin signalling by phosphorylating serine/threonine residues, on both IRS-1 and IRS-2, the insulin receptor and, possibly, other insulin-signalling molecules. The alternative phosphorylation of these insulin-signalling molecules alters their structure, affecting their molecular interactions and function (Boura-Halfon and Zick, 2009, Olefsky and Glass, 2010).
To intensify matters, nuclear factor-κB (NF-κB), is indirectly activated by IKKβ. NF-κB is activated when IKK phosphorylates the inhibitor of NF-κB (IκB), resulting in its degradation (Liu et al., 2012). This allows transport of NF-κB from the cytoplasm into the nucleus, where it acts as a transcription factor, driving the production of a plethora of pro-inflammatory mediators (Holt et al., 2011). Many of the cytokines produced as a result of NF-κB activation, such as TNF and IL-1β, actually activate NF-κB and JNK (Donath and Shoelson, 2011). Similarly, JNK targets activator protein-1 (AP-1), a transcription factor which also drives the production of pro-inflammatory mediators (Johnson and Olefsky, 2013). Thus, activation of IKKβ and JNK not only impairs insulin signalling through phosphorylation of insulin signalling molecules, but also amplifies the chronic inflammation which often underlies insulin resistance.

Accordingly, individuals with insulin resistance have been shown to have upregulation of IKKβ and JNK, in a range of tissues (Olefsky and Glass, 2010, Harford et al., 2011). Furthermore, depletion of IKKβ and JNK (particularly JNK1) has been demonstrated to be protective against insulin resistance (Hirosumi et al., 2002, Kalupahana et al., 2012). Interestingly, Arkan et al. (2005) found that the effect of IKKβ on insulin resistance is dependent on the tissue that it is expressed in; when mice lacked hepatic IKKβ, insulin sensitivity was only retained in the liver after mice were fed on a high fat diet (HFD), whilst insulin resistance developed in the muscle and fat tissue. Conversely, when myeloid cells lacked IKKβ, systemic insulin sensitivity was maintained. Similarly, JNK deficient macrophages were found to protect mice from HFD induced insulin resistance, despite developing an obese status comparable to wild type mice (Han et al., 2013).
In accordance, macrophages are said to be the main effector cells in the inflammation which drives insulin resistance (reviewed in Osborn and Olefsky, 2012). The macrophage’s existence in multiple tissues (including insulin-responsive tissues such as adipose tissue and the liver) and their ability to efficiently respond to stimuli in their surrounding environment may provide a rationale for the extent to which they contribute to local and systemic inflammation and associated insulin resistance (Osborn and Olefsky, 2012). Hence, although other cells may contribute to the inflammation which is said to be associated with insulin resistance, the main focus of the present thesis will be placed on macrophages and their monocyte precursors.

1.4. Monocytes; Classification and Inflammatory Role

Monocytes have long been considered as a reservoir of cells for the replenishment of tissue macrophages. Monocytes express adhesion and chemokine receptors, allowing them to migrate into tissue and differentiate into macrophages, which are renowned for their pronounced capabilities as immune effector cells (Geissmann et al., 2010). It is now known, however, that monocytes themselves play specific roles within the immune system, independent of their differentiation into macrophages (Ziegler-Heitbrock et al., 2010).

1.4.1. Monocyte Function and Classification

It is only in recent years that monocytes, as cells in their own right, have been appreciated for their contribution to homeostasis, immunity and tissue repair. As aforementioned, previous to this, the literature often suggested that the role of these cells was merely to serve as macrophage precursors (Robbins and Swirski, 2010, Ziegler-Heitbrock et al., 2010). It is now known that monocytes are able to produce inflammatory mediators and scavenge/phagocytose pathogens, lipids, dying cells and toxic compounds. They are, therefore, of importance in maintaining homeostasis and
eliciting immune responses (Auffray et al., 2009, Hoffman et al., 2013). Research has demonstrated, however, that monocytes are a heterogeneous group of cells that range in their ability to perform certain immunological function. Thus, monocytes can be divided into subsets depending on their function and phenotype (Appleby et al., 2013).

‘Classical’ monocytes account for 90% of all peripheral monocytes. They express high levels of CD14 but do not express CD16 (CD14\(^++\)/CD16\(^-\)). In contrast, ‘nonclassical’ and ‘intermediate’ monocytes account for the remaining 10% of circulating monocytes. Nonclassical monocytes express moderate levels of CD14 and high levels of CD16 and are, thus, described as CD14\(^++\)/CD16\(^++\) cells (Ziegler-Heitbrock et al., 2010). In comparison, intermediate monocytes express high levels of CD14 (similar to levels on classical monocytes) and lower levels of CD16 (CD14\(^++\)/CD16\(^+\)) (Auffray et al., 2009, Ziegler-Heitbrock et al., 2010, Wong et al., 2012).

1.4.2. Monocyte Subset Contribution to Inflammation and Associated Diseases

The contribution by each of the monocyte subsets to inflammation and associated diseases is a controversial topic. This conflict in findings may, in part, be due to the majority of the studies being carried out in mice models (Mehta and Reilly, 2012, Ziegler-Heitbrock and Hofer, 2013). Mice monocytes express varying levels of lymphocyte antigen 6C (Ly6C) on their surface, a protein which inversely correlates with CD16 expression on human monocytes. Cells expressing high levels of Ly6C (Ly6C\(^hi\)) share features with human classical, CD14\(^++\)/CD16\(^-\) monocytes, whereas mouse monocytes expressing low levels of Ly6C (Ly6C\(^lo\)) represent human CD16\(^+\) monocytes (Zimmermann et al., 2010, Shi and Pamer, 2011). However, human and mouse monocyte surface protein markers are not always comparable and may provide a reason for disparity within this research area (Ziegler-Heitbrock and Hofer, 2013).
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Nonetheless, in general, the literature appears to agree that CD16+ (nonclassical and intermediate) monocytes produce more pro-inflammatory mediators in response to lipopolysaccharide (LPS), including TNFα, IL-1β IL-6 and IL-8 and less of the anti-inflammatory cytokine IL-10, when compared to classical monocytes (Wong et al., 2011, Mehta and Reilly, 2012, Wong et al., 2012). In contrast, classical monocytes have been shown to produce IL-10, granulocyte colony-stimulating factor (G-CSF), MCP-1 and IL-6 (Wong et al., 2011). Concordantly, CD16+ cells have been shown to increase in number in infection and in inflammatory-linked diseases, such as in sepsis, asthma and inflammatory bowel disease (reviewed in Mehta and Reilly, 2012).

When CD16+ cells are subdivided further, nonclassical cells are thought to be involved in a phenomenon known as monocyte patrolling, whereby monocytes ‘crawl’ in blood vessels presumably scavenging oxidised lipids, dead cells and potential pathogens whilst intermediate cells are thought to be mainly responsible for the ‘pro-inflammatory’ status of CD16+ cells (Auffray et al., 2009, Shi and Pamer, 2011). In accordance, it has been suggested that intermediate monocytes have negative implications in a range of inflammatory-linked diseases (Ziegler-Heitbrock and Hofer, 2013).

With regards to T2D, a recent study by Fadini et al. (2013) found no difference in monocyte subsets in those with T2D when compared to healthy individuals. However, it has been found that mononuclear cells, which include monocytes, had higher levels of NF-κB activity when compared to controls, suggesting a more pro-inflammatory function for these cells (Ghanim et al., 2004). In accordance, Rogacev et al. (2010) found that the number of CD16+ monocytes was elevated in obese individuals. Furthermore, CD16+ monocytes have been shown to have implications in atherosclerosis, stroke and CVD, all of which are major complications associated with
T2D and account for the majority of deaths in this disease (Auffray et al., 2009, Donath and Shoelson, 2011, Rogacev et al., 2011, Mehta and Reilly, 2012, Ziegler-Heitbrock and Hofer, 2013, WHO, 2015). It is thought that these cells are more likely to play a role in CVD and atherosclerosis due to their increased expression of chemokine receptors, such as the fractalkine receptor (CX3CR1), and the macrophage inflammatory protein 1α (MIP-1α) receptor (CCR5) and/or MCP-1 receptor (CCR2), and adhesion molecules, such as very late antigen-4 (see figure (Fig.) 1.1), which increase the affinity of these cells to the vascular endothelium (Weber et al., 2000, Kashiwagi et al., 2010, Berg et al., 2012, Mehta and Reilly, 2012). Furthermore, the production of inflammatory mediators by CD16+ cells may promote the recruitment of additional monocytes and other inflammatory cells to the endothelium, intensifying the inflammatory processes which are associated with CVD, atherosclerosis and stroke (Ziegler-Heitbrock et al., 2010, Mehta and Reilly, 2012).

It must be noted, however, that classical monocytes have been shown to acutely respond to inflammation and infection and are increased with overnutrition (reviewed in Mehta and Reilly, 2012). They have elevated phagocytic ability when compared to CD16+ subsets and can induce cell-mediated cytotoxicity in an antibody-dependent manner. These cells were also found to have increased myeloperoxidase activity and release higher levels of superoxide. However, unlike CD16+ cells, classical monocytes have the ability to suppress activated lymphocytes (Passlick et al., 1989, Auffray et al., 2009, Robbins and Swirski, 2010).

As with CD16+ monocytes, classical monocytes have implications in CVD and atherosclerosis (Tacke et al., 2007). The expression of CX3CR1 and CCR2 by these cells (see Fig. 1.1) is thought to enhance their affinity to and migration into atherosclerotic plaques (Mehta and Reilly, 2012). In fact, in rodent models, the CD14+
equivalent, Ly6C$^{hi}$ monocytes were found to enter plaques more readily than the CD16$^+$ resembling Ly6C$^{hi}$ monocytes (Swirski et al., 2007). Moreover, Berg et al. (2012) recently found that classical monocyte cell count and percentage was an independent risk factor for cardiovascular events, although not for carotid atherosclerosis. Conversely, CD16$^+$ monocytes were not found to be predictive of cardiovascular events. However, this study has been criticised for being underpowered for the detection of associations between the significantly less abundant CD16$^+$ cells and CVD. Furthermore, CCR2, CCR5 and CX3CR1 were found not to be associated with cardiovascular events. These results raise concern since there is much evidence to suggest the opposite (reviewed in Mehta and Reilly, 2012). Nonetheless, the findings of Berg and his colleagues provide an insight into the role of classical monocytes in CVD and suggest that, in addition to CD16$^+$ cells, these cells may also contribute to the onset and progression of diseases associated with inflammation.

1.4.3. The Effect of Monocyte Subtypes on Macrophage Phenotype

As previously mentioned, there is growing evidence that macrophages have the capacity to majorly contribute to the chronic, low-grade inflammation which is implied in the pathology of insulin resistance. Thus, investigations into how the aforementioned monocyte subsets affect macrophage phenotype and function have been deemed important in elucidating ways in which to prevent diseases associated with inflammation, like insulin resistance and diabetes (Olefsky and Glass, 2010).

Research has suggested that Ly6C$^{hi}$ monocytes, analogous with classical monocytes in humans, differentiate into a pro-inflammatory macrophage, known as ‘M1’ macrophages, whereas macrophages derived from Ly6C$^{lo}$ monocytes appear to have regulatory ‘M2’ macrophage like features in that they contribute to wound healing, tissue remodelling and regulation of the immune system. Importantly, similar results
have been observed in human models (Auffray et al., 2007, Nahrendorf et al., 2007, Robbins and Swirski, 2010, Fadini et al., 2013). In contrast, Veremeyko et al. (2013) argue that classical monocyte-derived macrophages may be ‘dually activated’ into either M1 or M2 macrophages. In contrast non-classical monocytes are said to be destined for M1 macrophage polarisation whereas M2 macrophages derive from intermediate monocytes. The two macrophage phenotypes and their implications in health and disease will be discussed in more detail in subsequent sections.

Evidently, there is still much to be unveiled with regards to the relationship between monocyte subset and subsequent macrophage phenotype (Mehta and Reilly, 2012, Frantz and Nahrendorf, 2014). Despite this, it is clear that monocytes, as cells in their own right, may play a role in inflammation and associated diseases or, alternatively, that they may differentiate into macrophages, the latter of which are known to be key players in the inflammation associated with insulin resistance, T2D and CVD (Olefsky and Glass, 2010, Ziegler-Heitbrock et al., 2010).
Figure 1.1. Monocyte subtypes and their differentiation into M1 and M2 macrophages. Monocytes are classified depending on their level of CD14/CD16 expression. Furthermore, the chemokine receptors CCR2, CCR5 and CXCR1 are indicative of monocyte subtype and infer cell function. Alongside adhesion molecules, such as VLA-4, these receptors aid in the recruitment and infiltration of circulating monocytes into tissue, where they differentiate into macrophages. Macrophages may be polarised into ‘pro-inflammatory’ M1 cells or ‘alternatively activated’ M2 cells. However, it is not certain whether specific monocyte subtypes are destined for particular macrophage phenotypes or whether other factors, such as stimuli in the micro-environment, determine macrophage phenotype (adapted from Mehta and Reilly, 2012, Osborn and Olefsky, 2012).
1.5. Macrophage Function and Classification

Macrophages are found in almost every tissue and, depending on the tissue in which they reside, they can exist in a variety of phenotypes. In health, macrophages produce growth factors and clear apoptotic cells or cell debris; thus, these cells are required for tissue homeostasis. They are also able to recognise foreign objects via pathogen-recognition receptors and produce inflammatory mediators (as previously mentioned) and elicit immune responses by presenting antigen to other cells of the immune system (Geissmann et al., 2010, MacPherson and Austyn, 2012, Thiriet, 2013).

Our knowledge of macrophage ontology has recently changed; it was previously believed that tissue macrophages derive from circulating monocyte precursors but it is now evident that, in the steady state, the majority of adult tissue macrophages originate from embryonic development (Halder and Murphey, 2014). Embryonic macrophages (and erythrocytes) develop during the early stages of gestation, by a process known as primitive haematopoiesis which occurs in the yolk sac. Subsequently, definitive haematopoiesis in the foetal liver allows for the development of adult-like haematopoietic stem cells, including monocytes (Epelman et al., 2014). It is believed that most adult tissues contain resident macrophages which are foetal monocyte derived, with the exception of microglial cells which are yolk sac derived and Langerhans cells which are of dual origin (Ginhoux and Jung, 2014). Many resident macrophages may undergo self-replenishment and, thus, do not rely on circulating monocytes for their proliferation (Sieweke and Allen, 2013). This does not hold true for all tissue macrophages, however; Lamina propria of the intestine, dermal macrophages, marginal zone macrophages of the spleen and macrophages of the uterus require continual replenishment by adult, bone-marrow derived monocytes, perhaps due to the nature of these tissues whereby macrophages are chronically exposed to...
microorganisms or tissue remodelling is crucial. Therefore, macrophage ontology varies depending on tissue type (Ginhoux and Jung, 2014).

Additionally, adult monocytes may replenish macrophages under certain conditions, such as in acute inflammation whereby resident tissue macrophage numbers may be severely reduced or during experimental macrophage depletion, macrophage populations may be expanded via infiltration of circulating monocytes into tissues and differentiation into monocyte derived macrophages (MDM) (Ginhoux and Jung, 2014). MDM have potential to permanently integrate into the resident macrophage population, or alternatively, following inflammation they may undergo cell death or re-enter the circulation (Davies et al., 2013). Interestingly, in the steady state, adult monocytes have also been demonstrated to infiltrate tissues without differentiating into macrophages. It is thought that these ‘tissue monocytes’ may play a role in antigen transport to lymph nodes (Jakubzick et al., 2013). Thus, monocytes appear to not only be important for macrophage replenishment following macrophage depletion, but they may also play a role in tissue, independent of macrophages (Ginhoux and Jung, 2014).

In order to carry out specific effector functions, macrophages require activation, for example, by cytokines, metabolites, plasma proteins and microbial ligands. Macrophage phenotype may vary depending on the activation stimulus, thus macrophages may exist in many different states (Gordon and Martinez, 2010, Natoli and Monticelli, 2014). The two most reported macrophage phenotypes are M1 and M2 macrophages (reviewed in Sica and Mantovani, 2012). Further subclassification of the M2 phenotype into M2a, M2b and M2c macrophages has also been reported (Mantovani et al., 2004). Table 1 provides a detailed (although not exclusive) list of stimuli and markers of the M1 and M2 macrophage phenotypes, whilst the following section describes each phenotype in more detail.
1.5.1. M1 Macrophages – Characteristics and Activation

M1 macrophages are characteristically pro-inflammatory cells, otherwise known as ‘classically activated’ macrophages. They secrete pro-inflammatory mediators and reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). Thus, these cells have strong microbicidal and tumoricidal activity. They also recruit and activate specific pro-inflammatory T cell subsets, such as T helper (Th) 1 and Th17 cells, via chemokine production, including C-X-C motif chemokine 10 (CXCL10), and the expression of surface proteins, such as MHCII, CD40, CD80 and CD86. T cell recruitment by M1 cells, thus, brings about an inflammatory, type I response (Mantovani et al., 2004, Murray and Wynn, 2011a, Murray and Wynn, 2011b).

Classical activation of macrophages usually requires the cytokine, interferon-γ (IFN-γ), which alone may sufficiently promote M1 polarisation (Martinez, 2011). Alternatively, IFN-γ can work in combination with microbial ligands, such as LPS or alongside cytokines, such as TNFα or granulocyte-macrophage colony-stimulating factor (GM-CSF), to induce M1 polarisation (Galdiero et al., 2013). Independent of IFN-γ, LPS may activate macrophages to share similar features with M1 macrophages. However, LPS-induced M1 polarisation relies on the autocrine production of interferon-β (IFN-β), which activates similar molecular signalling pathways to IFN-γ. Similarly, certain stimuli, such as TNF, IL-1 and toll-like receptor (TLR) ligands, are able to induce an M1-like phenotype by promoting the production of pro-inflammatory mediators but they are unable to activate all of the molecular process required for complete M1 polarisation. Thus, there is massive heterogeneity within the M1 macrophage population, depending on the activation stimulus/stimuli (Lawrence and Natoli, 2011).
### Table 1.1. Stimuli and markers of macrophage polarisation.

Macrophages may be directed into the M1 or M2 phenotype by various stimuli. Once polarised, macrophages may be identified due to the expression of specific markers of polarisation. Some of these markers are shared between subsets, whilst others are exclusive to a particular phenotype. (ICs, Immune complexes; IL-1ra, IL-1 receptor antagonist; LIF, Leukocyte inhibitory factor; TGM2, Translutaminase 2; MR (CD206), Mannose Receptor; NO, Nitric oxide; iNOS, Nitric oxide synthase; SR, scavenger receptor; VEGF, vascular endothelial growth factor) (adapted from Duluc et al., 2007; Hao et al., 2012).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>M1</th>
<th>M2a</th>
<th>M2b</th>
<th>M2c</th>
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<td><strong>Stimulation</strong></td>
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<td>ICs</td>
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<td>IL-1R</td>
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1.5.2. Molecular Pathways for M1 Polarisation

Signal transducer and activator of transcription (STAT) 1 has been demonstrated to be vital for IFN-γ-induced M1 polarisation (Lawrence and Natoli, 2011). IFN-γ-induced activation of Janus-kinase (JAK) 1 and/or JAK2, causes phosphorylation of STAT1 and subsequent transcription of M1-associated genes (see Fig. 1.2) (Lawrence and Natoli, 2011, Knudsen and Lee, 2013).

Similarly, LPS binds to its respective receptor, TLR4, on the macrophage cell surface to induce activation of transcription factors, such as NF-κB and AP-1 (Lawrence and Natoli, 2011). These transcription factors drive the transcription of many pro-inflammatory mediators including TNFα, IL-12, IL-1β, IL-6 and IFN-β (see Fig. 1.2) (Biswas and Mantovani, 2010). Furthermore, via interferon regulatory transcription factor (IRF) 3, LPS upregulates the production of IFN-β which is able to activate IRF5, resulting in the production of the classical M1 cytokine, IL-12, and IL-23, whilst also activating STAT1 (see Fig. 1.2) (Lawrence and Natoli, 2011).

Likewise, GM-CSF binds to the colony stimulating factor 2 receptor α (CSF2Rα) to initiate M1 gene regulation. GM-CSF receptor binding causes JAK2 to phosphorylate and activate STAT5, resulting in the upregulation of IRF5 (Lawrence and Natoli, 2011). IRF5 has been shown to be associated with the production of pro-inflammatory cytokines, including IL-12, the activation of Th1 cells and suppression of IL-10 production by macrophages (Krausgruber et al., 2011).

Moreover, classical activation of macrophages inhibits M2 polarisation, for example via STAT1 or the production of suppressor of cytokine signalling (SOCS) molecules (see Fig. 1.2). Thus, not only does the activation of M1-inducing molecular pathways govern the pro-inflammatory and microbicidal/tumorcidal functions of M1
macrophages but it also inhibits M2 macrophage polarisation (Lawrence and Natoli, 2011).

1.5.3. M2 Macrophages – Characteristics and Classification

In contrast to M1 macrophages, M2 macrophages play anti-inflammatory and homeostatic roles in immunity. They are highly phagocytic and express high levels of mannose and galactose receptors, allowing them to act as scavenger cells. They also produce a range of chemokines and cytokines, mainly of an anti-inflammatory nature. Consequently, M2 macrophages aid in the clearance of parasites, regulate immune responses and reduce inflammation. Furthermore, these cells promote tissue remodelling and wound healing (Biswas and Mantovani, 2012).

M2 macrophages are also identified as ‘alternatively activated’ macrophages. As with M1 macrophages, and possibly to an even greater degree, there is vast heterogenicity amongst this group of cells depending on the type of stimuli by which they are activated (Lawrence and Natoli, 2011). Some have attempted to further divide the M2 macrophage population into subgroups; perhaps the most commonly reported M2 classification system used today was devised by Mantovani et al. (2004). Mantovani and colleagues subcategorised M2 macrophages as M2a, M2b or M2c macrophages, depending on activation stimuli, marker expression and their functional properties. M2a macrophages are said to undergo alternative activation, typically by IL-4 and/or IL-13, resulting in the increased expression of cell surface markers, such as major histocompatibility complex (MHC) II, non-opsonic receptors (such as mannose receptor (MR), and CD163) and secreted cytokines, such as the anti-inflammatory mediators IL-10 and IL-1 receptor antagonist (IL-1Ra) (Hao et al., 2012, Liu and Yang, 2013). Furthermore, IL-4 and IL-13 activated M2 macrophages produce the CC chemokine ligand (CCL) 17, CCL22 and CCL24, which promote a less inflammatory
type II response (when compared to the type I response elicited by M1 macrophages) by recruiting Th2 cells, eosinophils and basophils (Mantovani et al., 2004, Hao et al., 2012). CCL17 and IL-10 production by M2a macrophages is also important in inhibiting M1 polarisation, specifically that involving CpG islands (Katakura et al., 2004). CpG islands are genetic regions rich in CpG dinucleotides which promote the binding of transcription factors, including NF-κB, AP1 and IRFs, to promoter regions of the gene (Lawrence and Natoli, 2011). Hence, M2a macrophages are said to play specific roles in promoting type II inflammatory responses whilst dimming type I inflammation. They also participate in the killing and encapsulation of parasites and aid in wound healing and tissue repair (Mantovani et al., 2004, Kharraz et al., 2013).

M2b cells are activated by combined exposure to immune complexes, TLR ligands (including LPS) and/or IL-1 receptor ligands, such as IL-1Ra. They express MHCII and CD86 and produce a range of cytokines, including the M1-associated cytokines, TNF-α, IL-6 and IL-1 (Hao et al., 2012). However, unlike M1 macrophages, they do not express IL-12 and, instead produce high levels of the potent anti-inflammatory mediator, IL-10. They also express CCL1, a chemokine involved in the recruitment of Th2 cells, regulatory T (Treg) cells and eosinophils (Kharraz et al., 2013). Additionally, M2b cells are thought to induce Th2 cell propagation and activation (Mantovani et al., 2004, Liu et al., 2011). Thus, M2b macrophages are said to be involved in regulating inflammation whilst promoting a Th2 type response (Mantovani et al., 2004, Hao et al., 2012).

Similarly, M2c macrophages are also involved in immunoregulation and suppression but they have additional roles in matrix deposition and tissue remodelling (Mantovani et al., 2004, Hao et al., 2012). Macrophages may be activated (or ‘deactivated’, as Mantovani et al. (2004) term it) by IL-10, transforming growth factor-β (TGF-β) or
glucocorticoids to induce the M2c phenotype (Liu et al., 2012). Characteristically, M2c macrophages express the scavenger receptor CD163, pattern recognition receptors, such as MR, TLR1 and TLR8, and the chemokine receptor, CCR2. In contrast, they express little MHCII on their cell surface (Mantovani et al., 2004, George and Johnson, 2010, Hao et al., 2012). They also produce and secrete IL-10 and TGF-β. These cytokines have anti-inflammatory and wound healing functions (Behm et al., 2012, Hao et al., 2012). Furthermore, M2c macrophages produce the chemokines, CXC motif ligand 13 (CXCL13), CCL16 and CCL18 which elicit a type II response by recruiting eosinophils and naïve T cells, cells which suppress immune responses and aid in tissue remodelling (Hao et al., 2012). It is suggested that, when naïve T cells are recruited by high levels of IL-10, tolerance and immunoregulation may result. Similarly, although M2c cells produce CCR2 and CCR5, in the presence of high levels of IL-10, monocytes do not migrate or become activated in response to adequate stimuli, particularly LPS. Instead, it is assumed that M2c-associated CCR2 and CCR5 act as decoy receptors, scavenging chemokines and dimming the inflammatory response (Mantovani et al., 2004).

Other classifications for M2 macrophages have emerged since the publication by Mantovani et al. in 2004. For example, Grinberg et al. (2009) described an ‘M2d’ subset of macrophages which are activated by adenosine A2A receptor agonists following stimulation by TLR agonists and have increased production of IL-10, decreased secretion of pro-inflammatory cytokines (TNF-α and IL-12) and increased vascular endothelial growth factor (VEGF) production, the latter of which strongly promotes angiogenesis. Others use entirely separate terminology for the classification of M2 macrophage subtypes. For example, in 2008, Mosser and Edwards proposed classifying M2 macrophages based on their function, as opposed to marker expression.
IL-4/IL-13 activated macrophages are unable to efficiently present antigen or produce pro-inflammatory cytokines, RNI or ROI. They reiterate that they do, however, produce extracellular matrix components, suggesting a primary role in wound healing; hence naming these cells ‘wound healing’ macrophages. In contrast, ‘regulatory’ macrophages may be induced by a range of stimuli, including glucocorticoids, immune complexes, prostaglandins, apoptotic cells and IL-10, but only in the presence of a second stimulus, such as a TLR ligand. These cells produce IL-10 and prevent pro-inflammatory cytokine production, such as IL-12, whilst retaining phagocytic and antigen presentation abilities. Some even consider regulatory macrophages as distinct from M2 macrophages altogether (Cassetta et al., 2011).

Despite these differences in classification systems, it is generally agreed that M2 macrophage activation results in the upregulation and activation of an assortment of genes which determine the anti-inflammatory and homeostatic function of these cells (Biswas and Mantovani, 2010).

1.5.4. Molecular Pathways for M2 Polarisation

IL-4 and IL-13 are two of the most commonly investigated inducers of M2 macrophage polarisation (reviewed in Murray and Wynn, 2011b). Upon binding to their common receptor, the IL-4 receptor α-chain (IL-4Rα), IL-4 and IL-3 activate JAK1, JAK2 and/or JAK3, resulting in the phosphorylation and activation of STAT6 (Lawrence and Natoli, 2011). STAT6 is said to be a ‘master regulator’ of M2 genes (Szanto et al., 2010). STAT6 also directly and indirectly (via SOCS1) inhibits STAT1 which, as aforementioned, plays a major role in M1 polarisation (Sica and Mantovani, 2012).
STAT6 also induces the transcription of and acts as a cofactor for other transcription factors, including the peroxisome proliferator-activated receptors (PPARs), PPARδ and PPARγ (Szanto et al., 2010, Liao et al., 2011, Sica and Mantovani, 2012). When activated, PPARs may regulate inflammation by reducing the production of pro-inflammatory genes. PPARγ, in particular, has been shown to be highly involved in M2 macrophage polarisation. Bouhlel et al. (2007) found that this transcription factor was crucial in priming monocytes for M2 polarisation and that PPAR-γ activation resulted in greater inhibition of M1 macrophage function. One way in which PPARγ induces the immunosuppressive activity of M2 macrophages is via transrepression of NF-κB and AP-1 (Ricote et al., 1998, Soskic et al., 2011). Furthermore, PPARγ activation dictates the metabolic processes which occur within macrophages, most notably lipid metabolism within these cells (Chawla, 2010, Nagy et al., 2012). This transcription factor will be discussed in more detail in section 1.7.

Similarly, STAT6 activation results in Kruppel-like factor 4 (KLF4) activation and STAT6 also acts as a cofactor for this transcription factor. KLF4 is able to sequester co-activators of NF-κB, inhibiting the production of inflammatory mediators by this transcription factor whilst upregulating M2-associated genes (including PPARγ) (Mahabeleshwar et al., 2011, Sica and Mantovani, 2012).

In a similar manner to STAT6, STAT3 is responsible for inducing the transcription of M2-associated genes whilst being able to directly inhibit STAT1 and NF-κB activity. IL-10, for example, promotes M2 polarisation by activating STAT3, whilst also inducing p50 NF-κB homodimers which interfere with the functional activity of NF-κB (see Fig. 1.2). Thus, IL-10 plays important roles in suppressing the production of inflammatory mediators by M2 macrophages (Sica and Mantovani, 2012).
Phosphoinositide 3-kinase (PI3K) activation has also been demonstrated as having an important role in M2 macrophage polarisation. IL-4 is able to activate PI3K, perhaps further explaining its M2-polarising actions (Fruman et al., 1999, Lawrence and Natoli, 2011). Although the mechanism for PI3K-dependent M2 polarisation is not entirely known, SOCS1 appears to play an important role in this mechanistic pathway since blockade of SOCS1 inhibits PI3K activity (Whyte et al., 2011).

As with M1 polarisation, IRFs also have implications in M2 polarisation. IRF-4 is activated by IL-4 and chitin (a parasite/fungal cell wall component) via jumonji domain containing-3 (JMJD3) (see Fig. 1.2) (Satoh et al., 2010b, Lawrence and Natoli, 2011, Sica and Mantovani, 2012). JMJD3 is a histone 3 Lys27 (H3K27) demethylase which functions by removing H3K27me3, an inhibitory histone, allowing for the transcription of genes required for M2 polarisation (Satoh et al., 2010b). In contrast, although induced by pro-inflammatory mediators, JMJD3 does not seem to play such a substantial role in M1 polarisation (De Santa et al., 2009, Lawrence and Natoli, 2011). Furthermore, JMJD3-activated IRF-4 directly inhibits IRF-5, preventing the expression of pro-inflammatory M1 cytokines such as IL-12 and IL-23 (see Fig. 1.2) (Sica and Mantovani, 2012).

CCAAT-enhancer-binding proteins (C/EBP) have also been implied in M2 polarisation, but only in association with cyclic adenosine monophosphate- [cAMP] responsive element-binding (CREB) proteins. TLR ligands, such as LPS, activate the CREB-C/EBP axis resulting in elevated expression of IL-10 and dual specificity protein phosphatase 1 (DUSP1), both of which inhibit the production of pro-inflammatory, M1-associated genes (Ananieva et al., 2008, Kim et al., 2008a, Lawrence and Natoli, 2011). Additionally, CREB-deficient mice have been shown to
have impaired wound healing, indicating that this pathway is important in generating wound-healing macrophages (Ruffell et al., 2009, Harries et al., 2012).

Similarly, in the presence of myeloid differentiation primary response gene (88) (MyD88) activating molecules, such as IL-1 or LPS, immune complexes can activate macrophages so that they have an M2-like phenotype. Immune complexes, such as IgG, bind to their receptor to prevent TLR4 signalling whilst upregulating IL-10 and other suppressors of inflammation, including SOCS3, *tumour necrosis factor alpha-induced protein 3* (TNFAIP3, also known as A20) and A20-binding inhibitor of NF-κB activation 3 (ABIN3) (Wang et al., 2010). This ‘coordinated activation’ of M1 and M2 genes by M1-associated stimuli may aim to regulate macrophage polarisation to maintain a healthy balance of M1 and M2 macrophages.
Figure 1.2. Molecular mechanisms for M1 and M2 macrophage polarisation.

Classical stimuli, such as IFN-γ and LPS activate transcription factors, such as STAT1 and NF-κB and IRFs which upregulate the transcription of M1-associated genes. Conversely, M2 stimuli, such as IL-4, IL-13 and IL-10, activate STAT3 and STAT6 which upregulate M2-associated genes or, alternatively, activate PPARs and KLF4 which also induce M2 activation of macrophages. Furthermore, certain M1-inducing molecular pathways inhibit M2 polarisation and vice versa. Thus, some transcription factors, such as STAT, CREB and IRFs, are involved in reciprocal regulation of both M1 and M2 genes (adapted from Sica and Mantovani, 2012).
1.5.5. Other Macrophage Classification Systems

Although useful, the M1/M2 classification system has been criticised for being oversimplified. It is believed that true M1 or M2 macrophages actually represent the extremes of a continuum and that, in reality, ‘hybrid’ or ‘overlapping’ macrophage phenotypes exist (see Fig. 1.3). Consequently, in certain settings, macrophages have been found to express markers which are associated with more than one M2 subtype or both the M1 and M2 phenotype (Zeyda et al., 2007, Mosser and Edwards, 2008, Lopez-Castejon and Brough, 2011, Mantovani et al., 2013). For example, adipose tissue macrophages (ATMs) have been described as M2-like macrophages but they have also been shown to produce levels of M1-associated cytokines, including TNFα, IL-6 and MCP-1 (Zeyda et al., 2007).

Alternatively, macrophages may exist that are neither M1 nor M2 (Biswas and Mantovani, 2010). Tumour associated macrophages (TAMs) are distinct from M1 and M2 macrophages, although they may share features with both (Qian and Pollard, 2010). Cassetta et al. (2011) propose that there are at least six different types of macrophages which contribute to cancer progression, these include invasive, activated, immunosuppressive, angiogenic, metastasis-associated and regulatory macrophages, each with distinct phenotypes.

Similarly, ‘Mox’ macrophages, which are thought to account for 30% of all macrophages in advanced atherosclerotic lesions in mice, have been shown to express distinct gene expression patterns and have different functions to M1 and M2 macrophages (Kadl et al., 2010, Chinetti-Gbaguidi and Staels, 2011a). In the same way, ‘M4’ macrophages (supposedly atheroprotective cells induced by CXC chemokine ligand 4 (CXCL4)) express both M1- and M2-associated genes but do not have a gene pattern resembling either phenotype (Gleissner et al., 2010).
Figure 1.3. Diagrammatic representation of macrophage activation. The activation status of macrophages may be placed on a liner scale, ranging from M1 (classically activated) macrophages, to M2 (alternatively activated) macrophages (a). Alternatively, macrophage activation status may be viewed on a monochromatic diagram, with three distinguished groups of macrophages (classically activated (red), wound-healing (yellow) and regulatory (blue)) being represented by the primary colours with secondary colours representing hybrid phenotypes, such as TAMs (green) (adapted from Mosser and Edwards, 2008).
Nevertheless, although some macrophages do not neatly fit into the M1/M2 classification system, comparisons are still often made with M1 and M2 macrophages. Thus, the M1/M2 nomenclature will predominantly be used throughout this thesis.

1.6. The Role of Specific Macrophage Phenotypes in Inflammation and Associated Diseases

In inflammation, macrophages undergo four steps; recruitment, differentiation, activation and immunosuppression and tissue restoration. Thus, macrophage plasticity is critical in ensuring that a challenge is handled efficiently and health is re-established. The phenotype of tissue resident macrophages is dependent on their location in the body but, in general, they have an M2-like phenotype with intrinsic anti-inflammatory roles (Murray and Wynn, 2011a).

Upon challenge, macrophages respond to signals in their microenvironment by recruiting circulating monocytes into tissue. Subsequent activation of these newly recruited macrophages, via stimuli produced by other immune cells, damaged tissue and/or pathogens, promotes specific effector functions by these cells, including antimicrobial defence and T cell and neutrophil recruitment. Generally, recruited macrophages exhibit a pro-inflammatory, M1-like macrophage phenotype. However, these cells are said to participate in ‘orderly’ inflammation, a process which ensures that tissue damage is prevented whilst effector duties are efficiently carried out (Murray and Wynn, 2011a).

Once the challenge has been dealt with, macrophages may also aid in the resolution of inflammation (Lawrence and Natoli, 2011). It is thought that IL-4-induced macrophage proliferation may contribute to this process. IL-4 is produced by Th2 cells and, as previously mentioned, promotes M2 macrophage polarisation. IL-4 has also
been found to induce self-renewal of tissue macrophages, perhaps reducing the recruitment and activation of pro-inflammatory macrophages at late stages of inflammation and contributing to inflammation resolution. However, M1 macrophages have also been reported to proliferate in response to IL-4 (Jenkins et al., 2011, Murray and Wynn, 2011a). Nonetheless, as previously described, IL-4 signalling in macrophages has been shown to suppress molecular pathways associated with M1 polarisation and induce wound-healing and tissue repair (Sica and Mantovani, 2012).

Evidently, the fine balance in M1:M2 ratio dictates the beneficial role of these macrophages in maintaining health. However, in certain disease states, this ratio may become off-balance, allowing for the contribution of these cells to disease. In this section, the role of macrophages in a selection of diseases will be discussed.

1.6.1. Macrophages in insulin resistance and T2D

The M1:M2 ratio has been found to be increased in a T2D setting (Pradhan Nabzdyk et al., 2013, You et al., 2013). Concordantly, M1 macrophages appear to play a central role in local and systemic inflammation which drives insulin resistance and associated T2D (Olefsky and Glass, 2010). Furthermore, PPARγ, a key regulator of M2 but not M1 polarisation, majorly controls the metabolic function of macrophages. Thus, the skewing of macrophages to the M1 macrophage phenotype in T2D has been shown to alter metabolic function, impairing insulin sensitivity, glucose uptake and lipid metabolism by these cells (Murray and Wynn, 2011a). In fact, specific PPARγ deletion in macrophages has resulted in systemic insulin resistance (Odegaard et al., 2007).

Research involving insulin-sensitive tissues has provided further insight into the impact of pro-inflammatory macrophages, assumed to be of the M1 phenotype, on
insulin signalling and glucose metabolism (Shoelson et al., 2006). In adipose tissue, for example, macrophage numbers are said to be elevated in obese individuals (Osborn and Olefsky, 2012). Since, obesity accounts for 80-85% of overall risk for T2D, it is very common that individuals with T2D are also overweight or obese and, thus, have higher numbers of ATM (Donath and Shoelson, 2011, Holt et al., 2011). Not only is macrophage recruitment elevated in obesity, it has also been found that the ATMs of obese mice were skewed towards a pro-inflammatory, M1 phenotype, as opposed to those of lean mice, which were mainly of an M2-phenotype. This may, in part, explain the altered inflammatory status and increased risk of insulin resistance in obese individuals (Lumeng et al., 2007a, Chinetti-Gbaguidi and Staels, 2011a). Within adipose tissue, macrophages may activate adipocytes via direct cell-to-cell contact, causing them to produce a range of inflammatory mediators (Lumeng et al., 2007a). Of these mediators, TNFα has gathered much attention due to its impact on insulin signalling. Hotamisligil et al. (1993) found that elevated TNFα production by adipose tissue of obese rats caused a reduction in insulin-dependent glucose uptake. This disturbance in glucose metabolism could be improved by TNFα neutralisation, indicating a role for this cytokine in the pathogenesis of insulin resistance. Recent evidence suggests that ATM are almost entirely responsible for TNFα production by adipose tissue in obesity, whilst also contributing to IL-6 production (Weisberg et al., 2003, Xu et al., 2003). Furthermore, ATM have been shown to perturb local insulin-dependent glucose transport by GLUT-4 in adipocytes (Abel et al., 2001, Lumeng et al., 2007b, Harford et al., 2011). Since lipolysis is increased whilst lipogenesis is preserved by insulin-resistant adipocytes, it is thought that insulin resistance in adipocytes may contribute to adipose tissue growth and obesity (Rask-Madsen and
Kahn, 2012). Thus, it is evident that ATMs have key implications in prolonging the chronic inflammation which is associated with insulin resistance.

In the liver, insulin promotes glycogen synthesis, inhibits gluconeogenesis and regulates lipid metabolism by supporting lipogenesis (Monga and Cagle, 2010). As with adipose tissue, the liver contains parenchymal cells (hepatocytes) and resident macrophages (Kupffer cells). However, when compared to ATM in adipose tissue, the Kupffer cell population is much denser in the liver and their cell number does not increase in obesity. Instead, Kupffer cells become more activated in obesity, resulting in increased production of inflammatory mediators (Cai et al., 2005, Shoelson et al., 2006, Baffy, 2009). Additionally, it has recently been found that, as with adipose tissue, macrophages are recruited to the liver in obesity. These macrophages are distinct from Kupffer cells but also contribute to liver-derived inflammation (Obstfeld et al., 2010). In the liver, cells of myeloid origin (e.g. Kupffer cells) are thought to be mainly responsible for the inflammatory mediators produced by this organ (Donath and Shoelson, 2011). Huang et al. (2010) highlighted the importance of Kupffer cells in liver-derived inflammation when they demonstrated that depletion of Kupffer cells in the liver of rats fed a HFD or high sucrose diet improved insulin sensitivity. By using a range of cytokine neutralising antibodies, they also determined that Kupffer cell-derived TNFα was a key player in instigating insulin resistance in the liver and disturbances in hepatocyte-mediated lipid metabolism. Similarly, others have found that insulin resistance was improved when Kupffer cells were polarised into a less pro-inflammatory phenotype (Kang et al., 2008, Odegaard et al., 2008).

Interestingly, insulin-producing pancreatic β-cells are also susceptible to the negative effects of inflammation on insulin-responsiveness and increased numbers of macrophages have been found in the islets of those with T2D and in obese and diabetic
rodents (Maedler et al., 2002, Ehses et al., 2007, Campbell and Verbeke, 2013). Macrophage-derived IL-1β and TNFα has been found to induce the production of inflammatory mediators by β-cells, as well as causing β-cell dysfunction. It is suggested, however, that infiltrating macrophages, as opposed to resident tissue macrophages, are mainly responsible for the inflammation which is associated β-cell dysfunction and impaired insulin secretion. Nonetheless, since macrophage infiltration is increased in T2D, pancreatic macrophages appear to play a role in amplifying and prolonging pancreatic inflammation and β-cell dysfunction in individuals with T2D (Eguchi et al., 2012, Eguchi and Manabe, 2013).

Overall, therefore, it is clear that macrophage-derived inflammation promotes insulin resistance and impairs glucose uptake by insulin-sensitive tissues, whilst impairing β-cell function and insulin secretion (see Fig. 1.4). Therefore, research aimed at elucidating ways in which to alter inflammatory status so as to promote insulin sensitivity may be beneficial for the prevention and treatment of T2D.
Figure 1.4 Macrophage-derived inflammation in insulin-sensitive tissues is associated with hyperglycaemia and insulin resistance. Macrophage infiltration into key insulin-sensitive tissues (adipose tissue, the liver and the pancreas) enhances and prolongs the local and systemic inflammation which drives insulin resistance and associated hyperglycaemia, ultimately resulting in T2D (adapted from Osborn and Olefsky, 2012).
1.6.2. Macrophages in Atherosclerosis

The risk of atherosclerosis is elevated in those with diabetes, with its development often being accelerated and more severe with this condition (Barlovic et al., 2011). It is a chronic inflammatory disease which is caused by the accumulation of lipids, cells and extracellular matrix between the intima and the endothelial cell layer of arteries. This build-up of biological material forms a plaque, also known as a lesion, which may occlude blood flow through the blood vessel (Moore and Tabas, 2011). However, symptoms generally only display when plaques rupture, promoting platelet recruitment and thrombus (Bench et al., 2011, Moore and Tabas, 2011). Consequently, myocardial infarction, unstable angina, sudden cardiac death and stroke are all potential outcomes of advanced atherosclerosis (Moore and Tabas, 2011).

Macrophages are the first immune cells to migrate into lesions and appear to be involved in multiple stages of plaque development and progression, up to and including plaque rupture (Gui et al., 2012, Stoger et al., 2012). Both M1 and M2 macrophages have been found to exist in atherosclerotic plaques at all stages of plaque development (Bouhlel et al., 2007, Stoger et al., 2012, Medbury et al., 2013). It is thought that an imbalance in M1:M2 macrophage ratio may prevent the resolution of inflammation in atherosclerotic plaques, resulting in chronic inflammation and plaque progression (Gui et al., 2012).

There is much evidence to support the notion that M1 macrophages promote atherosclerosis (Buono et al., 2003, Oh et al., 2012). Concordantly, monocytes recruited more readily into plaques seem to be of a more pro-inflammatory nature, with a high likelihood that they may differentiate into pro-inflammatory, M1 macrophages (Swirski et al., 2007). Since some M1-associated inflammatory mediators are pro-atherosclerotic and pro-thrombotic, M1 macrophages are capable of
promoting the initiation and development of atherosclerosis, as well as plaque rupture and downstream thrombotic events (Huang et al., 2012, Moore et al., 2013, Tasaki et al., 2013). In accordance, M1 macrophages are said to be the predominant macrophage phenotype found in vulnerable areas of plaques (Stoger et al., 2012).

Additionally, M1 macrophages generate ROS and reactive nitrogen species (RNS), both of which contribute to oxidative stress within lesions. Upon exposure to oxidative stress, lipids are modified and these modified lipids are more regularly taken up by macrophages (Moore et al., 2013). Lipid-laden macrophages, otherwise known as foam cells, have major implications in the initiation and progression of plaques (Park et al., 2012). Interestingly, M2 macrophages have been shown to have increased lipid uptake due to elevated scavenger receptor expression, particularly that of scavenger receptor A1 (SR-A1) and CD36 and are, thus, more likely to develop into foam cells (Yakubenko et al., 2011, Oh et al., 2012, Rousselle et al., 2013). In addition, it is thought that resident adventitia macrophages, which display an M2 phenotype, may promote immune cell infiltration and plaque destabilisation by inducing neovascularisation in plaques (Stoger et al., 2012, Jetten et al., 2014). Therefore, a role has also been implied for M2 macrophages in atherosclerosis development and progression (Oh et al., 2012).

Nonetheless, there is still much support for a protective role of M2 macrophages in atherosclerosis; they are thought to inhibit inflammation and improve plaque stability (Moreno et al., 2012, Stoger et al., 2012, Medbury et al., 2013). Furthermore, some have found that PPARγ-associated M2 activation of human macrophages in atherosclerotic plaques results in a phenotype which demonstrates resistance to cholesterol loading, impairing their ability to transform into foam cells, and enhanced phagocytic abilities, which may promote the clearance of apoptotic cells in lesions and
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prevent plaque progression (Chinetti-Gbaguidi et al., 2011b). Thus, it is likely that the role of M2 macrophages in atherosclerosis may be stimuli dependent and, hence, the discrepancies in findings may be attributable to an inability to replicate the distinct atherosclerotic environment (which encompasses a range of macrophage polarising stimuli) outside of an in vivo setting (Stoger et al., 2012). Thus, it is evident that further research is required to determine the precise role of both M1 and M2 macrophages in atherosclerosis.

1.6.3. Rebalancing the M1:M2 Ratio as a Useful Strategy for Disease Prevention

The evidence outlined above demonstrates that both M1 and M2-like macrophages have implications in inflammatory-linked disease but they are also vital for maintaining health. Although certain macrophage subtypes contribute to the pathology of some diseases, simply the presence of M1 or M2 macrophages in a system does not necessarily dictate disease states - it is often the ratio of these cells which determines disease development (Barros et al., 2013). Therefore, there is much effort being placed on establishing ways to re-balance the M1:M2 ratio in order to prevent the onset and/or progression of disease.

In this thesis, particular focus is placed on elucidating mechanisms that promote M2 polarisation in a way that may prevent or revert the elevated M1:M2 ratio which is associated with T2D (Kalupahana et al., 2012). As outlined in section 1.5.4, there are a range of potential molecular pathways which may be targeted for M2 macrophage polarisation. Focus will be placed on PPARγ since this transcription factor is thought to be a key regulator of M2 polarisation, at both a macrophage and monocyte level (Bouhlel et al., 2007, Murray and Wynn, 2011b). Furthermore, macrophage-specific PPARγ has been shown to be highly influential on systemic insulin resistance.
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(Odegaard et al., 2007). Hence, this transcription factor has gained much interest with regards to macrophage polarisation in the prevention of insulin resistance and associated T2D.

1.7. PPARγ as a Target for Insulin Resistance and T2D

A beneficial role for PPARγ in T2D was first suggested in 1995, when Lehmann et al. (1995) found that a group of anti-diabetic drugs, namely TZDs, were high affinity ligands for this transcription factor. Via PPARγ, TZDs improve insulin sensitivity by promoting fatty acid uptake by adipose tissue. Additionally, TZDs may also promote adiponectin secretion by adipose tissue, also lowering free fatty acid levels. As a consequence, insulin-sensitive tissues other than adipose tissue (e.g. the muscle, the liver and the pancreas) are not exposed to metabolically-damaging levels of free fatty acids and, thus, insulin sensitivity is improved, gluconeogenesis by the liver is inhibited and blood glucose levels are reduced. Furthermore, blood insulin levels are also reduced due to a reduction in insulin secretion by the pancreas (Yki-Jarvinen, 2004). In accordance, the direct activation of PPARγ is known to systemically improve lipid metabolism and insulin resistance, whilst regulating adipogenesis in adipose tissue (Ahmadian et al., 2013).

1.7.1. The Role of Macrophage-Specific PPARγ in M2 Polarisation

PPARγ exists as two isoforms, PPARγ1 and PPARγ2, the latter being exclusively expressed by adipocytes (Wadosky and Willis, 2012). PPARγ is highly expressed in macrophages where it has implications in suppressing inflammation and altering lipid metabolism (Ahmadian et al., 2013). The impact of PPARγ activation on macrophage-derived inflammation was first recognised in 1998, when it was found that a range of
endogenous and exogenous PPARγ ligands inhibited pro-inflammatory responses by macrophages (Jiang et al., 1998, Ricote et al., 1998).

Subsequently, more sophisticated techniques, such as macrophage-specific depletion of PPARγ, have provided useful insight into this area. Hevener et al. (2007) found that macrophage-specific depletion of PPARγ in mice resulted in the upregulation of markers of inflammation and, accordingly, the disruption of insulin signalling in adipose tissue, the muscle and the liver. Consequently, whole body glucose intolerance and systemic insulin resistance ensued, suggesting an important role for macrophage PPARγ in whole body insulin resistance. Of relevance to these findings, macrophage-specific deletion of PPARγ in Balbc, Th2-oriented mice was also shown to impair alternative activation of these cells upon treatment with IL-4. As a result, mice were more prone to become obese in response to a HFD. Surprisingly, however, the increase in adipose tissue mass was associated with a decrease in ATM number, primarily due to decreased M2 macrophage density. In accordance, macrophage-PPARγ deficient mice developed insulin resistance and glucose intolerance. Therefore, these findings also suggest that macrophage-specific PPARγ activation may decrease insulin resistance and T2D risk, potentially due to its ability to drive M2 polarisation (Odegaard et al., 2007). Confounding results were found by Marathe et al. (2009), who used a Th1-biased mouse strain (C57BL/6) to investigate the effects of macrophage-specific PPARγ depletion and found no effect on obesity, insulin resistance or glucose tolerance, despite beneficial alterations in inflammation. These results suggest that genetic background may have an impact on the beneficial actions of macrophage-specific PPARγ. Nonetheless, studies involving PPARγ agonists have generally shown that PPARγ activation skews macrophage polarisation in favour of an M2 phenotype (Chinetti-Gbaguidi and Staels, 2011a). Moreover, investigations
with murine ATM, which were found to differentially express PPARγ amongst the two subsets (F4/80lo and F4/80hi), have been used to further support the notion that PPARγ activation favours an M2 phenotype in macrophages whilst PPARγ-deficiency evokes an M1-like phenotype (Bassaganya-Riera et al., 2009).

1.7.2 Evidence for PPARγ Activation in the Priming of Monocytes for M2 Polarisation

Interestingly, Bouhlel et al. (2007) found that PPARγ expression positively correlated with M2 marker expression in human carotic atherosclerotic lesions. Although they found that PPARγ activation in already differentiated macrophages did not alter M2 marker expression in response to IL-4, PPARγ was shown to increase M2 marker expression in MDM which had been differentiated after treatment with either of the PPARγ agonists, rosiglitazone or GW1929, and IL-4. Thus, PPARγ is thought to be important in the ‘priming’ of monocytes for differentiation into the M2 macrophage phenotype in response to IL-4.

The phenomenon of PPARγ-induced M2 polarisation in monocytes was further investigated by specifically isolating monocytes from patients receiving treatment with the PPARγ agonist, pioglitazone. In accordance, it was found that pioglitazone increased both M2-marker (IL-10 and CD163) messenger RNA (mRNA) and protein expression, whilst decreasing M1 marker (IL-6) expression in circulating monocytes (Satoh et al., 2010a).

In comparison, upon monocyte treatment with the PPARα agonist, GW7647, and the PPARβ/δ agonist, GW501516, IL-4-induced M2 marker expression was unaltered following cell differentiation, yet treatment of monocytes with the PPARγ agonist, GW1929, amplified IL-4-induced M2 marker expression in MDM (Bouhlel et al.,
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2009). On the contrary, others have suggested a role for PPARα in the priming of human monocytes; adiponectin was found to increase M2 marker expression in peripheral blood monocytes which had been differentiated in the presence of IL-4, compared to treatment with IL-4 alone, potentially in a PPARα-dependent manner. Nonetheless, PPARγ was also shown to participate in the priming of M2 macrophages by adiponectin (Lovren et al., 2010).

Altogether these findings suggest that, although cytokines such as IL-4 and IL-13 are important in the induction of M2 polarisation, PPARγ may be vital for the acquisition and maintenance of the M2 macrophage phenotype (reviewed in Chawla, 2010). More specifically, PPARγ activation in monocytes, rather than macrophages, induces M2 marker expression and may predict macrophage phenotype prior to tissue infiltration (Bouhlel et al., 2007, Satoh et al., 2010a). This exciting area of research still requires much attention, however, since questions still remain as to whether PPARγ-induced monocyte priming efficiently promotes a stable M2 macrophage phenotype following cell differentiation or whether subsequent exposure to additional stimuli within tissue may allow for a switch in macrophage phenotype.

1.7.3. Ligand-Dependent Mechanisms of PPARγ Activation

PPARγ may be activated upon ligand binding to the protein’s ligand-binding domain (LBD). PPARγ heterodimerises with the nuclear receptor, retinoid X receptor (RXR) to form a complex which constitutively binds to peroxisome proliferator response elements (PPREs) in target genes (Harmon et al., 2011). In the presence of a ligand PPARγ/RXR heterodimerisation and binding to PPREs is enhanced. Furthermore, ligand-binding promotes the loss of repressors of transcription and the induction of co-activators and, consequently, upregulation of PPARγ-RXR target genes (see Fig. 1.5). Additionally, corepressors may be induced upon PPARγ activation, resulting in
the suppression of inflammatory pathways, an important feature of M2 polarisation (Harmon et al., 2011, Ahmadian et al., 2013).

1.7.3.1. Exogenous PPARγ Ligands

Synthetic exogenous ligands are available for the activation of PPARγ. TZDs, such as rosiglitazone and pioglitazone, are well known for their ability to act as high affinity ligands for PPARγ (Norris and Sigmund, 2012). They are also potent enhancers of insulin sensitivity and multiple studies have demonstrated their effectiveness in preventing T2D onset (DREAM Trial Investigators et al., 2006, DeFronzo et al., 2011, Cariou et al., 2012). Additionally, they have been shown to improve markers of CVD risk, such as endothelial function, lipid metabolism and the development of atherosclerosis (Harmon et al., 2011). As previously mentioned, TZDs have also been found to promote M2 polarisation (Chinetti-Gbaguidi and Staels, 2011a). Rosiglitazone has been shown to promote M2 macrophage polarisation in cultured MDM and ATM, whilst pioglitazone has been shown to do the same in circulating monocytes obtained from obese diabetic individuals (Bouhlel et al., 2007, Satoh et al., 2010a, Prieur et al., 2011). However, a meta-analysis carried out by Nissen and Wolski (2010) revealed that there is an unfavourable link between rosiglitazone and myocardial infarction. When compared to pioglitazone, rosiglitazone was also associated with a higher risk of congestive heart failure and death (Loke et al., 2011). It is thought that these detrimental outcomes may be as a result of disrupted renal sodium reabsorption, prompting fluid retention upon rosiglitazone use (Harmon et al., 2011). TZDs have also been associated with bone loss and fractures and bladder cancer (Schwartz, 2008). Thus, alternative methods of PPARγ activation, are currently being sought (Harmon et al., 2011).
Figure 1.5 Ligand-dependent activation of PPARγ. Upon ligand binding, co-repressor proteins (e.g. nuclear receptor co-repressor (N-CoR)) are released from the PPARγ-RXR heterodimer to form an active transcription complex which is able to bind to the PPRE on target genes. Additionally, co-activators, such as PPARγ co-activator (PGC-1) may also aid in the assembly of an effective PPARγ-RXR complex involving histone acetyltransferases (HATs) and steroid receptor co-activator-1 (SR-1). Subsequently, active PPARγ-RXR is able to upregulate or repress transcription of target genes (adapted from Murphy and Holder, 2000).
1.7.3.2. Endogenous PPARγ Ligands

Although natural ligands tend to bind with lower affinity to PPARγ when compared to synthetic ligands, they have the potential to activate PPARγ without the detrimental side effects associated with therapeutic agents (Harmon et al., 2011). Endogenous ligands for PPARγ exist as fatty acids and their derivatives (Wahli and Michalik, 2012). For example, the arachidonic acid derivative, 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂), is an endogenous ligand for PPARγ, which is said to bind specifically and with high affinity to PPARγ’s LBD (Harmon et al., 2011, Georgiadi and Kersten, 2012). Upon treatment with IL-13, monocytes were found to have elevated expression of CD36, potentially via PPARγ activation due to IL-13-induced increases in 15d-PGJ₂ production. These findings show potential for 15d-PGJ₂ in PPARγ activation and subsequent M2 polarisation (Berry et al., 2007). However, it is thought that the anti-inflammatory actions of 15d-PGJ₂ may, in part, be due to PPARγ-independent mechanisms (Varga et al., 2011).

Similarly, the enzyme 15-lipoxygenase (15-LOX) in human macrophages may promote production of the endogenous PPARγ ligands, 9-hydroxy-10E,12Z-octadecadienoic acid (9-HODE) and 13-hydroxy-9Z,11E-octadecadienoic acid (13-HODE) (Harmon et al., 2011). 9-HODE and 13-HODE are modified derivatives of linoleic acid, and are found as components of oxidised low density lipoprotein (oxLDL) which induce CD36 expression in a PPARγ-dependent manner, potentially explaining increased LDL uptake and foam cell formation by M2 macrophages (Canton et al., 2013). Interestingly, IL-4 has been shown to strongly increase the expression of 12/15-lipoxygenase, the murine homologue of 15-LOX, in macrophages (Huang et al., 1999). In accordance, 15-LOX gene expression was found to be increased in M2 macrophages (Chinetti-Gbaguidi and Staels, 2011a).
Other endogenous PPARγ ligands include 15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HETE) and 13-OXO-9Z,11E-octadecadienoic acid (13-oxoODE) which have been shown to have anti-inflammatory and anti-atherogenic properties (Harmon et al., 2011). Of note, it has been suggested that 15-HETE, alongside 13-HODE and lysophosphatidic acid (LPA, another endogenous PPARγ ligand) is induced in macrophages following IL-4 treatment, suggesting a role for these endogenous PPARγ ligands in M2 polarisation (Czimmerer et al., 2012). In relation, nitroalkene fatty acids, such as nitrolinoleic acid (LNO2) and nitrol-oleicacid (OA-NO2) and 15-keto-prostaglandin E2 (15-keto-PGE2) show less promising roles in promoting M2 polarisation since, both LNO2 and OA-NO2 are likely to have short half-lives and, therefore, their potential to act as endogenous PPARγ ligands in vivo is questionable and 15-keto-PGE2 is not expressed in macrophages (Harmon et al., 2011).

1.7.4. PPARγ Activation via Post-Translational Modification

Independent of ligand-binding PPARγ activity may also be regulated by post-translational modification. For example, mitogen-activated protein kinase (MAPK)-induced phosphorylation of serine 82 in the A/B-domain (the N-terminal region) of PPARγ has been shown to inhibit PPARγ activation (Harmon et al., 2011). Similarly, cyclin-dependent kinase (cdk) 5-induced PPARγ phosphorylation at serine 273 (S273) has been shown to alter PPARγ activity in adipocytes. S273 phosphorylation inhibits the production of the insulin sensitising hormone, adiponectin by adipocytes. Since adiponectin has been shown to induce the M2 macrophage phenotype, cdk5 inhibition may prove useful in preventing an increase in the M1:M2 ratio which is implied in the development of insulin resistance and T2D (Choi et al., 2010, Lovren et al., 2010). Conversely, PPARγ-activation has been enhanced upon cdk7- and cdk9-induced
phosphorylation, suggesting a kinase-specific role for the activation or inhibition of PPARγ via phosphorylation (Harmon et al., 2011).

Similarly, small ubiquitin-like modifier (SUMO) proteins may be covalently attached to PPARγ in a process known as sumoylation, altering PPARγ activity (Wadosky and Willis, 2012). SUMO-1 may bind to lysine 107 in the transcriptional activation function domain (AF-1) of the A/B domain, potentially inhibiting PPARγ activity (Ohshima et al., 2004). In contrast, sumoylation of the lysine residue K395 has been shown to promote the anti-inflammatory activity of PPARγ via suppression of NOS2 in murine macrophages (Pascual et al., 2005, Anbalagan et al., 2012). Thus, it is possible that K395 sumoylation may induce an M2-like phenotype in macrophages. However, further research is required to prove the role of sumoylation in M2 polarisation.

Additionally, ubiquitination (the covalent attachment of ubiquitin to lysine residues) of PPARγ can fate the protein for degradation. In adipocyte specific PPARγ2, this process usually occurs following ligand-induced PPARγ activation in a regulatory manner (Hauser et al., 2000, Wadosky and Willis, 2012). However, it has also been shown that IFN-γ may also promote PPARγ ubiquitination and subsequent degradation in adipocytes (Waite et al., 2001). Thus, it may be possible that IFN-γ may promote M1 macrophage polarisation whilst suppressing M2 macrophage polarisation by inducing PPARγ degradation.

To summarise, it is clear that there are several mechanisms by which PPARγ may be activated in order to induce M2 macrophage polarisation and associated improvements in insulin resistance and T2D risk. Exogenous ligands, such as TZDs, have proven useful in the treatment of insulin resistance and T2D by binding with high affinity to
PPARγ and inducing its activation (reviewed in Harmon et al., 2011). They have also been shown to enhance M2 polarisation (reviewed in Chinetti-Gbaguidi and Staels, 2011a). However, TZDs have been linked to deleterious side effects, raising concerns regarding their use. On the other hand, the promotion of PPARγ activation via naturally occurring events, such as endogenous ligand production and post-transcriptional modifications, may eliminate the risk of detrimental side-effects (Harmon et al., 2011). Therefore, much effort is currently being placed on elucidating novel ways in which to modulate the internal pathways which drive PPARγ activation, and subsequent M2 polarisation, without the use of therapeutic agents. Importantly, within our research group, it has been that physical activity may fit the desired criteria (Butcher et al., 2008, Yakeu et al., 2010, Thomas et al., 2012).

1.8. Physical Activity for the Prevention and Management of Insulin Resistance and T2D

Physical activity is defined as bodily movement involving skeletal muscle contraction which results in a substantial rise in energy expenditure. In contrast, exercise is defined as repetitive, planned and structured physical activity, carried out with the ultimate aim of maintaining or improving physical fitness (De Feo and Schwarz, 2013). Current physical activity guidelines advise that adults should partake in 150 minutes of moderate-intensity exercise per week, in bouts of 10 minutes or more. Alternatively, 75 minutes of vigorous activity per week or a combination of moderate and vigorous intensity exercise is also advised (DOH, 2011). However, a recent survey revealed that less than 50% of the UK adult population partook in the recommended levels of physical activity in 2012 (based on the 2004 physical activity guidelines, recommending 30 minutes of physical activity on at least 5 days per week) (BHF, 2012). These statistics are highly concerning considering the increasing prevalence of
diseases associated with physical inactivity, including obesity and T2D (Danaei et al., 2011, Malik et al., 2013).

Participation in exercise has been associated with many health benefits, including reduced risk of T2D and CVD (reviewed in Warburton et al., 2006). There is a substantial amount of evidence to support the notion that exercise may be useful for the prevention and management of T2D. Several key studies, including the Diabetes Prevention Study (DPS) in Finland and the Diabetes Prevention Program (DPP) found significant reductions in diabetes incidence (58%) in participants who underwent lifestyle interventions. Moreover, in the DPP study, the lifestyle intervention was found to reduce diabetes incidence to a greater extent than use of the anti-diabetic agent, metformin (850mg twice daily), which reduced incidence by 31% when compared to a placebo group. Both the DPS and the DPP used combined lifestyle interventions of diet counselling and exercise or weight loss and exercise, respectively (Eriksson et al., 1999, Diabetes Prevention Program Research, 2002). In contrast, the Da Qing IGT and Diabetes Study investigated the effects of exercise alone on T2D onset over 6 years and similarly found a 46% decreased risk of developing T2D, when compared to control subjects. The Da Qing study also investigated the effect of diet only and diet plus exercise interventions on T2D incidence and found that each of these interventions resulted in lesser reductions in T2D incidence when compared to the exercise only cohort, with a 31% and 42% decrease in risk of T2D development, respectively (Pan et al., 1997).

1.8.1. The Effect of Physical Activity on Inflammation and Monocyte/Macrophage Polarisation

Although exercise has long been known to aid in the prevention and management of insulin resistance and T2D, it is likely that multiple mechanisms lie behind its
beneficial actions, and these are still under investigation (Colberg et al., 2010). One potential mechanism involves the effects of exercise on inflammation. Exercise elicits a short-term inflammatory response which is followed by anti-inflammatory events (Golbidi et al., 2012). In doing so, exercise may constitute as an example of hormesis, a process whereby low-dose stress to a biological system may induce a beneficial response in the long term (Ji et al., 2006, Radak et al., 2008). For example, the exercise-induced increases in ROS (as a by-product of aerobic metabolism) are known to act as a systemic stressor which may induce synthesis of inflammatory cytokines, including IL-6 by contracting muscle cells (Pan et al., 2012, Davies et al., 2015). Hence, myocyte-derived IL-6 has been shown to majorly account for acute elevations in IL-6 observed following exercise (Petersen and Pedersen, 2005, Golbidi et al., 2012, Pan et al., 2012, Dishman et al., 2013). As previously mentioned, chronic elevations in IL-6 are often associated with the insulin resistant state (Gustafson, 2010, Olefsky and Glass, 2010). However, perhaps because exercise may not induce a ‘typical’ inflammatory state (whereby IL-6 is often elevated alongside other pro-inflammatory cytokines, such as TNFα) and because the response to IL-6 is thought to be dependent on the combination of other external stimuli, exercise-associated transient increases in IL-6 may not elicit the same response as that produced during an immune response, for example, but may actually beneficially impact upon insulin sensitivity and glucose homeostasis (Febbraio and Pedersen, 2002, Kristiansen and Mandrup-Poulsen, 2005, Scheller et al., 2011). Muscle-derived IL-6 is known to improve glucose and lipid metabolism in muscle and also in the liver and adipose tissue in an endocrine manner (Febbraio and Pedersen, 2002). Additionally, exercise-induced IL-6 synthesis has been found to inhibit TNFα, whilst inducing anti-inflammatory cytokines, such as IL-4, IL-10 and IL-1Ra which are also known to downregulate TNFα and other
inflammatory mediators, such as IL-1, IL-8 and MIP-1α (Petersen and Pedersen, 2005). Thus, although IL-6 is probably not the only player in the exercise hormesis theory, the initial inflammatory stress provided by myocytic generation of this cytokine appears to induce a compensatory biological response via production of regulatory cytokines and inhibition of pro-inflammatory cytokines, including TNFα. To this end, exercise-induced IL-6 may alter systemic inflammation so as to inhibit the actions of pro-inflammatory mediators, and hence bring about benefits with regard to chronic inflammatory conditions such as insulin resistance, T2D and CVD (Febbraio and Pedersen, 2002, Petersen and Pedersen, 2005, Golbidi et al., 2012).

Additionally, exercise has been shown to modify immune cell infiltration into tissues. For example, an investigation into the effects of a diet and exercise intervention in obese individuals revealed that inflammation was greatly reduced post-intervention, particularly in adipose tissue. Interestingly, this reduction in inflammatory cytokine expression was mainly attributed to decreased macrophage infiltration, since macrophage markers were also shown to decrease in adipose tissue following the intervention (Bruun et al., 2006). In accordance with these findings, Kawanishi et al. (2010) concluded that exercise attenuated the pro-inflammatory effects of HFD feeding in the adipose tissue of murine models and reduced M1 marker expression whilst increasing mRNA levels of M2 macrophage markers. They attributed these changes to reduced M1 macrophage infiltration and macrophage switching from the M1 phenotype to the M2 phenotype. In contrast, Oliveira et al. (2013) found that a single bout of mild to moderate exercise (swimming) did not alter macrophage number in white adipose tissue of rats which had been previously fed on a HFD for 12 weeks. However, there was an improvement in the inflammatory profile and insulin signalling within these rats, potentially due to a shift in macrophage polarisation, favouring the
M2 phenotype. In accordance, M2 macrophage marker expression was found to be upregulated in white adipose tissue from exercised rats. Therefore, it was suggested that a switch in macrophage phenotype, rather than a decrease in macrophage recruitment, may be responsible for the reduction in inflammation resulting from exercise participation. IL-10 was also found to be upregulated in white adipose tissue and in the circulation following exercise. Additionally, following LPS treatment after exercise, rats were found to have increased levels of IL-4 and similar LPS levels when compared to non-exercised animals. Thus, exercise was found to upregulate the M2 stimuli, IL-10 and IL-4, and attenuate levels of the M1 stimulus, LPS, potentially explaining elevated M2 polarisation upon exercise (Oliveira et al., 2013). Similarly, a single bout of exercise (treadmill running at a 10° incline, at 20m/min for 90 minutes) induced M2 macrophage marker expression in murine muscle, resulting in increased glucose uptake and insulin sensitivity. Intriguingly, upon whole body macrophage depletion, the effect of exercise on M2 macrophage accumulation in skeletal muscle and insulin responsiveness was abrogated, despite GLUT4 expression being unaltered. Thus, it is suggested that exercise-induced M2 macrophage polarisation may play an important role in the prevention of insulin resistance and T2D (Ikeda et al., 2013).

1.8.2. PPARγ Activation as a Potential Mechanism for Physical Activity-Induced M2 Polarisation

Exercise-induced oxidative stress is known to have an impact on lipid oxidation, increasing levels of circulating oxLDL (Golbidi et al., 2012). As aforementioned, the oxidised lipid components of oxLDL, 9-HODE and 13-HODE, may act as PPARγ ligands (Canton et al., 2013). In their study, Butcher et al. (2008) aimed to determine whether PPARγ was upregulated in circulating leukocytes from previously sedentary individuals following an 8 week walking programme. Fascinatingly, they found that
PPARγ mRNA expression was upregulated in these leukocytes. In a similar study, a gene reporter assay was used to determine the availability of activators of PPARγ in plasma from exercised individuals. In support of Butcher and colleague’s suggestion, it was proposed that exercise induced PPARγ ligand generation (activating PPARγ to a similar degree as did the natural PPARγ ligand, 13-HODE). However, the mode of PPARγ activation was not fully elucidated; despite PPARγ phosphorylation being shown not to be responsible for the increase in PPARγ activity, other post-translational modifications were not investigated and, thus, there is potential for PPARγ activation to have occurred in a ligand-independent manner (Thomas et al., 2012). Nonetheless, exercise was shown to induce PPARγ activation in leukocytes, potentially providing a further mechanism for its anti-inflammatory effects.

In the same leukocyte samples as those obtained by Butcher et al. (2008), the effects of exercise-induced PPARγ activation on M2 marker expression was investigated. Accordingly, gene expression of markers of M2 polarisation were upregulated, as were the expression of the PPARγ co-activators, PGC-1α and PGC-1β. These novel results support a role for PPARγ in exercise-induced M2 polarisation (Yakeu et al., 2010). Additionally, they suggest that exercise-induced PPARγ activation may promote an athero-protective phenotype in leukocytes since receptors for lipid uptake and reverse cholesterol transport were also found to be upregulated. Thus, these cells may aid in cholesterol sequestration and clearance, which has been shown to prevent atherosclerosis and CVD (Butcher et al., 2008). Interestingly, PPARα and PPARβ/δ expression did not change, indicating that other PPARs may not impact on leukocyte phenotype following exercise (Yakeu et al., 2010).

In summary, exercise has been shown to induce markers of M2 polarisation in circulating leukocytes, potentially via PPARγ ligand generation and subsequent
PPARγ activation (Butcher et al., 2008, Yakeu et al., 2010, Thomas et al., 2012). It is, therefore, suggested that exercise-induced PPARγ activation may represent a novel mechanism by which exercise aids in the prevention and management of insulin resistance, T2D and associated CVD (Thomas et al., 2012). However, the evidence largely relies on data based on leukocyte studies, hence it is not possible to definitively state that the alterations seen in PPARγ and/or certain M2 markers upon exercise solely occur within monocytes since other leukocytes may also express these (Wahli and Michalik, 2012; see Appendix, Fig. A1.1). Therefore, further research is required to elucidate the effects of exercise on PPARγ expression and activity and subsequent M2 marker expression specifically within monocytes/macrophages, so as to provide additional evidence for the mechanisms by which exercise exerts its beneficial actions in relation to insulin resistance and T2D. Consequently, exercise may be shown to be an efficient and safe alternative to the current anti-diabetic, PPARγ-targeting therapies that are available for the treatment of insulin resistance, T2D and its cardiovascular complications.

1.9. Research Rationale and Aims

Inflammation is heavily implied in the development of insulin resistance and subsequent T2D (reviewed in Gregor and Hotamisligil, 2011). Elevated levels of pro-inflammatory M1 macrophages in comparison to inflammation-suppressing M2 macrophages may contribute to the chronic inflammation which drives insulin resistance (Kalupahana et al., 2012). PPARγ is an important transcription factor which promotes M2 macrophage polarisation (reviewed in Chawla, 2010). PPARγ has also been shown to enhance IL-4/IL-13-driven M2 polarisation by ‘priming’ circulating monocytes for the M2 phenotype (Bouhlel et al., 2007). However, the mechanisms behind PPAR-γ-induced monocyte priming are yet to be elucidated, since the majority
of research within this area has been carried out in macrophages. Also, it is yet to be revealed whether the polarisation status of monocytes remains stable upon differentiation into macrophages or whether intra-tissue stimuli may induce a phenotypic switch. Additionally, both PPARγ and M2 marker expression have been found to be elevated in leukocytes upon participation in exercise (Butcher et al., 2008, Yakeu et al., 2010). However, exercise-induced PPARγ activity and associated M2 marker expression have not been investigated in isolated monocytes. Furthermore, it has been implied that exercise generates PPARγ ligand production, however, limited research has been carried out in this area with the possibility of ligand-independent activation of PPARγ still existing.

Thus, the main aims of this research are to:

1) Evaluate the effects of an 8 week moderate-intensity exercise programme on systemic inflammation, insulin sensitivity and markers of T2D and CVD risk.

2) Determine the effects of participation in a moderate-intensity, 8 week walking programme on PPARγ expression and activity and on markers of M2 polarisation in circulating monocytes. Further, to assess whether PPARγ ligand generation and/or PPARγ activation occurs during exercise.

3) Investigate the ability of exercise to induce M2 polarisation stimuli in serum and investigate the effects of these stimuli on the priming of monocytic cells for M2 polarisation in vitro.

4) Assess the effect of PPARγ activation, either alone or in combination with IL-4 and IL-13, on the priming of monocytic cells for M2 macrophage polarisation in vitro and the mechanisms underlying any observed alterations in monocyte phenotype. Also to assess the ability for LPS challenge to induce a phenotypic switch in PPARγ and/or IL-4/IL-13-primed macrophages.
CHAPTER 2

The Effects of an 8 Week, Moderate-Intensity Exercise Programme on Type 2 Diabetes and Cardiovascular Disease Risk Factors

2.1. Introduction

Participation in physical activity has been associated with many health benefits, including improvements in cardio-respiratory fitness, musculo-skeletal fitness, body composition and weight, bone health and mental health (Kotecki, 2011). Important to this research, physical activity has also been found to reduce the risk of metabolic disease, including T2D, and CVD (Danaei et al., 2011, Malik et al., 2013). Ominously, however, less than half of the UK adult population partake in the recommended levels of physical activity (150 minutes of moderate-intensity aerobic exercise per week, in bouts of 10 minutes or more, 75 minutes of vigorous activity per week or a combination of the two) (DOH, 2011, BHF, 2012).

Several large-scale studies, including the Diabetes Prevention Study (DPS) in Finland, the Diabetes Prevention Program (DPP) and the Da Qing IGT and Diabetes Study found significant reductions in diabetes incidence using lifestyle interventions which included exercise participation (Pan et al., 1997, Eriksson et al., 1999, Diabetes Prevention Program Research, 2002). Importantly, the Da Qing IGT and Diabetes Study, which investigated the effects of exercise alone on T2D incidence found greater improvements in diabetes risk markers as compared to diet only and also diet plus exercise interventions (Pan et al., 1997).

Despite these findings, there still appears to be a reliance on methods other than exercise for the prevention and treatment of T2D, as outlined by Hordern et al. (2012);
“Exercise training, is a recognised, although relatively underutilised strategy that is central to the prevention, care and management of T2DM and pre-diabetes”. Instead, therapeutic agents are often prescribed for the treatment of T2D, some of which (most notably the TZDs) have been associated with detrimental side-effects, including increased CVD and mortality rates (Nissen and Wolski, 2010). Exercise may provide a safer alternative to these anti-diabetic drugs. Furthermore, exercise may aid in the prevention of CVD, a T2D complication which accounts for the majority of deaths within these individuals (Morrish et al., 2001). Thus, considerable interest and effort is being placed on providing evidence to promote exercise prescription for the prevention and treatment of T2D and its cardiovascular co-morbidities.

It is possible that a lack of knowledge with regards to the optimal type of exercise required for T2D prevention and management may be a factor in the low implementation rates associated with this intervention. Although, the beneficial effects of exercise on glycaemic control, insulin resistance and insulin sensitivity are known, these effects are variable depending on mode, duration and/or intensity of the exercise being undertaken. Similarly, the ability for exercise to beneficially alter CVD risk factors, such as blood lipid levels, body composition and blood pressure is also dependent on the type of exercise administered (Colberg et al., 2010).

Thus, in the present study, an 8 week, moderate-intensity, brisk walking intervention was carried out with the aim of evaluating the impact of participation on T2D and CVD risk. This chapter presents the observational findings from the exercise study with the aim of evaluating its effect on aerobic capacity and physiological and biochemical measures associated with T2D/CVD. Most notably, systemic insulin sensitivity was also analysed to determine the effects of this specific exercise programme on insulin resistance and T2D risk.
2.2. Methods

2.2.1. Participants

A power calculation was carried out using Minitab v16 and was used to determine sample size required, using the change in PPARγ-dependent gene expression as a primary outcome (for relevance of this measure to the current research, see Chapter 3). The power calculation assumed at least an 80% increase in PPARγ-dependent gene expression with a standard deviation (SD) of 50%, a required power of 0.9 (90%) and using a two-sample t-test for comparisons (Butcher et al., 2008). Consequently, a sample size of at least 11 was required for this study. To account for a 25-30% attrition rate, it was decided to recruit at least 15 and no more than 20 individuals.

Thus, a cohort of 19 healthy, yet sedentary females were recruited onto the study, mean age of 42 ± 11 years; body weight 76.54 ± 12.54 kg; body mass index (BMI) 29.7 ± 5.1 kg/m²; waist circumference 91.74 ± 13.36 cm; systolic blood pressure 126.3 ± 9.9 mmHg; diastolic blood pressure 78.5 ± 8.8 mmHg (means ± SD). Seca® electronic scales and height monitor (Birmingham, UK) were used to measure weight and height, respectively. BMI was calculated using the formula BMI = (weight kg/(height m x height m)). Waist circumference was measured using a standard anthropometric tape measure to measure the circumference of the natural waste; the narrowest waist circumference found between the lowest rib margin and the iliac crest.

Blood pressure was measured using an MX2 digital automatic blood pressure monitor (Oron, Milton Keynes, UK). Measurements were taken in triplicate after participants had been supine and silent for at least 5 minutes, with a 5 minute period between each reading. The mean of the two closest measurements (within ± 10mmHg of each other) was calculated to give mean systolic and diastolic blood pressure values for each individual.
2.2.2. Inclusion and Exclusion Criteria

Participants were included if they were over the age of 18 and sedentary (inactive). Participants were assessed for activity levels using the short version of the International Physical Activity Questionnaire (IPAQ). Those who scored as category 1, i.e. less than 5 days of moderate-intensity exercise/walking 30 minutes per day, less than 3 days of vigorous activity for 20 minutes per day or less than 5 days of a combination of walking, moderate- and vigorous-intensity activities achieving 600 metabolic equivalent (MET)-min/week, were included in the study. Those who scored above category 1 were excluded from the study. Additionally, individuals with a history of CVD or those taking lipid-lowering or metabolism altering drugs were also excluded from participation. Although both males and females were eligible for recruitment, convenience sampling meant that only females were recruited.

2.2.3. Ethical Approval

Ethical approval was granted by the School of Health Sciences’ School Research Ethics Committee (SREC) at Cardiff Metropolitan University, Cardiff, UK. Informed consent was obtained from each individual participating in the study.

2.2.4. Pre-Health Screening

A health questionnaire was completed by participants and anthropometric measures were obtained in order to ascertain risk of any cardiovascular or respiratory complications which may be contraindicative for the exercise programme. Additionally, participant fitness was estimated using the submaximal Rockport Fitness Walking Test (Kline et al., 1987), a test used to estimate maximal oxygen consumption ($VO_{2\text{max}}$), which is safer to conduct than maximal tests and is suitable for those with low activity levels which may impact on running ability (American College of Sports, 2013). Following a 4 minute familiarisation/warm up period, participants were
required to walk as fast as possible for one mile (1.6 kilometres) on a treadmill (Woodway Desmo, Waukesha, USA). Heart rate (HR) upon completion was measured using a Polar S410 HR monitor (Polar Electro, Finland). Time of completion was also noted. VO\textsubscript{2max} was estimated using following formula (Kline \textit{et al.}, 1987):

\[
\text{VO}_{2\text{max}} = 132.853 - (0.0769 \text{ (body mass in pounds)}) - (0.3877 \text{ (age in years)}) + (6.315 \text{ (gender; male = 1, female = 0)}) - (3.2649 \text{ (time to walk 1 mile in minutes and 100ths of minutes)}) - (0.1565 \text{ (heart rate upon mile completion in beats per minute)}).
\]

VO\textsubscript{2max} was estimated at baseline and after completion of the exercise programme, ensuring a 24h rest period between the final exercise session and the VO\textsubscript{2max} test.

\textbf{2.2.5. Exercise Programme Design}

The exercise programme consisted of three, 45 minute walking sessions on a treadmill (Woodway Desmo, Waukesha, USA) per week for 8 weeks, totalling 24 fully supervised walking sessions. A maximum of two walking sessions were carried out consecutively. This exercise programme has previously been used by our research group and has been shown to elicit health benefits when compared to a control group who did not partake in the programme (Butcher \textit{et al.}, 2008, Yakeu \textit{et al.}, 2010). Thus, it was decided to make comparisons on a within-group basis (ie. by comparing ‘pre-exercise’ versus ‘post-exercise’ readings), as opposed to using a non-exercised control group. Participants were asked not to carry out additional physical activity to that provided within the study or reported as part of their regular routine at baseline.

In the first session, participants selected a speed at which they were comfortable walking at a brisk rate for 45 minutes. Researchers also aided in speed selection to ensure that participants exercised at moderate-intensity. Exercise intensity was based on the percentage of maximum HR (HR\textsubscript{max}; calculated using the formula 220-age in
years), with moderate-intensity physical activity falling within 55-69% of HRmax (Bagchi and Preuss, 2012). HR was monitored using a Polar S410 HR monitor (Polar Electro, Finland) which was fitted prior to the commencement of any exercise. HR measurements were taken at rest and then at 5, 15, 30 and 45 minutes into the exercise session. HR was monitored on a weekly basis, on every third exercise session. To maintain exercise intensity throughout the programme, walking speed was altered according to changes in HR i.e. as HR levels declined, walking speed was increased.

2.2.6. Sampling Techniques

2.2.6.1. Collection of Blood Samples

Participants were fasted for 12 hours prior to blood collection. Blood was collected at four time points; immediately prior to (‘baseline’) and immediately following (‘post 1’) the first exercise session and immediately prior to (‘8 weeks’) and immediately following (‘post 8 week’) the final exercise session. A 24h rest period was left between the penultimate exercise session and procurement of the ‘8 weeks’ sample. In this chapter, only ‘baseline’ and ‘8 weeks’ blood samples were used for analysis (‘post 1’ and ‘post 8 week’ samples will be covered in Chapter 3). Blood was collected by venepuncture of the antecubital vein in plain tubes for serum collection. All phlebotomy equipment was provided by Greiner VACUETTE (Gloucester, UK) and all phlebotomy was conducted by trained phlebotomists, following completion of a blood donation questionnaire to ensure safe practice. Samples were treated according to the Cardiff Metropolitan University’s human tissue act (HTA) guidelines.

2.2.6.2. Serum Collection

For serum collection, whole blood collected in plain blood tubes was allowed to clot by bringing samples to room temperature (RT) and leaving to stand for 60 minutes.
Blood was then centrifuged for 10 minutes at 3000 revolutions per minute (RPM). Serum was carefully removed and transferred into cryotubes for immediate storage at -80°C

2.2.7. Biochemical Analysis of Serum Samples

Following serum procurement, as described in section 2.2.6.2., sample aliquots were transferred to the Diabetic Research Network Wales Laboratories (Swansea, UK) for analysis. Samples were transferred in compliance with the HTA and analysed within one month of study completion.

2.2.7.1. Cholesterol, HDL and Triglyceride Analysis

The Instrumentation Laboratory Cholesterol kit, HDL-Cholesterol kit and Triglyceride kit were used to determine total serum cholesterol, HDL and triglyceride levels, respectively. Measurements were made on an iLab 300 Plus analyser (Instrumentation Laboratories UK ltd, Warrington, UK). A range of calibrators and quality controls were run with every test.

2.2.7.2. LDL Analysis

LDL was quantified indirectly using the Friedewald (Friedewald et al., 1972) equation, as follows (all values in mmol/l):

\[ [LDL] = [Total \ cholesterol] - [HDL] - (Triglycerides/2.2) \]

The Friedewald calculation for LDL concentration is only valid when triglyceride levels are <4.5 mmol/l (Crook, 2012). None of the samples analysed in this study contained triglyceride concentrations > 2.2 mmol/l.
2.2.7.3. Glucose Analysis

Glucose determination was carried out on an iLab 300 analyser using the IL Test Glucose Oxidase assay as per manufacturer’s instruction.

2.2.7.4. Insulin Analysis

Serum insulin was measured using the Invitrogen Insulin Assay Kit, as per manufacturer’s instruction (Invitrogen Ltd, Paisley, UK). This assay measures ‘true’ insulin concentrations since it does not cross-react with other insulin-like products. The Berthold CentroPlate Luminometer (Berthold Technologies, Herts, UK) to read plates and insulin concentrations were determined using the MikroWin software.

2.2.8. Calculations for Insulin Resistance/Sensitivity

The updated homeostasis model assessment (HOMA2) was used to assess insulin resistance. HOMA2 was developed by Levy et al. (1998) and is based on the HOMA1 model, developed by Matthews et al. (1985). The HOMA1 model acknowledges both insulin resistance and β-cell function as predictors for fasting glycaemia levels. HOMA1 provides linear approximations of HOMA_insulin resistance, an estimate of tissue insulin resistance and HOMA_%B, an estimate of pancreatic β-cell activity, relative to a reference sample. HOMA_insulin resistance (mmol/L X μU/ml) and HOMA_B% (mU/L X mmol/L) are calculated using the following formulae:

\[
\text{HOMA_insulin resistance} = \frac{\text{fasting glucose (mmol/L)} \times \text{fasting insulin (μU/ml)}}{22.5}
\]

\[
\text{HOMA_B}% = 20 \times \frac{\text{fasting insulin (mU/L)}}{\text{fasting glucose (mmol/L)} - 3.5}
\]

Unlike HOMA1, HOMA2 also takes into account hepatic and peripheral glucose resistance, the contribution of proinsulin on insulin resistance and increases in insulin secretion associated with plasma glucose concentrations >10 mmol/L (Levy et al.,
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1998). The HOMA2 calculator (www.dtu.ox.ac.uk/homacalculator/download.php) was used to provide model-derived estimates of HOMA_insulin resistance, HOMA_%B, and also the reciprocal of HOMA_insulin resistance, HOMA_%S, using fasting serum insulin and glucose concentrations. HOMA_%S is an estimated measure of insulin sensitivity and is useful for the determination of β-cell function when used in combination with HOMA_%B (Wallace et al., 2004). A HOMA_insulin resistance value of 1 and HOMA_%B or HOMA_%S values of 100% were deemed healthy.

Additionally, the McAuley’s Score for measuring the insulin sensitivity index (McAuley’s ISI) was used as a surrogate measure of insulin resistance. This calculation has been found to be suitable for estimations of insulin resistance in normoglycaemic individuals, such as the participants within this study (McAuley et al., 2001, Ascaso et al., 2003). McAuley’s ISI was calculated as follows:

\[ \text{McAuley’s ISI} = \exp[3.29 - 0.25 \ln(I_0) - 0.22 \ln(BMI) - 0.28 \ln(TG)] \]

Where \( I_0 \) = fasting insulin (mU/l), \( BMI \) = body mass index (kg/m\(^2\)), \( TG \) = fasting triglycerides (mmol/l).

**2.2.9. Statistical Analysis**

All data were expressed as mean ± standard error of the mean (SEM), unless otherwise stated. Where comparisons were made between the means of two samples, t-tests or Wilcoxon’s pairwise analysis were used, depending on data distribution (the D’Agostino & Pearson omnibus normality test was used to test for normal distribution of data). Alternatively, one-way analysis of variance (ANOVA) with Tukey’s post-hoc analysis was used for multiple comparisons within groups of normally distributed data. Statistical analysis was performed using Minitab®16 or GraphPad Prism®5 software and results were deemed significant at \( p<0.05 \).
2.3. Results

These results demonstrate the effects of participation in an 8 week, moderate-intensity exercise programme. The baseline values for each individual were used as comparators, with specific time-points for comparison stated in the text.

2.3.1. Physiological Measures and Estimated VO$_{2\max}$

Anthropometric data was collected immediately prior to the final exercise session (‘8 weeks’) and comparisons were made to individual baseline values. Mean body mass was significantly reduced (baseline: 76.54 ± 12.54 kg, 8 weeks: 75.68 ± 12.38 kg, $p<0.05$) upon study completion. Accordingly, BMI was also significantly reduced following the intervention (baseline: 29.7 ± 5.1 kg/m$^2$, 8 weeks: 29.4 ± 4.9 kg/m$^2$, $p<0.05$, see Fig.2.1). Similarly, as demonstrated in Fig.2.1, waist circumference was significantly reduced following exercise participation (baseline: 91.74 ± 13.36 cm, 8 weeks: 86.17 ± 12.03 cm, $p<0.01$). Furthermore, as shown in Fig.2.2, both systolic (baseline: 126.3 ± 9.9 mmHg, 8 weeks: 121.2 ± 12.0 mmHg) and diastolic (baseline: 78.4 ± 7.8 mmHg, 8 weeks: 77.8 ± 10.9 mmHg) blood pressure were decreased, although non-significantly, post intervention. Additionally, VO$_{2\max}$ was significantly increased following study completion (baseline: 26.79 ± 8.24 ml/kg/min, 8 weeks: 34.68 ml/kg/min, $p<0.01$), as shown in Fig.2.3.
Figure 2.1. Alterations in body mass, BMI and waist circumference following participation in the 8 week, moderate-intensity exercise programme. Body mass (A) was obtained and BMI (B) calculated for individuals who took part in a moderate-intensity walking study for 8 weeks, prior to participation (baseline) and before their final exercise session (8weeks). Additionally, waist circumference (C) was also measured at (baseline) and before the final exercise session (8weeks) (n=19 in all cases; values expressed as mean ± SEM; *p<0.05, **p<0.01, paired t-test).
Figure 2.2. Alterations in blood pressure following participation in the 8 week, **moderate-intensity exercise programme**. Both A) systolic blood pressure and B) diastolic blood pressure were measured in participants, at baseline and following 8 weeks of exercise participation (n=19 in all cases; values expressed as mean ± SEM, paired t-test).
Figure 2.3. Alterations in VO$_{2\text{max}}$ following participation in the 8 week, moderate-intensity exercise programme. The submaximal Rockport Fitness Walking Test was employed to estimate VO$_{2\text{max}}$ as an assessment of aerobic capacity in individuals prior to (‘baseline’) and following participation (‘8 weeks’) in an 8 week, moderate-intensity brisk walking programme (n=15 in all cases; values expressed as mean ± SEM; **p<0.01, paired t-test).
2.3.2. Blood Lipid Levels

Blood lipid levels were detected in n=17 samples. No significant changes were identified in serum total cholesterol (baseline: 4.8 ± 0.9 mmol/L, 8 weeks: 5.0 ± 1.1 mmol/L), LDL (baseline: 2.6 ± 0.7 mmol/L, 8 weeks: 2.9 ± 0.8 mmol/L) or HDL (baseline: 1.6 ± 0.4 mmol/L, 8 weeks: 1.6 ± 0.4 mmol/L) following participation in the exercise programme (see Fig.2.4.).

Conversely, median levels of serum triglycerides were significantly reduced post intervention, when compared to baseline (baseline: 1.3 ± 0.5 mmol/L (95% CI: 1.10 → 1.60), 8 weeks: 1.1 ± 0.5 mmol/L (95% CI: 0.83 → 1.43), p<0.01, Wilcoxon’s paired analysis), as shown in Fig.2.4.

2.3.3. Glucose, Insulin and Insulin Resistance

Both fasting plasma glucose (baseline: 4.8 ± 0.1 mmol/L, 8 weeks: 4.7 ± 0.1 mmol/L) and fasting plasma insulin (baseline: 49.9 ± 12.4 pmol/L, 8 weeks: 34.2 ± 4.6 mmol/L) did not alter significantly (see Fig. 2.5).

Furthermore, HOMA_insulin resistance (baseline: 1.1 ± 0.3, 8 weeks: 0.8 ± 0.1) HOMA_%B (baseline: 103.4% ± 13.8%, 8 weeks: 90.9% ± 8.1%) and HOMA_%S (baseline: 127.6% ± 12.6%, 8 weeks: 154.1% ± 15.7%) although a decreasing trend was shown in each of these parameters (see Fig.2.6). HOMA results are applicable to n=14 participants since the HOMA2 calculator is only compatible with insulin values ranging from 20-400pmol/L and, thus, some samples were omitted from the analysis.

In contrast, there was a significant increase in the McAuley’s ISI value (baseline: 8.2 ± 0.5, 8 weeks: 9.1 ± 0.6, p<0.05) 8 weeks into the exercise study, when compared to baseline (n=17 due to loss of triglyceride results during analysis, see Fig. 2.7).
Figure 2.4. Serum lipid levels at baseline and upon completion of an 8 week, moderate-intensity brisk walking programme. Lipid levels (Total cholesterol, white; LDL, light grey; HDL, dark grey; triglycerides diagonal striped grey) were analysed in fasting serum samples obtained pre-exercise intervention (baseline) and after 8 weeks of moderate-intensity exercise, immediately prior to the final exercise session (8 weeks) (n=17 in all cases; values expressed as median with interquartile range and min to max; **p<0.01, Wilcoxon’s paired analysis).
Figure 2.5. The effects of participation in an 8 week, moderate-intensity exercise programme on fasting serum glucose and insulin levels. A) glucose and B) insulin levels were measured in fasting plasma samples obtained from participants pre-exercise intervention (baseline) and after 8 weeks of moderate-intensity exercise, immediately prior to the final exercise session (8weeks) (n=19 in all cases; values expressed as median with interquartile range and min to max; \( p > 0.05 \), Wilcoxon’s paired analysis).
Figure 2.6. Analysis of insulin sensitivity via HOMA2 calculations following participation in an 8 week, moderate-intensity exercise programme. HOMA2 analysis of insulin sensitivity and A) HOMA_insulin resistance and B) HOMA_%B and HOMA_%S values were calculated at baseline (using samples obtained pre-exercise) and following participation in the exercise intervention (‘8weeks’; using samples obtained immediately prior to the final exercise session) (n=14 in all cases; values expressed as mean ± SEM, paired t-test).
Figure 2.7. Analysis of insulin sensitivity via McAuley’s ISI calculations following participation in an 8 week, moderate-intensity exercise programme. Fasting serum insulin, glucose and triglyceride levels, and BMI, were obtained from participants pre-exercise intervention (baseline) and after 8 weeks of moderate-intensity exercise, immediately prior to the final exercise session. This data was used to analyse insulin sensitivity via the McAuley’s ISI. Comparisons were made at baseline and following participation in an 8 week, moderate-intensity exercise intervention (‘8 weeks’) (n=17 in all cases; values expressed as mean ± SEM; *p<0.05, paired t-test).
2.4. Discussion

The present study demonstrates that participation in an 8 week moderate-intensity exercise programme beneficially altered key predictors for T2D and CVD risk, which include body weight, BMI, waist circumference, triglyceride levels, aerobic capacity and insulin sensitivity in high risk, sedentary females.

Excess body fat is the strongest predictor of T2D risk; hence interventions aimed at weight loss are thought to be beneficial for T2D prevention (Hu et al., 2001, Goldstein and Mueller-Wieland, 2007). However, the effect of exercise alone on body weight is a controversial topic and it is generally agreed that participation in the recommended levels of physical activity has little impact on body mass (Colberg et al., 2010, Thorogood et al., 2011). Instead, approximately 1 hour of exercise per day is thought to be required for reductions in weight; a volume of exercise which is considerably higher than that recommended for reductions in T2D risk and that used within this study (Ross et al., 2000, Ross et al., 2004, Colberg et al., 2010). In accordance, where similar exercise interventions have been used to the 30 minute per day, thrice weekly brisk walking programme administered in this study, no changes have been found in body mass when compared to baseline (Donnelly et al., 2003, Ross et al., 2004, Villareal et al., 2011). However, in contrast with these findings, significant alterations in body mass (see Fig.2.1) and, consequently BMI (see Fig.2.1) were observed in the female cohort used within this study, following participation in the 8 week moderate-intensity exercise intervention. Nonetheless, participants remained in the overweight category of the BMI scale (BMI = 25.0-29.9) and, thus, their BMI-associated risk of T2D development was unaltered following participation in the exercise study. It is possible that caloric restriction may have contributed to the significant reductions in body weight and BMI which were observed in this study. Despite asking participants
not to alter their diet throughout the exercise study, the lack of calorie regulation provides a limitation to this research since it has been found that caloric restriction plays a considerable role in weight loss, either in combination with exercise or independently (Swift et al., 2014). Thus, in future exercise studies, it may be useful to regulate diet, for example by providing food diaries throughout the duration of the study.

Similarly, waist circumference has been found to be a strong predictor for obesity-associated co-morbidities, such as hypertension, dyslipidaemia, hyperinsulinaemia and cardiometabolic risk, with some stating that it is a better marker of T2D risk than BMI (Janssen et al., 2004, Poirier et al., 2005, Chaput et al., 2011, Tchernof and Despres, 2013). However, others have demonstrated that waist circumference may only be superior for predicting T2D risk in females with a BMI>28 (Kodama et al., 2012). Nonetheless, in the present study, waist circumference was used as a surrogate measure of central obesity. Central obesity is thought to have greater implications in T2D risk than total obesity which is often measured via BMI. Visceral fat, particularly intra-abdominal fat, has been shown to play a major role in insulin resistance and T2D development since it is has high metabolic activity, contributing to lipid metabolism, fatty acid production and insulin resistance-inducing inflammation (Patel and Abate, 2013). Furthermore, central obesity has been shown to positively correlate with liver fat content which, in excess, is associated with increased intrahepatic insulin levels and increased glucose and triglyceride output, all of which contribute to insulin resistance and T2D development (Despres, 2012). Interestingly, mean waist circumference was found to be significantly reduced following participation in the 8 week moderate-intensity exercise intervention, from 91.74 ± 13.36 cm at baseline to 86.17 ± 12.03 cm post exercise (p<0.01, see Fig.2.1). Importantly, women with a waist
circumference >88 cm are said to be at increased risk of T2D development, hypertension and CVD. Thus, with a mean BMI between 25.0 and 29.9, at time of commencement the participants in this study were classified as being at ‘high’ risk of disease development, with their risk status beneficially changing to ‘increased’ risk upon completion of the intervention, despite remaining in the ‘overweight’ BMI category (Kotecki, 2011). It must be noted that the use of anthropometric tape to measure waist circumference has its limitations in that it is a fairly subjective measure, allowing for large variation between the measurements obtained (Samuel et al., 2010). However, participation in the 8 week, moderate-intensity exercise programme seemingly altered fat storage, in a way which may reduce T2D risk.

Hypertension is also often associated with T2D risk and is greatly implied in the vascular complications associated with this disease. Thus, T2D treatment is often aimed at reducing blood pressure (Colberg et al., 2010). Many studies have reported significant reductions in blood pressure following exercise participation, potentially due to exercise-induced endocrine effects and nitric oxide synthesis (Kadoglou et al., 2007, Loimaala et al., 2009, Colberg et al., 2010, Frisoli et al., 2011). In the present study, however, upon completion of the exercise intervention, a non-significant trend toward reductions in both systolic and diastolic blood pressure was observed (see Fig.2.2). These non-significant reductions in blood pressure may be attributed to the non-hypertensive state of participants upon study commencement. Instead, participants were classified as prehypertensive, having a mean systolic blood pressure >120 mmHg but <140 mmHg at baseline, placing them at a greater risk of developing hypertension (Kotecki, 2011). Accordingly, a recent meta-analysis revealed moderate reductions in blood pressure (similar to those found within this study) in normotensive and prehypertensive individuals following participation in aerobic exercise, whereas
hypertensive individuals were found to have more pronounced reductions in blood pressure (Thorogood et al., 2011). Nonetheless, the data suggests that participation in the exercise study may be beneficial for the regulation of hypertension. Interestingly, Balducci et al. (2010) found significant reductions in mean systolic and diastolic blood pressures (-4.2 mmHg and -1.7 mmHg, respectively) in 606 participants with T2D or the metabolic syndrome who partook in a mixed aerobic and resistance training exercise programme, bi-weekly for 12 months. The reduction in systolic blood pressure observed by Balducci and colleagues was very similar to that observed in this study, suggesting that a significant reduction in systolic blood pressure may have been observed if a larger cohort was used or the study duration was increased. Of note, the decrease in systolic blood pressure observed in this study following exercise participation neared significance ($p=0.059$) when compared to baseline, supporting this suggestion.

An estimate of VO$_{2\text{max}}$ was also obtained at the commencement and following completion of the study in order to analyse the effect of the exercise programme on aerobic capacity. VO$_{2\text{max}}$ may be used to predict the risk of cardiovascular and all-cause mortality, with more deaths occurring in those with a low VO$_{2\text{max}}$ (Reusch et al., 2013). Importantly, individuals with insulin resistance and T2D are likely to have defects in functional exercise capacity when compared to non-diabetic obese/sedentary individuals (Bauer et al., 2007, Nadeau et al., 2010). However, aerobic capacity has been found to improve with exercise participation (Reusch et al., 2013). Several large scale studies, including the HERITAGE Family Study, DREW, INFLAME and STRRIDE have demonstrated beneficial improvements in VO$_{2\text{max}}$ following participation in supervised aerobic exercise programmes (Bouchard et al., 1995, Kraus et al., 2001, Morss et al., 2004, Thompson et al., 2008). Notably, these
observed improvements in aerobic capacity following exercise have also been found with a diabetic cohort, suggesting that exercise may prevent the development of cardiovascular co-morbidities and reduce mortality rates in individuals with diabetes (Reusch et al., 2013). In the non-diabetic, sedentary cohort of the current study, estimated VO$_{2\text{max}}$ was also found to significantly increase (see Fig.2.3) in 15 participants following completion of the 8 week moderate-intensity exercise intervention programme, when compared to baseline (in the remaining participants, baseline VO$_{2\text{max}}$ levels were below the measurable range and, therefore, comparisons could not be made with post-exercise levels). Hence, it is assumed that an exercise intervention similar to the one used in this study is sufficient to improve aerobic capacity and, consequently, reduce the risk of cardiovascular associated mortality.

Similar to VO$_{2\text{max}}$, blood lipid levels are modifiable cardiovascular risk factors. Dyslipidemia has implications in both T2D and CVD; the typical profile contributing to these diseases consists of elevated serum levels of LDL and triglyceride, combined with low HDL levels (DeFronzo et al., 2011). In particular, elevated LDL (particularly small dense LDL particles) and decreased HDL has been found to majorly contribute to CVD, whereas triglycerides appear to have less of an impact (Bitzur et al., 2009). On the other hand, triglycerides have been found to be associated with accelerated T2D development. It is thought that elevated circulating free fatty acids contribute to impaired glucose metabolism by insulin sensitive tissues, resulting in decreased glycogen synthesis and glucose uptake in muscle and increased glucose output from the liver. In the liver, free fatty acid metabolites, mainly diacylglycerol, contribute to the production of triglycerides. Thus, triglycerides act as a marker of insulin resistance-inducing fatty acid metabolite levels (Samuel et al., 2010).
The reported effects of exercise on blood lipid levels are varied, with most reporting reductions in LDL levels (Colberg et al., 2010). Others have reported increases in HDL levels following exercise participation (Butcher et al., 2008, Balducci et al., 2010). Furthermore, a recent meta-analysis revealed that isolated aerobic exercise may reduce total cholesterol and triglyceride levels (Thorogood et al., 2011). In contrast, many have found no change in blood lipid levels upon exercise participation (Colberg et al., 2010). In this study, serum total cholesterol, LDL and HDL levels were not found to alter significantly following exercise participation, when compared to baseline levels (see Fig.2.4). Surprisingly, these results are in disparity with Butcher et al. (2008) (from which the exercise programme used in this study was devised) who found significant reductions in total cholesterol and increases in HDL, with no change in LDL. There is a possibility that gender differences may have played a role in these conflicting results. Whereas this study used a solely female cohort, Butcher and colleagues carried out their research on both males and females. Women are known to have higher levels of HDL when compared to males due to hormonal differences (Perez-Lopez et al., 2010). Thus, it is likely that marked improvements in HDL may not have been observed in the female participants upon exercise completion of this study due to already elevated levels. On the other hand, serum triglyceride levels were found to reduce significantly upon study completion (see Fig.2), correlating with the findings by Kelley et al. (2006) and Thorogood et al. (2011), in their meta-analyses of results from studies investigating the effects of aerobic exercise on lipid levels. These results suggest that participation in an 8 week, moderate-intensity exercise programme may be beneficial in preventing T2D onset and the development CVD by inhibiting the production of insulin resistance-inducing fatty-acid metabolites and, thus, hepatic triglyceride synthesis. In order to further investigate this, it may be interesting to
determine hepatic levels of fatty acid metabolites, such as diacylglycerol, following exercise participation.

In order to assess the impact of the 8 week, moderate-intensity exercise programme on T2D risk, serum glucose and insulin levels were analysed and used to calculate insulin sensitivity at commencement and completion of the study. Non-significant changes in serum glucose and insulin levels were found following study participation (see Fig. 2.5). These findings are not unexpected in non-diabetic individuals upon participation in non-glycogen depleting exercise (such as that used in this study), where elevated glucose uptake is usually matched by hepatic glucose synthesis and secretion, allowing for maintenance of blood glucose levels (Colberg et al., 2010, Jenkins and Hagberg, 2011). However, although individuals were not considered insulin resistant throughout the duration of the study (baseline: 49.9 ± 12.4 pmol/L, 8 weeks: 34.2 ± 4.6 mmol/L) as defined by mean fasting insulin values >84.7 pmol/L, mean insulin sensitivity, as calculated using the McAuley’s ISI, was shown to improve significantly upon study completion when compared to baseline (see Fig. 2.7) (McAuley et al., 2001). In contrast, when calculated via HOMA2, non-significant changes were found in HOMA_insulin resistance values, HOMA_%B and HOMA_%S (see Fig. 2.6), although HOMA_%S neared significance (p=0.07). In diabetic studies, the HOMA index is the most widely used surrogate marker for insulin sensitivity, due to its high correlation with results obtained by ‘gold standard’ methods (Antuna-Puente et al., 2009). Nonetheless, the efficacy of the HOMA index for estimations of insulin sensitivity is reduced in non-diabetic cohorts (Katz et al., 2000). In correlation, others have found less pronounced improvements in HOMA indices in non-diabetic individuals when compared to those with diabetes following an aerobic exercise intervention (Jenkins and Hagberg, 2011). In contrast, the McAuley’s ISI has
been found to be a more specific and sensitive surrogate measure of insulin sensitivity in normoglycaemic individuals, in whom fasting triglyceride levels and BMI strongly correlated with insulin sensitivity (McAuley et al., 2001, Ascaso et al., 2003). This may explain the conflicting results obtained in this study with regards to insulin sensitivity measurements whilst also supporting the use of the McAuley’s ISI over HOMA calculations to demonstrate the beneficial impact of the exercise intervention on insulin sensitivity and the associated reduction in T2D risk. Since improvements in insulin sensitivity are known to be majorly beneficial in terms of T2D prevention and treatment, this finding is hugely relevant in a T2D context and supports the use of a similar exercise regime in order to prevent the onset and progression of this disease.

In conclusion, participation in the 8 week moderate-intensity exercise intervention beneficially altered key predictors for T2D and CVD risk, including body weight, BMI, waist circumference, triglyceride levels, aerobic capacity and insulin sensitivity in high risk, sedentary females. Thus, despite the need to repeat these investigations in a male cohort, it is suggested that the prescription of a moderate-intensity brisk walking programme, similar to the one used in this study, may be beneficial for the prevention of T2D and its cardiovascular complications in the largely sedentary and obese UK population. Importantly, however, these risk factor measurements do not provide insights into the mechanisms by which the observed exercise-associated beneficial effects were brought about. Therefore, given the body of evidence reviewed in Chapter 1 that identifies M2 monocyte-macrophage polarisation to be beneficial in the context of T2D/CVD, the next chapter will focus on the impact of the current exercise intervention on the expression of markers of polarisation in monocytes isolated from exercising participants.
CHAPTER 3

Participation in Regular Exercise Alters Markers of M1/M2 Polarisation in Monocytes: A Potential Role for PPARγ and IL-6

3.1. Introduction
The specific mechanisms by which physical activity elicits its beneficial effects with regard to insulin resistance and T2D are not entirely known, however one potential mechanism involves the impact of physical activity on inflammation. The chronic low-grade inflammation, which accompanies inactivity and obesity, has been shown to promote insulin resistance (Gleeson et al., 2011). Inflammatory mediators, including TNFα, MCP-1, IL-1β and IL-6, have been indicated to directly contribute to insulin resistance by disrupting insulin signalling, thus impairing its function (Gustafson, 2010, Olefsky and Glass, 2010). However, there is evidence that in some contexts, IL-6 may also have anti-inflammatory effects and, accordingly, it has been shown to play a beneficial role in preventing obesity-induced insulin resistance (Petersen and Pedersen, 2005, Ropelle et al., 2010, Mauer et al., 2014). Interestingly, physical activity has been associated with an initial rise in serum levels of IL-6, which is followed by an increase in anti-inflammatory cytokine synthesis, including IL-10, IL-1Ra and soluble TNF-α receptor (sTNF-R). These late-phase cytokines function by resolving inflammation and diminishing the initial pro-inflammatory response (Petersen and Pedersen, 2005, Golbidi et al., 2012).

Further to its effect on circulating cytokines, physical activity has been shown to have a beneficial impact on immune cell content of tissues, and subsequent local and systemic inflammation. More specifically, physical activity is thought to play an
important role in influencing macrophages away from the ‘M1’ phenotype (Ikeda et al., 2013, Oliveira et al., 2013). ‘M1’, pro-inflammatory macrophages are said to majorly contribute to the inflammation which drives insulin resistance by producing insulin resistance-inducing inflammatory mediators, including TNFα and MCP-1, and promoting immune cell infiltration into tissues (Osborn and Olefsky, 2012). Interestingly, the number of M1 macrophages has been shown to be elevated in the adipose tissue of obese individuals, perhaps explaining the pronounced increase in T2D risk in this cohort (Murray and Wynn, 2011a). Depletion of these cells or inhibition of pro-inflammatory pathways within these cells, such as NF-κB and JNK pathways, has been shown to reduce both local and systemic inflammation and, correspondingly, insulin resistance status (Huang et al., 2010, Romeo et al., 2012). M1 macrophages have also been implied in the development and progression of T2D-associated complications, such as atherosclerosis and CVD (Oh et al., 2012). On the contrary, following participation in exercise, macrophages have been found to adopt a less inflammatory, ‘M2’ phenotype which may be beneficial in the prevention of insulin resistance and, subsequently, T2D and its complications (Ikeda et al., 2013, Oliveira et al., 2013).

The exact mechanisms behind the ability for physical activity to induce M2 macrophage polarisation remains to be determined but it is possible that this phenomenon may occur as a result of changes in systemic inflammatory cytokine production (Balducci et al., 2010). In general it has been found that systemic levels of activators of M2 macrophage polarisation, such as IL-4 and IL-13, do not tend to change in response to exercise (Suzuki et al., 2000, Malm et al., 2004, Peake et al., 2005). Although, a recent study found that a combination of high-intensity aerobic and resistance exercise administered over a 12 month period significantly increased
circulating levels of IL-4 when compared to baseline values in individuals with T2D (Balducci et al., 2010). Furthermore, IL-4 mRNA expression was found to increase in mixed mononuclear cells following participation in low-intensity exercise (Yakeu et al., 2010). Thus, there is a potential for exercise to induce M2 polarisation by increasing IL-4 synthesis.

Another way in which physical activity may induce M2 polarisation is via activation of the nuclear transcription factor, PPARγ in monocytes/macrophages (Butcher et al., 2008, Yakeu et al., 2010, Thomas et al., 2012). Activation of PPARγ in monocytes has been deemed ‘critical’ for the priming of these macrophage precursors for the M2 phenotype (Bouhlel et al., 2007). Furthermore, PPARγ activation in macrophages has been suggested to be essential for M2 polarisation, with the deletion of this transcription factor resulting in impaired alternative activation of monocytes/macrophages (Odegaard et al., 2007). Thus, PPARγ is believed to be a key regulator of M2 polarisation (Murray and Wynn, 2011b). Interestingly, PPARγ expression and activity has been found to increase in mixed peripheral mononuclear cells (PMNCs; cells which include monocytes) following exercise participation (Butcher et al., 2008, Yakeu et al., 2010, Thomas et al., 2012). Concordantly, Yakeu et al. (2010) found that, following exercise participation, human PMNCs expressed higher levels of markers of M2 polarisation alongside elevated levels of PPARγ associated genes. It is yet to be elucidated, however, whether activation of PPARγ upon exercise occurs in a ligand dependent manner (and, if so, the type of ligands produced) and/or whether PPARγ-activating post-translational modifications are induced via physical activity participation. Additionally, the effects of physical activity on PPARγ activity specifically within monocytes have, to the author’s
knowledge, not yet been shown. Nonetheless, these findings suggest a potential role for physical activity-induced PPARγ activation in M2 macrophage polarisation.

Ultimately, M2 macrophage polarisation (and PPARγ activation) has been associated with improved insulin sensitivity, leading to the conclusion by Odegaard et al. (2007) that “Macrophage polarisation towards the alternative state might be a useful strategy for treating insulin resistance and Type 2 diabetes”. Thus, further research into this area may aid to elucidate the effects of physical activity on monocyte/macrophage phenotype, so as to provide additional evidence for the impact of physical activity on macrophage polarisation and the mechanisms by which physical activity beneficially alters inflammation, thus improving insulin resistance and T2D risk. In line with this, the present research aimed to determine the effects of an 8 week, moderate-intensity exercise programme on markers of M2 polarisation in isolated human monocytes and also to evaluate the role of PPARγ in this process.
3.2. Methods

Refer to sections 2.2.1 - 2.2.5 for details of participants, inclusion/exclusion criteria, ethical approval, pre-health screening and exercise programme design.

3.2.1. Sampling Techniques

3.2.1.1. Collection of Blood Samples

Participants were fasted for 12 hours prior to blood collection. Blood was collected at four time points; immediately prior to (‘baseline’) and immediately following (‘post 1’) the first exercise session and immediately prior to (‘8 weeks’) and immediately following (‘post 8 week’) the final exercise session. Blood was collected by venepuncture of the antecubital vein in ethylenediaminetetraacetic acid (EDTA) or in plain tubes for serum collection. All phlebotomy equipment was provided by Greiner VACUETTE (Gloucester, UK) and all phlebotomy was conducted by trained phlebotomists, following completion of a blood donation questionnaire to ensure safe practice. Samples were treated according to the University of Wales Institute’s human tissue act (HTA) guidelines.

3.2.1.2. Serum Collection

Serum was collected as described in section 2.2.6.2.

3.2.2. Blood Fractionation

Blood collected in EDTA was fractionated using density gradient centrifugation; Histopaque®-1077 (Sigma-Aldrich, Dorset, UK) was carefully layered on top of whole blood samples and centrifuged at 400 times gravity (xg) for 30 minutes at RT to fraction. The mononuclear cell layer was transferred into a centrifuge tube, washed several times in isotonic phosphate buffered saline (PBS) solution and centrifuged at 250 xg for 10 minutes to pellet mononuclear cells. Contaminating platelets were
removed by centrifuging at 200 xg for 10 minutes, following which the cell pellet was used in subsequent monocyte isolation procedures.

3.2.3. Monocyte Isolation

Monocytes were isolated from mononuclear cells via magnetic cell isolation using CD14 MACS MicroBeads (Miltenyi Biotec, Germany). These magnetic beads were used to directly label monocytes by binding to CD14 molecules on their cell surface prior to passing cells through a MACS Column (containing a matrix of ferromagnetic spheres) which is held in a magnetic separator, causing labelled cells to be retained in the column. In this way, purified isolated monocytes were obtained for use in this study (see Appendix, Fig. A1.2).

CD14 MACS MicroBeads were used according to manufacturer’s instructions. Briefly, mononuclear cells (obtained as per section 3.2.2) were incubated with CD14 MicroBeads before being passed through a 30μm nylon mesh filter directly into a LS MACS Columns held in a QuadroMACS™ Separator. Labelled cells were removed from the column and retained for ribonucleic acid (RNA) and protein isolation.

3.2.4. RNA Extraction

TRI Reagent® (Applied Biosystems, Warrington, UK), a commercially available product used for total RNA isolation, was used to induce cell lysis whilst inhibiting RNase activity due to its phenol and guanidine thiocyanate components. Following cell lysis, a phenol/chloroform based method was used for RNA extraction. Nuclease-free pipette tips, and microcentrifuge tubes were used throughout RNA extraction and all procedures were carried out in sterile working conditions.

Briefly, following monocyte isolation (as described in section 3.2.3), cells were resuspended in 1 ml TRI Reagent®. The homogenate was mixed with chloroform to
produce an aqueous RNA phase which was subsequently mixed with isopropanol to precipitate RNA. RNA pellets were washed with 75% ethanol, dried and resuspended in nuclease-free water prior to storage at -80°C.

3.2.4.1. RNA Quantification and Assessment of Purity
RNA samples were quantified and purity was assessed using the NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Leicester, UK). Measurements of absorbance at 260nm allowed for the quantification of RNA. RNA concentrations of <10ng/μl were considered too low for use in reverse transcription polymerase chain reaction (RT-PCR) assays and were not processed.

Measurements were also taken at 280nm in order to calculate the ratio of absorbance at 260nm and 280nm, to assess nucleic acid purity. For RNA, a 260:280 ratio of approximately 2.0 is considered ‘pure’ RNA, with lower ratios indicating contamination with products that absorb at 280nm, including proteins or phenol (NanoDrop Technologies, 2007). Only samples with a 260:280 ratio of >1.8 were used in this study to enhance PCR efficiency (Filion, 2012). Additionally, the spectral pattern created following spectrophotometry of RNA samples was analysed to ensure one main peak at approximately 260nm to further confirm RNA purity.

3.2.5. Conversion of RNA to cDNA
RNA was converted into single-stranded complementary deoxyribonucleic acid (cDNA) using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Warrington, UK). Briefly, RNA samples were normalised based on concentration and MultiScribe™ Reverse Transcriptase, random primers, deoxyribonucleotide (dNTP) Mix and buffer, were added at a ratio of 1:1 to each RNA sample. Samples were converted to cDNA using a 96-Well GeneAmp PCR system 9700 (Applied
Biosystems, Warrington, UK) thermal cycler under the following thermal cycle: 25°C for 10 minutes; 37°C for 120 minutes; 85°C for 5 minutes; 4°C for ∞. cDNA samples were stored at -20°C.

3.2.6. Gene Expression Analysis via RT-PCR

RT-PCR was carried out using Fast SYBR® Green or TaqMan® Fast Universal, No AmpErase® UNG assays in combination with an Applied Biosystems Fast 7500 Real-Time PCR System, as per manufacturer’s instructions (Applied Biosystems, Warrington, UK).

3.2.6.1. Primer Design (Fast SYBR® Green)

For assays using Fast SYBR® Green, primers for genes of interest and housekeeping genes were designed using the National Center for Biotechnology Information’s (NCBI’s) primer designing tool, ‘Primer-BLAST’. All primers were designed to be specific for the gene of interest/housekeeping gene, producing an amplicon between 50-150 base pairs in length. Primer length was designed to be between 18-22 base pairs, GC content was between 30-80% and melting temperature (T_m) was between 58-60°C. Primers contained less than four di-nucleotide repeats and runs of identical nucleotides were avoided, particularly G residues of which 4 or less consecutive residues were ensured. Additionally, the last five nucleotides at the 3’ end contained no more than 2 G and/or C bases. Primer pairs were also separated by at least one intron on corresponding genomic DNA to ensure amplification of the cDNA template only. Primers were made to order (Sigma Aldrich, Dorset, UK) and reconstituted in 1X Tris-EDTA (TE) buffer, pH 8.0 (Invitrogen Ltd, Paisley, UK) to obtain a concentration of 100 mM. Primers aliquots were stored at -20°C. The sequences of primers used within this chapter are given in table 3.1.
3.2.6.2. Primer Optimisation (Fast SYBR® Green)

Primer optimisations were carried out to determine optimal forward (F) and reverse (R) primer volumes required for efficient RT-PCR via Fast SYBR® Green. The primer volumes giving the highest fluorescence signal i.e. delta Rn (ΔRn), and greater amplicon (lower Cycle threshold (Ct) value) were deemed optimal (see Appendix, A2.1). Additionally, dissociation curves were generated for each PCR reaction to ensure specificity of gene amplification (see Appendix, Fig. A2.2).

3.2.6.3. PCR Efficiency (Fast SYBR® Green and TaqMan®)

PCR amplification efficiency was analysed for both Fast SYBR® Green and TaqMan® methods. This was determined by using serial dilutions of cDNA over at least a 3-log (or 6-log where possible) dilution range to generate Ct values for the gene of interest/housekeeping gene. Ct values were plotted against log [cDNA] and the equation of the semi-log regression line was used to calculate PCR efficiency (E), using the following equation:

$$E = (10^{(-1/slope)} - 1) \times 100$$

Efficiency of 100 ±10% were used (see exemplar data in Appendix, Fig. A2.3).

The comparative C_T method was employed to calculate relative gene expression. This method relies on use of a housekeeping gene to standardise the RNA concentration prior to deducing gene expression relative to a reference or calibrator sample. Using this method, relative gene expression was calculated using the following formula:

$$2^{-\Delta\Delta C_T}$$

Where ΔC_T is the difference between Ct values of the gene of interest and housekeeping gene, and ΔΔC_T is the difference between ΔC_T of relative samples.
When using the comparative C<sub>T</sub> method, PCR efficiencies of the gene of interest and housekeeping gene must be equivalent (Biosystems, 2008); thus, validation was carried out for all genes of interest and housekeeping genes.

3.2.6.4. RT-PCR using Fast SYBR® Green and TaqMan® Chemistries

Fast SYBR® Green RT-PCR was used to analyse gene expression of markers of M2 activation, Dectin-1, IL-10 and IL-1Ra, the marker of M1 activation, TNFα, and the PPARγ-associated genes, CD36, LXRα, ABCA1, PGC-1α and COX-2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. β-actin was also used to normalise data, giving similar results as GAPDH (data not shown) (see Table 3.1 for primer sequences).

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CATTGACCTCAACTACATG</td>
<td>TCTCCATGGTGTTGGAAGAC</td>
</tr>
<tr>
<td>β-actin</td>
<td>TCTCTGTGGCATCCACGAA</td>
<td>GAAGCATTTGCGGTTGAC</td>
</tr>
<tr>
<td>Dectin-1</td>
<td>GGAAGCAACACATTTGGAATGG</td>
<td>CTTTGCTAGGAGTCACACTGTC</td>
</tr>
<tr>
<td>IL-10</td>
<td>ACGGCCTGTGATCATCGATT</td>
<td>TTGGACCTTATTAAGGACTTCTTC</td>
</tr>
<tr>
<td>IL1Ra</td>
<td>GGCTCCGCACGTACCTAATCAC</td>
<td>GACAGGCACTCCTCCTCCCTAT</td>
</tr>
<tr>
<td>TNFα</td>
<td>TGCTCTGTCATCCTGAGTA</td>
<td>CTGGCCAGAGGCTGATAGACA</td>
</tr>
<tr>
<td>CD36</td>
<td>GGAAGTGATGATGAAACAGGC</td>
<td>GAGACTGTGTTGTCTCAGCGT</td>
</tr>
<tr>
<td>LXRα</td>
<td>CAGACTACATCGCCACAGG</td>
<td>TGAGCAGGATCTGTCTCTCTCT</td>
</tr>
<tr>
<td>ABCA1</td>
<td>GCACTGAGGAAGATGCTGAAA</td>
<td>AGTTCCGGAGGTTCTGCTGTCT</td>
</tr>
<tr>
<td>PGC1α</td>
<td>TCGCTCCTCGTGAGGGACA</td>
<td>TGCTTCGTCGTCAAAAAACAG</td>
</tr>
<tr>
<td>COX-2</td>
<td>TGAACCCACTTCAACACA</td>
<td>GAGAAAGGCTCCAGCTTT</td>
</tr>
</tbody>
</table>

Table 3.1: Primer sequences used in SYBR® Green RT-PCR assays.
TaqMan® RT-PCR was used to analyse gene expression of the M1 marker, MCP-1 and of PPARγ. The housekeeping genes GAPDH and glucuronidase β (GUSB) were used to standardise cDNA input values. PrimeTime® Assays (Integrated DNA Technologies, Iowa, US), composed of two primers and a hydrolysis probes were selected for use in TaqMan® assays and only those which detected all genetic variants but not genomic DNA and those in which exon boundaries were consolidated were selected for use (Assay Configuration: 6-FAM/ZEN/IBFQ, P:P 2.0). PrimeTime® Assays were resuspended in TE buffer to a final stock concentration of 20X for PrimeTime Std qPCR Assay, according to manufacturer’s instructions, for a working concentration of 500nM primers and 250nM probe and stored at -20ºC.

Fast SYBR® Green and Taqman® RT-PCR was carried out as per manufacturer’s instructions, using the Applied Biosystems Fast 7500 Real-Time PCR System (Applied Biosystems, Warrington, UK). Thermal cycling conditions were set as follows:

\[
\text{95ºC for 20 seconds; 95ºC for 3 seconds; 60ºC for 30 seconds}
\]

\[\text{HOLD}\]

\[\text{40 CYCLES}\]

Amplification plots were used to generate Ct values for genes of interest and housekeeping genes and ΔCt was calculated. The comparative C\(_T\) method (see section 3.2.6.3) was then used to calculate gene expression relative to the reference sample. Baseline samples, i.e. those taken prior to the 8 week exercise programme, were used as reference samples. A non-template control (NTC; nuclease free water instead of cDNA) was included for each housekeeping gene/gene of interest. Only Ct values of <35 were used in analysis.
3.2.7. Serum Cytokine Analysis

Enzyme-linked immunosorbant assay (ELISA) was used to determine cytokine levels in serum samples; the Human IL-4 High Sensitivity ELISA (eBioscience, Vienna, Austria) was used to quantify IL-4 and The RayBio® Human IL-13 ELISA Kit (Insight Biotechnology Ltd, Middlesex, UK) was used to quantify IL-13 in ‘baseline’ and ‘8 weeks’ serum samples in order to determine the chronic effects of participation in the exercise study. The Quantikine® HS ELISA Human IL-6 Immunoassay (R&D Systems, Abington, UK) was used to quantify serum IL-6 in ‘baseline’ and in ‘post 1’ serum samples so as to determine the acute effects of participation.

All assays were carried out according to manufacturer’s instructions in undiluted serum samples, with all samples, standards and blanks analysed in duplicate. Spectrophotometry was carried out on a Tecan Infinite 200 (Tecan, Reading, UK). The average absorbance value from blank wells was removed from those obtained from standard/sample wells. A calibration curve was generated from standard absorbances and used to quantify cytokine concentrations in unknown samples.

3.2.8. Western Blotting

The expression of PPARγ in isolated monocyte samples was determined by Western blot. PPARγ-transfected human embryonic kidney cells (HEK-293T) were used as positive controls. All reagents were obtained from Sigma Aldrich (Dorset, UK) and antibodies from New England Biolabs (Herts, UK), unless otherwise stated.

3.2.8.1. Protein Extraction

Following monocyte isolation (as described in section 3.2.3), cells were centrifuged and resuspended in 112μl Radio-Immunoprecipitation Assay (RIPA) Buffer (with phosphatase and protease inhibitors) for 30 minutes, mixing every 10 minutes, to
extract cellular proteins. Samples were sonicated using a VCX 500 Ultrasonic Processor (Sonics & Metrials (UK) Ltd., Suffolk, UK) to shear DNA and particulate material was removed by centrifugation. Protein lysates were stored at -80°C.

3.2.8.2. Quantification of Protein Concentration

The Bio-Rad DC Protein Assay (Bio-Rad, Hertfordshire, UK) was used to quantify protein in samples. Briefly, bovine serum albumin (BSA) standards or samples were added to the wells of a microtitre plate. Reagent A was prepared and added to each well of the plate. Reagent B was then added to each well and the plate was incubated for 15 minutes, following which absorbance was measured at 620nm. A standard curve was used to determine the concentration of protein in the unknown samples.

3.2.8.3. Protein Separation by SDS-PAGE Electrophoresis

Samples and the control were normalised to a concentration of 15μg/μl protein by diluting accordingly in lysis buffer (RIPA) to a total volume of 15μl. 3.75μl of 4X NuPAGE lithium dodecyl sulphate (LDS) Sample Loading Buffer (Invitrogen Ltd, Paisley, UK) and 1.5μl of 10X Dithiothreitol (DTT; reducing reagent, Invitrogen Ltd, Paisley, UK) was added to samples prior to mixing and denaturing proteins by heating to 80°C for 5 minutes in a 96-Well GeneAmp PCR system 9700 (Applied Biosystems, Warrington, UK) thermal cycler, cooling samples to 4°C before removal.

NuPAGE® 12-well, 10% Bis-Tris, 1.0mm Gels (Invitrogen Ltd, Paisley, UK) were used to separate proteins. NuPAGE® gels were inserted into an XCell SureLock™ Mini-Cell Electrophoresis System tank (Invitrogen Ltd, Paisley, UK) and the inner chamber was filled with 1X 3-(N-morpholino) propane sulphonic acid (MOPS) running buffer (Invitrogen Ltd, Paisley, UK). Wells were washed with 1X MOPS buffer prior to loading samples. Additionally, a molecular weight standard marker,
MagicMark™ (Invitrogen Ltd, Paisley, UK), was loaded in the first well of each gel. The outer chamber was filled with 1X MOPS buffer and electrophoresis was carried out at 200V for 1h and 10 minutes at 4°C.

3.2.8.4. Electrophoretic Transfer of Proteins

The iBlot® Dry Blotting System (Invitrogen Ltd, Paisley, UK) was used to transfer proteins from the gel to nitrocellulose membranes. All reagents were obtained from Invitrogen Ltd (Paisley, UK), unless otherwise stated.

Following protein separation, the gel was equilibrated in 2X NuPAGE® Buffer plus 10% methanol and 1:1000 NuPAGE® antioxidants. The ‘Bottom Stack’ (composed of a copper anode and a 0.2μM nitrocellulose membrane) was inserted into the iBlot® Dry Blotting System. The gel was then layered onto the nitrocellulose membrane and covered with filter paper which had been pre-soaked in distilled water. Air bubbles were removed using a blotting roller and the ‘Top Stack’, (composed of a copper cathode) was layered on top. Air bubbles were removed and a sponge was applied to the system to firmly sandwich the transfer stack. Protein transfer was carried out at 20V for 7 minutes.

3.2.8.5. Immunoblotting of Proteins

In order to determine the presence of PPARγ in isolated monocyte samples, a PPARγ (81B8) rabbit monoclonal antibody was used at a 1:1000 dilution. Primary antibody dilutions were made in 5% BSA diluted in Tris-buffered saline containing 0.1% Tween-20 (TBS-T, Tween-20 obtained from Thermo Fisher Scientific, Leicester, UK). The PPARγ antibody recognises both PPARγ isoforms 1 and 2, producing molecular bands at 53 and 57 kDa, respectively. A goat anti-rabbit IgG, HRP-linked
antibody was used as a secondary antibody at a 1:1000 dilution in 0.25% non-fat dried milk in TBS-T.

Briefly, the membrane was washed in TBS-T and blocked with 5% non-fat dried milk diluted in TBS-T for 1 hour at RT, on an orbital shaker. The membrane was washed again and incubated with the PPARγ primary antibody overnight at 4°C. Following this, the membrane was washed in TBS-T and incubated with the secondary antibody for 1 hour at RT, whilst shaking. Subsequently, the membrane was washed and incubated 6ml (0.1ml/cm²) of Amersham™ ECL Select™, as per manufacturer’s instructions. To visualise immunoreactive bands, the membrane was placed between two acetate sheets, with air bubbles being removed using a blotting roller and exposed to Kodak® BioMax® (Sigma Aldrich, Dorset, UK) X-Ray film in an autoradiography hypercassette (GE Healthcare Life Sciences, Buckinghamshire, UK).

3.2.8.6. Peptide Competition Assay

To confirm specific band reactivity of the PPARγ (81B8) rabbit monoclonal antibody used in Western blotting (see section 3.2.8.5), a blocking peptide specific to the primary antibody was used.

Monocyte protein samples were isolated quantified and denatured, as described in section 3.2.8.1 – 3.2.8.3. Selected ‘baseline’, ‘Post 1’ and ‘8 weeks’ samples were loaded into the wells of a NuPAGE® 10-well, 10% Bis-Tris, 1.0mm Gels, as described in section 3.2.8.3. Additionally, differentiated 3T3-L1 cell lysate (obtained ready to load, in sodium dodecyl sulfate (SDS) sample buffer; New England Biolabs, Herts, UK) was loaded as a positive control. Samples were loaded so that wells 2 to 5 and wells 7 to 10 were identical, with MagicMark™ being loaded in the first well and a pre-stained protein ladder (PageRuler; Thermo Fisher Scientific, Leicester, UK) being
loaded in well 6. Protein Separation and transfer was carried out as described in sections 3.2.8.3 and 3.2.8.4, except that separation was carried out at 200V for 1h 30 minutes to ensure adequate separation of PPARγ isoforms. The membrane was washed in TBS-T and blocked with 5% non-fat dried milk diluted in TBS-T for 1 hour at RT, whilst shaking. The membrane was washed again and cut down the middle (using the pre-stained ruler as a visual guide), in order to obtain two identical membranes. One half of the membrane was incubated with a 1:1000 dilution of the PPARγ primary antibody, diluted in 5%BSA in TBS-T, as described in section 3.2.8.4. Simultaneously, the other half of the membrane was incubated with 1:1000 PPARγ antibody solution which had been pre-incubated with an equal dilution (1:1000, diluted in 5% BSA in TBS-T) of blocking peptide for at least 2 hours at RT. Membranes were incubated with the primary antibody or primary antibody-blocking peptide solution overnight at 4°C. The membrane was washed, incubated with the secondary antibody, washed again and bands were visualised, as described in section 3.2.8.4. Comparisons were made between membranes incubated with the PPARγ antibody alone or in combination with the blocking peptide.

3.2.9. Analysis of PPARγ Activation via Dual Gene Reporter Assay

Serum obtained from study participants was used to investigate the effects of the exercise intervention on PPARγ binding to the PPARγ response element (PPRE) and on the availability of PPARγ ligands. Dual gene reporter assays were employed to investigate these cellular processes. These assays make use of an ‘experimental’ reporter; a DNA sequence (plasmid) cloned with a reporter gene, producing a measurable signal which correlates with the cellular process to be measured (in this instance PPARγ binding to the PPRE or ligand binding to the LBD). Additionally, a ‘control’ reporter is used as an internal control which is indicative of transfection
efficiency and provides a baseline reporter signal. In this study, the experimental constructs and control constructs were co-transfected into HEK293T cells. Furthermore, PPARγ expression vectors were also transfected into cells for the detection of exercise induced changes in PPARγ-to-PPRE or ligand-to-LBD binding.

N.B. Gene reporter assays were also attempted with the human monocytic cell line, THP-1 cells (see Appendix, Fig. A3.1). However, these cells proved too difficult to transfect and were therefore deemed not suitable for use with the gene reporter assay systems.

3.2.9.1. HEK293T Cell Culture

HEK293T (ATCC, Middlesex, UK) are epithelial cells with high transfectability. All tissue culture consumables were manufactured by Corning® (Sigma Aldrich, Dorset, UK) and all reagents were obtained from Invitrogen (Invitrogen Ltd, Paisley, UK), unless otherwise stated. Cells were grown in complete Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% US Defined, Irradiated and Heat inactivated Fetal Bovine serum (FBS; Thermo Fisher Scientific, Leicester, UK), 100U/ml penicillin/100µg/ml streptomycin mix and 4mM L-glutamine.

Briefly, vials containing HEK293T cells were removed from liquid nitrogen and thawed rapidly by briefly immersing in a water bath set at 37°C, with constant agitation. Vial contents were transferred into a centrifuge tube and pre-warmed DMEM was added. Cells were centrifuged at 200 xg for 10 minutes, following which cells were resuspended in fresh pre-warmed DMEM and transferred into a 25 cm² cell culture flask. The culture was then placed in a 37°C, 5% CO2, humidified incubator overnight. The next day, cells were examined under a microscope to ensure healthy
growth and adherence. Subsequently, medium was aspirated from cells to remove
dead cells and fresh pre-warmed DMEM added.

Cells were split every 2-4 days, when 70-80% confluency was reached, plating at a
density of 100 cells/mm. Medium was removed and the cells washed with pre-warmed
sterile PBS (calcium and magnesium free). Cells were incubated with 0.25% trypsin-
EDTA for 1-2 minutes at 37°C. DMEM was added at thrice the volume of trypsin used
to stop trypsinisation. Cells were centrifuged and resuspended in fresh DMEM. Cells
were counted using a haemocytometer, checking for cell viability using the trypan blue
exclusion method, whereby 0.4% trypan blue (Sigma Aldrich, Dorset, UK) was added
to an equal volume of cells. Since viable cells with intact cell membranes do not take
up the dye but dead cells do, cells stained blue could be detected under a microscope
and percent cell viability was determined. Only cultures with >5% cell viability were
used for investigations. The desired number of viable cells was then transferred to a
25 cm² culture flask and DMEM was added (0.25 ml/cm²). The plate was incubated
in a 37°C, 5% CO2, humidified incubator.

In order to maintain the HEK293T stock, frozen cultures were prepared frequently.
Cells were trypsinised and counted as explained above. Cell suspensions were
dispensed into a cryotube at 2x10⁶ cells, centrifuged and the cell pellet was
resuspended on ice in 500μl of cold FBS. Subsequently, 500μl of FCS+20% Dimethyl
Sulfoxide (DMSO) was slowly added to cells, bringing the freezing medium to a final
concentration of 10% DMSO in FBS. Cells were then slowly frozen (1°C per minute)
in a ‘Mr Frosty’ Cryo Freezing Container (Sigma Aldrich, Dorset, UK), at -80°C,
overnight, following which vials were transferred to liquid nitrogen for storage.
3.2.9.2. Plasmid Preparation

The PureYield™ Plasmid Maxiprep System (Promega, Southampton, UK) was used to prepare plasmids from frozen glycerol stocks of *E. coli* (JM109 bacterial strain), from working plasmid stocks or from DNA spots on filter paper, for eukaryotic transfection. Kanamycin (100μg/ml) was used to select transformed cultures. All media and culture vessels were sterilised prior to use and bacterial culture techniques were carried out aseptically, with negative controls being used to confirm this. Plastic culture vessels were obtained from Greiner Bio-one (Stonehouse, UK) and all reagents were obtained from Sigma-Aldrich (Sigma-Aldrich, Dorset, UK), unless otherwise stated.

Agar plates were prepared by dissolving 1.2% (w/v) Agar (EMD Millipore, Darmstadt, Germany), 25g of Luria broth (LB) containing 15mM magnesium chloride in 1L of deionised water. For suspension cultures, LB medium was prepared by dissolving 25g LB in 1L deionised water. In both cases, media was sterilised by autoclaving at 121°C and allowed to cool to <50°C before adding Kanamycin to a working concentration of 100μg/ml.

Where a glycerol stock of transformed *E. coli* JM109 was available, transformed cells were streaked onto the agar plates and incubated for 24 hours at 37°C. A single bacterial colony was then re-streaked on a new plate and incubated at 37°C for a further 24 hours. Plates with single transformed bacterial colonies were then stored at 4°C for a maximum of 1 week.

Alternatively, where plasmids were stored as dry spots on filter paper or working plasmid stocks were available, an *E. coli* JM109 transformation protocol was used. Briefly, *E. coli* JM109 were first streaked onto LB agar + magnesium chloride media.
and incubated at 37°C, overnight. Meanwhile, the dry plasmid spot was cut out and incubated with TE buffer (Invitrogen Ltd, Paisley, UK) to obtain a rehydrated plasmid solution. A single bacterial colony was then applied to the bottom of a microcentrifuge tube and incubated on ice. Following this 10μl of the working plasmid stock or the rehydrated plasmid solution was added to the bacteria suspension. Alternatively, 0.1ng of pGem3Z (Promega, Southampton, UK) was added to the bacteria suspension as a positive control or 10μl of water was added as a negative control. Bacteria were heat shocked for 90 seconds at 42°C exactly. Bacterial suspensions were returned to ice for 2 minutes, after which they were added to SOC medium (Sigma Aldrich, Dorset, UK). Cultures were incubated at 37°C for 1.5 hours, shaking at 200rpm. Eppendorf tubes were centrifuged at 13,000xg for 3 minutes and the cell pellet was resuspended in LB. Cultures were spread on agarose media containing antibiotics and plates were incubated for 24 hours at 37°C. Single colonies were re-streaked to obtain single colonies of transformed cells. Transformed cells were stored at 4°C on the cell culture plate for up to 1 week.

Prior to use, plates were returned to an incubator for 30 minutes. Following this, a suspension of transformed bacteria (obtained from glycerol stocks or via the transformation protocol described above) was made by inoculating 5ml of LB + Kanamycin with a single colony of bacteria. Starter cultures were grown for 8 hours at 37°C with vigorous shaking at 250rpm in an orbital shaker (Eppendorf® 5350 Thermomixer; Sigma Aldrich, Dorset, UK). Subsequently, a 1:1000 dilution of starter culture was made using fresh LB medium and incubated for 16-21 hours at 37°C with shaking at 250rpm. The optical density (OD) of cultures was read at 600nm using a spectrophotometer (Jenway 6705 UV/Visible Spectrophotometer; Thermo Fisher Scientific, Leicester, UK) and only cultures with an OD of 1.5-4 were used in
subsequent plasmid purification steps. Alternatively, bacterial suspensions were diluted in glycerol to a final concentration of 25% glycerol and stored at -80°C, creating glycerol stocks for long term storage.

For plasmid purification, cultures were transferred into a centrifuge tube and centrifuged at 5000xg for 10 minutes at RT. Bacterial pellets were frozen at -20°C or used immediately. Plasmids were purified using the PureYield™ MaxiPrep system (Promega, Southampton, UK) as per manufacturer’s instructions. Briefly, cell pellets were completely resuspended in Cell Resuspension Solution and Cell Lysis Solution. Neutralization Solution was then added to lysed cells and lysates were centrifuged at 14,000xg for 20 minutes at RT. The lysate was removed from cell debris and centrifuged at 7,000xg for 30 minutes. DNA was purified by applying the lysate onto a PureYield™ Clearing Column and then through a PureYield™ Maxi Binding Column under maximum vacuum (650mm Hg/25.6 inches Hg of pressure). After slowly releasing the vacuum and removing the filtration device, Endotoxin Removal Wash was added to the Maxi Binding Column and the vacuum was turned back on, allowing the wash to run through the column. Column Wash was then added to the column, following which the membrane was allowed to dry under vacuum. Subsequently, the column was removed from the vacuum and plasmids were eluted into nuclease-free water by centrifugation.

DNA was quantified using the NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Leicester, UK), as described in section 3.2.4.1 and DNA was considered pure if the 260/280 ratio was approximately 1.8. DNA quality was also assessed via agarose gel electrophoresis. Briefly, a 0.8% agarose gel was made by dissolving 0.8g of ultrapure agarose (Invitrogen Ltd., Paisley, UK) in Tris-borate-EDTA solution (1XTBE; made by diluting 10.8g Tris, 5.5g Boric acid and 0.93g
EDTA in 1L of distilled water), heating in a microwave. After cooling, the agarose solution was poured into a horizontal electrophoresis gel tank, fitted with a comb for well formation. The gel was set at RT before covering with 1XTBE. Meanwhile, plasmids were diluted to a concentration of 200ng in nuclease free water, to a total volume of 10μl. 2μl of 6XDNA loading buffer (New England Biolabs, Herts, UK) was added to 10μl of the plasmid sample and samples were carefully loaded into wells of the gel, alongside 6μl of the 1kb DNA ladder (New England Biolabs, Herts, UK). Gel electrophoresis was carried out at a constant voltage of 80V for 1.5 hours, the gel was stained with 0.5μg/ml ethidium bromide for 20 minutes, with gentle agitation and destained with deionised water overnight. Bands were visualised using the UVP BioSpectrum® Imaging System (VWR International Ltd., Leicestershire, UK).

3.2.9.3. PPRE-Luciferase Gene Reporter Assay

PPARγ-binding to the PPRE was measured via gene reporter assay and used to assess exercise-induced PPARγ activation. Comparisons were made to individual baseline levels. Furthermore, a Renilla luciferase reporter plasmid (Pr6tk; Promega, Southampton, UK) was used as an internal control to account for variation in transfection efficiency. On all plates, a non-transfected control (NTC) was included and transfected cells were treated with rosiglitazone (a known activator of PPARγ) as a positive control.

HEK293T were cultured in 25 cm³ flasks, as described in section 3.2.9.1, in media without antibiotics. When confluence was reached, cells were washed with PBS and trypsinised. Cells were counted using a haemocytometer and transferred to wells of an opaque white 96 well plate (Greiner Bio-One CELLSTAR plate, Sigma Aldrich, Dorset, UK) at a cell density of 2x10⁵ cells/well and incubated in a 37°C, 5% CO₂, humidified incubator for 24 hours. Cells were transfected; 100ng of each of the PPRE-
luciferase reporter construct and the PPARγ expression vector (both kindly supplied by Dr M. van Bilsen, Maastricht University, Belgium) and 2ng of the Renilla plasmid were diluted in Opti-MEM® I Reduced-Serum Medium (Invitrogen Ltd, Paisley, UK). Lipofectamine® LTX Reagent (Invitrogen Ltd, Paisley, UK) was added and the plasmid-Lipofectamine® mix was incubated for 30 minutes at RT. Media was aspirated from wells and replaced with 100μl fresh media. 20μl plasmid-Lipofectamine® mix was then added to each well in a dropwise manner. Plates were incubated in a 37°C, 5% CO2, humidified incubator for 24 hours.

The protein concentration of serum samples was quantified as described in section 3.2.8.2 in order to normalise samples to the lowest protein concentration measured (77.85mg/ml) by diluting in media. Normalised serum was added to fresh media at 10% v/v (instead of 10% FBS). Media was removed from cells, replacing with media + 10% normalised serum and cells were incubated in a 37°C, 5% CO2, humidified incubator for 24 hours. Subsequently, the media was removed and wells were gently washed with pre-warmed PBS. 20μl of 1 x passive lysis buffer was added to each well and the plate was incubated for 15 minutes at RT, with gentle rocking. Using a Tecan Infinite 200 plate reader (Tecan, Reading, UK), 100μl of Luciferase Assay Reagent II (LAR II) was auto-injected into a single well to generate a signal from the firefly luciferase reporter. Luminescence was read prior to the reaction being quenched by auto-injection of Stop&Glo® Reagent. Simultaneously, the Renilla luciferase reaction was initiated and luminescence was read. The sequential addition of LAR II and Stop&Glo® was repeated for each well until all wells were read. Luminescence values were normalised with regard to transfection efficiency by use of a ratio of Luciferase-to-Renilla luminescence in each case, and reported as relative light units (RLU).
3.2.9.4. PPARγ-LBD Gene Reporter Assay

Ligand binding to the PPARγ-LBD was also measured via gene reporter assay and used to assess exercise-induced PPARγ activation specifically as a result of ligand binding, hence indicating alterations in PPARγ ligand generation upon exercise. Comparisons were made to individual baseline levels. Furthermore, a β-galactosidase (β-gal) reporter plasmid was used as an internal control (pSV; Promega, Southampton, UK) to account for variation in transfection efficiency. A non-transfected control (NTC) was included and transfected cells were treated with rosiglitazone as a positive control.

HEK293T were cultured in 25 cm³ flasks, as described in section 3.2.9.1, in supplemented medium without antibiotics. When cells became 70-80% confluent, cells were transfected; a TransFast™-plasmid mix was made by adding 2μg of a fusion vector carrying the PPARγ-LBD fused with the GAL4 DNA-binding domain (DBD) (PPARγ-LBD/GAL4-DBD, obtained from Dr J.S. Flier, Harvard Medical School, USA), 2μg of an Upstream Activation Sequence (UAS; GAL4 response element)-luciferase reporter construct (UAS, obtained from Professor V.K. Chatterjee, University of Cambridge, UK) and 0.5μg of β-gal to 1.5ml unsupplemented DMEM and mixing with TransFast™ Transfection Reagent (Promega, Southampton, UK) in a 3:1 charge ratio of TransFast™ (μl) to DNA (μg). The TransFast™-plasmid mix was incubated for 10 minutes. Meanwhile, cells were prepared by removing media and washed in unsupplemented DMEM. DMEM was removed by gentle aspiration and the TransFast™-plasmid mix was applied to cells. Cells were incubated with the plasmids in a 37°C, 5% CO2, humidified incubator for 1 hour, following which 2.5ml of supplemented media (minus antibiotics) was added to cells. Cells were incubated with plasmids in a 37°C, 5% CO2, humidified incubator for a further 24 hours. Following
this, media was removed from cells and cells were trypsinised, as described in section 3.2.9.1. Cells were counted and transferred to wells of a transparent 96-well cell culture plate (Corning® Costar®, Sigma Aldrich, Dorset, UK), at a cell density of 2x10^5 cells/well. Cells were incubated in a 37°C, 5% CO2, humidified incubator for 24 hours. The next day, serum samples normalised to a protein concentration of 77.85mg/ml by diluting in media were added to fresh media at 10% v/v (instead of the addition of 10% FBS). Media was removed from cells and replaced with media + 10% normalised serum. The plate was incubated in a 37°C, 5% CO2, humidified incubator for 24 hours. Subsequently, the media was removed from wells and wells were gently washed with pre-warmed PBS. 120µl of 1 x passive lysis buffer was added to each well and the plate was flash frozen at -80°C for 1 minute prior to storage at -20°C for a minimum of 2 hours. Following this, lysates were thawed for 30 minutes with gentle shaking and centrifuged at 1000rpm for 3 minutes. For firefly luciferase (UAS-luciferase) luminescence readings, 40µl of each lysate was transferred to the wells of an opaque white 96 well plate. Subsequently, using a Tecan Infinite 200 (Tecan, Reading, UK) plate reader, 50µl of LAR II was auto-injected into each well and luminescence readings were taken. Simultaneously, for internal control (β-gal) readings, 40µl of each lysate was transferred into wells of a transparent 96-well cell culture plate and an equal volume of o-Nitrophynyl-beta-D-galactopyranosidase (ONPG; β-gal substrate) was added to wells, incubating for 30 minutes at RT, or until a yellow colour developed. Absorbance was read using the Tecan Infinite 200 plate reader, set at 435nm. The ratio of Luciferase luminescence -to-β-gal OD was used to normalise values, and values were reported as RLU.
3.2.10. Statistical Analysis

All data were expressed as mean ± SEM, unless otherwise stated. Where comparisons were made between the means of two samples, the t-tests analysis was used. One-way ANOVA with Tukey’s post-hoc analysis was used for multiple comparisons within groups of normally distributed data. Statistical analysis was performed using Minitab®16 or GraphPad Prism®5 software and results were deemed significant at $p<0.05$. 
3.3. Results

The data presented represents the effects of participation in an 8 week, moderate-intensity exercise programme. Where possible, the baseline values for each individual were used as comparators, with specific time-points for comparison stated in the text. In some cases, RNA quality or quantity was not sufficient for use in RT-PCR. Similarly, some genes of interest were expressed at levels below the acceptable range of the assay (Ct>35 or <7Ct difference from the NTC) and so were excluded from analysis. All RT-PCR results are displayed as fold relative to GAPDH, however, comparable results were found with the housekeeping gene GUSB or β-actin (data not shown).

3.3.1. Gene Expression of Markers of M1 and M2 Polarisation in Isolated Monocyte Samples

mRNA expression of the marker of M1 polarisation, MCP-1, was significantly reduced to approximately 0.83 ± 0.1 fold \((p<0.05)\) of baseline expression values, following participation in the exercise intervention (see Fig. 3.1). Conversely, as also shown in Fig. 3.1, TNFα was significantly upregulated by 2.2 ± 0.4 fold \((p<0.01)\).

As demonstrated in Fig. 3.2, the M2 marker genes Dectin-1 and IL-10 were also significantly upregulated by 2.6 ± 0.5 fold \((p<0.01)\) and 3.0 ± 0.9 fold, respectively \((p<0.05)\). Also displayed in Fig. 3.2, the mRNA expression of another M2 marker, IL-1Ra was also elevated by 1.3 ± 0.2 fold, showing a non-significant trend towards increased expression \((p=0.099)\).
Figure 3.1. Effect of an 8 week, moderate-intensity exercise intervention on gene expression of the M1 markers, MCP-1 and TNFα. Monocytes were isolated from human blood obtained from sedentary individuals prior to commencement of an 8 week, moderate-intensity walking programme (‘baseline’) and again immediately prior to the final exercise session (‘8 weeks’). RT-PCR was used to analyse mRNA expression markers of M1 polarisation, MCP-1 and TNFα (MCP-1, n=8; TNFα, n=14; values expressed as fold gene expression relative to GAPDH ± SEM;* \( p<0.05 \), **\( p<0.01 \), t-test).
Figure 3.2. Effect of an 8 week, moderate-intensity exercise intervention on the gene expression of the M2 markers, IL-10, Dectin-1 and IL-1Ra. The expression of markers of M2 polarisation (IL-10, Dectin-1 and IL-1Ra) in monocytes, obtained from individuals prior to commencement of an 8 week, moderate-intensity walking programme (‘baseline’) and again prior to the final exercise session (‘8 weeks’), were analysed by RT-PCR (IL-10, n=10; Dectin-1, n=14; IL-1Ra, n=14; values expressed as fold gene expression relative to GAPDH ± SEM; *p<0.05, **p<0.01, t-test).
3.3.2. Serum IL-4 and IL-13 Following Chronic Exercise

IL-4 levels were non-detectable in serum samples, both at baseline and in the ‘8 weeks’ samples (minimum detection levels 0.25 pg/ml; data not shown). Alternatively, IL-13 levels were measurable in n=17 samples but did not alter significantly when comparisons were made between baseline and prior to the final walking bout of the exercise programme (baseline: 10.52 ± 3.43 pg/ml; 8 weeks: 11.20 ± 3.47 pg/ml; see Fig. 3.3).

3.3.3. Serum IL-6 Levels Following Acute Exercise

The acute effects of exercise on serum IL-6 levels were measured via ELISA. Serum IL-6 concentrations increased significantly immediately post exercise (baseline: 1.20 ± 0.21 pg/ml, post 1: 1.63 ± 1.32 pg/ml, p<0.01, see Fig. 3.3).
Figure 3.3. Serum IL-13 and IL-6 concentration in response to an 8 week, moderate-intensity brisk walking intervention. Serum IL-13 (A) and serum IL-6 (B) were measured via ELISA. Serum IL-13 concentration was measured in samples taken at baseline and immediately prior to the final bout of an 8 week, moderate intensity exercise intervention (‘8 weeks’), whereas serum IL-6 was measured in samples taken at baseline and following an acute bout of moderate intensity exercise (‘post 1’) (IL-13, n=17; IL-6, n=19; results expressed as individual data points; significance related to mean values; **p<0.01, paired t-test).
3.3.4. Gene Expression of PPARγ and PPARγ-Associated Genes in Isolated Monocyte Samples

PPARγ mRNA expression was analysed via RT-PCR. As shown in Fig 3.4, PPARγ mRNA expression did not alter significantly in monocytes obtained after 8 weeks of study participation (prior to the final exercise bout), when compared to baseline values (0.9 ± 0.1 fold).

PPARγ-associated genes were also analysed; when compared to baseline, in monocyte samples obtained after 8 weeks of study participation, prior to the final exercise session, the PPARγ coactivator, PGC-1α, was non-significantly increased (1.5 ± 0.6 fold, see Fig 3.4). However, the PPARγ downstream genes, CD36 (1.9 ± 0.5 fold, p<0.05) and Liver X receptor α (LXRα; 5 ± 1.9 fold, p<0.01) were significantly increased (Fig 3.4). In contrast, as shown in Fig 3.4, expression of the ATP-binding cassette transporter, ABCA1, was non-significantly increased as a result of the intervention (1.5 ± 1.9 fold, relative to baseline). In addition, the expression of cyclooxygenase-2 (COX-2), an enzyme associated with PPARγ activation, was also significantly upregulated in monocyte samples obtained prior to the final exercise session, when compared to baseline (3.6 ± 1.1 fold, p<0.01, see Fig 3.4).

3.3.5. Protein Expression of PPARγ in Isolated Monocyte Samples

PPARγ protein expression was analysed in ‘baseline’, ‘post 1’ and ‘8 weeks’ samples. A band corresponding to the PPARγ isoform 2 (at ~ 57 kDa) was detected in isolated monocyte samples, at all exercise time points analysed. Simultaneously, bands corresponding to both PPARγ isoforms 1 and 2 (at ~53 kDa and ~57 kDa, respectively) were detected in the positive control (see Fig 3.5). However, when a PPARγ blocking peptide was used, the ~57 kDa band remained visible in isolated monocyte samples, whilst both PPARγ isoforms disappeared in the positive control (see Fig. 3.5).
Figure 3.4. Effect of an 8 week, moderate-intensity exercise intervention on monocyte expression of PPARγ and PPARγ-associated genes. Monocytes were obtained at baseline and immediately prior to the final exercise session (‘8 weeks’) of an 8 week, moderate-intensity exercise intervention and gene expression of PPARγ and PPARγ-associated genes, PGC-1α, CD36, LXRα, ABCA1 and COX-2 was analysed via RT-PCR (PPARγ, n=10; PGC-1α, LXRα and ABCA1, n=9; CD36, n=16; COX-2, n=12; values expressed as fold gene expression relative to GAPDH ± SEM, *p<0.05, **p<0.01, t-test).
Figure 3.5. PPARγ protein expression in monocytes in response to a moderate-intensity exercise intervention. Western blot (A) was used to investigate protein expression of PPARγ (isoform 1 (PPARγ1) and isoform 2 (PPARγ2)) in monocytes isolated at baseline (BL), immediately following the initial exercise bout (1) and immediately prior to the final exercise bout (8) of an 8 week, moderate intensity exercise intervention, with PPARγ transfected HEK-293T cells being used as a positive control (C). A peptide competition assay (B), using a PPARγ blocking peptide was used to confirm specific band reactivity and PPARγ protein expression in monocyte samples obtained throughout the exercise study (n=6; figures show representative immunoblots).
3.3.6. PPAR\(\gamma\) Activation Properties of Exercised Serum

Gene reporter assays were used to analyse the PPAR\(\gamma\)-activating properties of serum obtained throughout the exercise study. When multiple comparisons were made, PPAR\(\gamma\) activation, measured using a PPRE-luciferase reporter assay, was significantly increased by samples obtained immediately after exercise (‘post 1’: 1.16 ± 0.19 fold RLU, relative to baseline; ‘post 8 week’: 1.17 ± 0.25 fold RLU, fold values relative to baseline, \(p<0.05\)). Conversely, the ‘8 weeks’ samples were not shown to significantly alter PPAR\(\gamma\) activity (1.14 ± 0.24 fold RLU, fold relative to baseline) when compared to other exercise time points (see Figure 3.6).

In contrast, ligand generation upon exercise, as measured by the PPAR\(\gamma\)-LBD gene reporter assay, did not alter significantly at any sample point during the exercise intervention, as demonstrated in Figure 3.7 (‘Post 1’: 1.09 ± 0.26 fold RLU; ‘8 weeks’: 0.94 ± 0.30 fold RLU; ‘Post 8 week’: 0.97 ± 0.26 fold RLU, fold values relative to baseline).

In both gene reporter assays, the positive control (rosiglitazone) was found to significantly upregulate PPAR\(\gamma\) activity (PPRE-luciferase reporter assay; PPAR\(\gamma\)-LBD gene reporter assay, 67.85 ± 23.85 fold RLU; \(p<0.01\) for both).
Figure 3.6. Effect of an 8 week, moderate-intensity exercise intervention on the PPARγ activating properties of serum. A gene reporter assay was used to analyse the PPARγ activating properties of serum obtained at baseline, immediately following the initial exercise bout (‘Post 1’) and immediately prior to the final exercise bout (8 weeks) and immediately following the final exercise bout (‘Post 8 week’) of an 8 week, moderate intensity exercise intervention. Serum or rosiglitazone (RSG; positive control) was applied to HEK-293T cells transfected with a PPARγ expression vector and a PPRE-luciferase construct and luminescence was measured. Luminescence values were normalised to a Renilla internal control in order to obtain RLU values corresponding to PPARγ activity (n=19 for serum, n=3 for RSG; values expressed as fold RLU ± SD, *p<0.05, one-way ANOVA).
Figure 3.7. Effect of an 8 week, moderate-intensity exercise intervention on PPARγ ligand availability in serum. PPARγ ligand binding was quantified by transfecting HEK293T cells with a fused PPAR-γ LBD/GAL4 construct and a UAS (GAL4 response element)-luciferase reporter construct prior to incubation with serum samples obtained baseline, immediately following the initial exercise bout (‘Post 1’) and immediately prior to the final exercise bout (8 weeks) and immediately following the final exercise bout (‘Post 8 week’) of an 8 week, moderate intensity exercise intervention, or rosiglitazone (RSG; positive control). Luminescence values were normalised to a β-gal internal control in order to obtain RLU values corresponding to ligand binding to the PPARγ-LBD and, hence, ligand availability (n=19 for serum, n=3 for RSG; values expressed as fold RLU ± SD, one-way ANOVA).
3.4. Discussion

The current data demonstrate that participation in the 8 week, moderate-intensity brisk walking intervention altered monocyte phenotype, with a tentative role for both PPARγ and IL-6 as putative mediators of this exercise-induced effect.

There are several well-defined markers of polarisation that are regularly used to identify macrophage phenotype (see Table 1.1). However, not all of these markers are expressed within monocytes, such as the regularly reported M2 macrophage marker, MR (Kjaergaard et al., 2014, Martinez and Gordon, 2014). To further complicate matters, some markers of polarisation are species specific and not applicable to human-based research (Raes et al., 2005). In this study, IL-10, Dectin-1 and IL-1Ra were found to be suitable M2 markers expressed by human monocytes, whereas MCP-1 and TNFα were recruited as M1 markers.

Both IL-10 and IL-1Ra regulate pro-inflammatory mediator production and contribute to the anti-inflammatory properties of M2 macrophages, whereas Dectin-1 is a major β-glucan receptor for the recognition and clearance of several fungal species (Petersen and Pedersen, 2005, Gales et al., 2010, Martinez and Gordon, 2014). When compared to baseline values, all three M2 markers were found to increase following the exercise intervention (see Fig. 3.2); mRNA expression of IL-10 and Dectin-1 were significantly elevated, whereas the increase in IL-1Ra expression neared significance. Several others have previously demonstrated that markers of M2 polarisation increase upon participation in exercise training (Kawanishi et al., 2010, Yakeu et al., 2010, Ikeda et al., 2013, Oliveira et al., 2013). In particular, Yakeu et al. (2010) used a very similar exercise intervention with human participants and observed comparable findings with regards to markers of M2 polarisation to the ones in the current study. However, Yakeu and colleagues used mixed leucocyte samples to conduct their research; thus, it was...
not possible to exclusively attribute observed changes in M2 marker gene expression to monocyte/macrophages (Butcher et al., 2008, Yakeu et al., 2010). Similarly, others have used adipose tissue or muscle samples, as opposed to monocyte/macrophages, to investigate the effects of exercise on M2 polarisation (Kawanishi et al., 2010, Ikeda et al., 2013, Oliveira et al., 2013). Hence, to the best of the author’s knowledge, this study is the first to demonstrate that participation in exercise training increases markers of M2 polarisation specifically within monocytes in a way which may drive them into the anti-inflammatory M2 macrophage phenotype that has been shown to be beneficial in the prevention of insulin resistance and T2D and its cardiovascular complications (Ikeda et al., 2013, Oliveira et al., 2013).

In contrast, gene expression of the pro-inflammatory M1 marker, MCP-1, significantly decreased by ~0.8 fold but TNFα expression was significantly elevated by ~2 fold (see Fig. 3.1). Several others have demonstrated exercise-induced reductions in M1 markers, such as MCP-1 (Kawanishi et al., 2010, Yakeu et al., 2010, Oliveira et al., 2013). However, TNFα expression has also been found to be reduced in response to exercise (Kawanishi et al., 2010, Oliveira et al., 2013). Notably, Kawanishi et al. and Oliveira et al. conducted their research on rodent models and utilised considerably different exercise protocols to the one used in this study, perhaps explaining this disparity in findings. Nonetheless, TNFα is known to have implications in the pathogenesis of insulin resistance and T2D (Donath and Shoelson, 2011). Thus it is important to elucidate whether the observed exercise-induced elevation in monocyte gene expression of TNFα is also evident at a protein level. Additionally, it may be interesting to investigate the effects of a similar exercise programme on the TNF-α inhibitor, sTNF-R, which has been shown to increase upon exercise and, hence, may neutralise the elevations in TNFα observed in this study (Venkatasamy et al., 2013).
Interestingly, the profile of polarisation markers expressed by monocytes following exercise training is representative of the M2b macrophage subset. These cells are said to produce M1-associated cytokines (including TNFα) but also high levels of IL-10, a potent anti-inflammatory cytokine with Th1 inhibitory functions, causing M2b cells to be immuno-regulatory and to induce a Th2 response (Mantovani et al., 2004, Hao et al., 2012, Kharraz et al., 2013). In this study, the exercise intervention induced a ~2 fold upregulation of TNFα but also a ~3 fold upregulation of IL-10 expression in monocytes, when compared to baseline levels, thus it is possible that exercise training drives monocytes into an M2b phenotype. To further clarify this, the expression of other markers of the M2b phenotype (such as IL-6, IL-1 and CCL1, with the absence of IL-12) should be analysed at both a gene and protein level (Mantovani et al., 2004; reviewed in Hao et al., 2012; reviewed in Kharraz et al., 2012).

To elucidate the mechanisms behind exercise-induced M2 polarisation, circulating levels of M2-stimuli, IL-4 and IL-13, were measured in serum. IL-4 was non-detectable in serum and IL-13 was unaltered upon completion (prior to the final exercise bout) of the exercise programme (see Fig. 3.3). Accordingly, the majority of the literature states that IL-4 and IL-13 serum levels do not alter following exercise participation (Suzuki et al., 2000, Malm et al., 2004, Peake et al., 2005). Thus, the exercise programme is not thought to have elicited its M2 polarising effects via these established activators of the alternative macrophage phenotype.

IL-6, on the other hand, is a cytokine which is known to be elevated upon exercise participation (Pedersen and Febbraio, 2008). Transient increases in systemic IL-6 during exercise are mainly attributed to secretion by contracting muscle (Gleeson et al., 2011). Following exercise, however, IL-6 levels have been shown to rapidly return to baseline, within 1-2 hours of exercise cessation (Moldoveanu et al., 2000, Gleeson
et al., 2011). Hence, in the present study, only the acute response of serum IL-6 to exercise participation was analysed, with chronic changes not being measured. In accordance with the literature, IL-6 was found to be significantly elevated in serum following participation in an acute exercise bout of the exercise intervention administered in this study (see Fig. 3.3). Despite being deemed as pro-inflammatory, IL-6 signalling upregulates the synthesis of regulatory cytokines, such as IL-10, IL-1Ra and sTNF-R and has been shown to prevent obesity-induced insulin resistance and promote insulin sensitivity in individuals with T2D (Petersen and Pedersen, 2005, Ropelle et al., 2010, Szostak and Laurant, 2011, Mauer et al., 2014). Recently, an interesting role for IL-6 in the alternative activation of monocytes has been revealed, providing a potential mechanism for its insulin resistance-preventing ability; Mauer et al. (2014) demonstrated that IL-6 was actually required for sufficient IL-4-dependent M2 polarisation and that depletion of the IL-6Rα chain of the IL-6 receptor in myeloid cells induced insulin resistance and impaired glucose homeostasis. More specifically, IL-6 was shown to operate via STAT3 to upregulate IL-4 receptor expression in macrophages, making these cells more sensitive to IL-4 stimulation. Thus, these results indicate that although circulating IL-4 levels may not have increased upon exercise participation, signalling via the IL-6-STAT3 axis may have rendered monocytes more sensitive to IL-4, thus inducing an M2 phenotype in these cells.

Since exercise has been shown to induce PPARγ, a transcription factor thought to play an important role in priming monocytes for M2 polarisation, it was considered relevant to investigate the expression levels and activity of PPARγ upon participation in the exercise intervention (Bouhlel et al., 2007, Butcher et al., 2008, Yakeu et al., 2010, Thomas et al., 2012). As aforementioned, several others have detected exercise-induced increases in PPARγ expression and/or activity levels in mixed mononuclear
cells upon participation in acute and chronic exercise (Butcher et al., 2008, Yakeu et al., 2010, Thomas et al., 2012). However, to the best of the author’s knowledge, this has not yet been investigated within isolated monocytes. The findings from the current study reveal that PPARγ mRNA expression did not significantly alter in monocytes following exercise training, when compared to baseline expression (see Fig. 3.4). Furthermore, detection of PPARγ at the protein level was questionable in monocytes at all time points analysed (‘baseline’, ‘post 1’ and ‘8 weeks’). As shown in Fig. 3.5, immunoblotting of samples with a PPARγ antibody generated bands at ~57 kDa, supposedly corresponding to the PPARγ isoform 2. However, this isoform is only known to be expressed in adipocytes, leading to concerns regarding result validity (Wadosky and Willis, 2012). Therefore, to confirm specific band reactivity, a blocking peptide was used to detect non-specific binding. As also shown in Fig. 3.5, the blocking peptide prevented visualisation of the ~53kDa and ~57kDa bands in the positive control, corresponding to PPARγ isoforms 1 and 2, respectively. However, the ~57kDa band was still visible in monocyte samples from the exercise-intervention, thus, it seems likely that these bands do not represent PPARγ protein expression.

PPARγ is a marker of macrophage differentiation since its expression is significantly enhanced in macrophages compared to monocytes and, thus, it is possible that PPARγ levels in the monocyte samples utilised in this study were below the detection limit of the Western blot assay utilised (Moore et al., 2001, Szanto and Nagy, 2005). Despite these findings, PPARγ activation was shown to be elevated in monocytes upon completion of the exercise intervention. The gene expression of the PPARγ co-activator, PGC-1α, was found to be non-significantly upregulated following participation in the exercise intervention (see Fig. 3.4). Due to the low copy number of this gene, upon RT PCR analysis, many participant samples had a Ct value >35 and
were, thus, not used in the analysis. Thus, it is possible that with a larger cohort number or with the use of more concentrated cDNA in analysis, significance may have been met. In accordance with this theory, PPARγ downstream genes, namely CD36 and LXRα were also significantly after the 8 week intervention when compared to baseline (see Fig. 3.4). Similar results have previously been shown in mixed mononuclear cells following exercise participation (Butcher et al., 2008, Yakeu et al., 2010, Thomas et al., 2012). The lipid scavenger receptor, CD36, is regulated by PPARγ and associated with the M2 macrophage phenotype (Bouhlel et al., 2007, Oh et al., 2012). Thus, CD36 may be used to further support the beneficial impact of the exercise intervention on monocyte/macrophage polarisation by acting as an M2 marker, whilst also being indicative of an increase in PPARγ activation. Similarly, LXRα is a PPARγ-regulated transcription factor with roles in lipid management (Chawla et al., 2001, Yakeu et al., 2010). Hence, its upregulation also suggests an increase in PPARγ activity, with beneficial improvements in lipid handling. However, in contrast with findings in mixed mononuclear cells, gene expression of the LXRα downstream gene, ABCA1 underwent only a non-significant increase in isolated monocytes upon participation in the moderate-intensity exercise programme. ABCA1 plays a role in cholesterol efflux and removal from cells. Thus, upregulation of CD36 without upregulation of ABCA1 may indicate increased lipid content within these cells. These findings correlate with the literature that suggests that M2 macrophages are more prone to foam cell formation due to increased uptake and retention of modified lipids (Oh et al., 2012). Controversially, these data may lead to concern with regards to the impact of exercise-induced M2 polarisation on atherosclerotic risk, since foam cells play a major role in the progression of this disease (Gleissner et al., 2010). However, there is a potential that, as with PGC-1α, if more samples were available in the analysis a significant
elevation in ABCA1 may have been observed in monocytes. Additionally, it is possible that other non-measured regulators of cholesterol efflux, such as the PPARγ-LXR mediated molecule, ATP-binding cassette transporter G1 (ABCG1), may have been upregulated following the exercise intervention, as shown in previous studies (Butcher et al., 2008). Importantly, there is much support for a protective role for exercise participation (and M2 macrophage polarisation) in atherosclerosis and CVD development (Moreno et al., 2012, Stoger et al., 2012, Schuler et al., 2013). In conclusion, therefore, further research is required to confirm the role of exercise-induced M2 monocytes/macrophages in lipid uptake and atherosclerotic/CVD risk.

Following on, exercise-induced PPARγ ligand generation was investigated as a potential mechanism for the elevation in PPARγ activity observed. Participation in the 8 week, moderate-intensity exercise programme was found to significantly upregulate the expression of COX-2 mRNA (see Fig. 3.4). Although often reported as an M1 marker, this inducible enzyme is responsible for the production of endogenous PPARγ ligands, including 15d-PGJ2 and, in association with this it has been shown to induce M2 macrophage polarisation (Heusinkveld et al., 2011, Sica and Mantovani, 2012, Díaz-Gandarilla et al., 2013). In a similar way, exercise-induced COX-2 upregulation may play a beneficial role in promoting M2 polarisation via 15d-PGJ2 production. However, further research is required to fully elucidate the impact of COX-2 upregulation on PPARγ ligand production and M2 polarisation upon exercise.

As an additional mechanism for PPARγ activation, PPARγ ligand generation in response to exercise was also investigated via gene reporter assay. Notably, PPARγ ligand availability in serum did not alter throughout the duration of the exercise study (see Fig. 3.7). However, the PPARγ activating properties of serum (also assayed via gene reporter assay) was shown to increase acutely upon exercise, in samples obtained
immediately following the initial and final exercise bout (see Fig. 3.6). These results are in accordance with those obtained in a previous study within our research group and further indicate that exercise promotes PPARγ activation (Thomas et al., 2012). They also demonstrate that this effect is independent of exogenous ligand production. However, identification of the specific PPARγ activating factor(s) was beyond the scope of this study. Similarly, a recent investigation by Szanto et al. (2010) demonstrated that IL-4/STAT-6 signalling resulted in augmented PPARγ activation in macrophages independent of changes in ligand availability. Thus, it is possible that IL-4 may act as a PPARγ-activating factor in serum and that increased monocyte-specific IL-4 signalling following exercise participation (as a result of IL-6-induced upregulation of the IL-4 receptor and, subsequent increased sensitivity to IL-4) may be, at least in part, responsible for the increased PPARγ activity observed in monocytes as a result of the exercise intervention, despite the lack of exogenous ligand generation. Alternatively, there is a potential for exercise to trigger ligand-independent post-translational modifications of PPARγ, such as phosphorylation or sumoylation, resulting in PPARγ activation (Olefsky and Glass, 2010, Harmon et al., 2011). It is also possible that the model system used in these experiments (HEK293-T cells) do not express the relevant ligand uptake/processing machinery and, thus, the in vivo situation may not be accurately reflected by these results. Thus, future work may benefit from using cell models which more closely resemble human monocytes.

Regardless of the specific PPARγ-activating factor(s), the acute increase in PPARγ-activating properties of serum upon exercise may contribute to systemic effects of the exercise programme on insulin sensitivity observed in this study (data shown in Chapter 2, Fig 2.8). From the results presented, it is proposed that exercise participation may increase levels of blood-borne PPARγ activating factors, allowing
for sustained systemic PPARγ activation in a range of cells and tissues, including insulin-sensitive tissues such as skeletal muscle, the liver and adipose tissue (and not only monocytes). Exposure to these factors may activate PPARγ and beneficially impact on glucose metabolism, lipid management and inflammation within the aforementioned tissues, hence promoting insulin sensitivity and inhibiting insulin resistance (Harmon et al., 2011, Wahli and Michalik, 2012). Thus, with regards to insulin resistance and T2D prevention, exercise may provide similar beneficial outcomes as PPARγ targeting anti-diabetic agents without eliciting the deleterious side effects associated with some of these medications, namely the TZDs (Harmon et al., 2011). Thus, these findings may further support the use of exercise prescription in the management and treatment of T2D as a safe alternative to current therapies available.

In summary, this study demonstrates that participation in an 8 week, moderate-intensity exercise programme might induce M2 marker expression in monocytes, potentially priming them for the M2 macrophage phenotype, thought to be beneficial for the prevention/management of insulin resistance, T2D and its associated complications. It is suggested that PPARγ activation and/or IL-6 generation may be partly responsible for exercise-induced changes in monocytes M2 marker expression. Of course a major assumption of this research is that the M2 monocyte phenotype is retained upon cell infiltration and differentiation in tissues, whereas the possibility of intratissue ‘switching’ of macrophage phenotype in response to stimuli in the microenvironment has been previously reported (Dalmas et al., 2011, Lee et al., 2013). Thus, further investigations are required to elucidate the effects of similar exercise interventions on M1:M2 ratio in various tissue environments so as to fully explore the potential for exercise to alter macrophage phenotype in a way that may be beneficial for the prevention of diseases, such as T2D and its cardiovascular complications.
CHAPTER 4

The Impact of IL-6 on M1/M2 Marker Expression in Monocytic Cells and Peripheral Mononuclear Cells

4.0. Introduction

The strong correlation between chronic elevations in systemic IL-6 and insulin resistance has implied a role for this cytokine in the pathogenesis of T2D. In accordance, levels of circulating IL-6 are predictive of T2D and its cardiovascular complications (Vozarova et al., 2001, Danesh et al., 2008, Patterson et al., 2010, Daniele et al., 2014). Upon closer inspection, however, the association between IL-6 and insulin resistance may not show a causative link but may instead reflect adiposity and fat mass in subjects. At rest, adipose tissue cells, in particular adipose tissue macrophages, are a major source of IL-6, accounting for approximately 33% of circulating IL-6 (Weisberg et al., 2003, Makki et al., 2013). Since IL-6 production has been found to increase with obesity, and obesity is a major risk factor for insulin resistance, fat mass and adiposity may provide the link between IL-6 levels and insulin resistance (Jiang et al., 1998, Makki et al., 2013, Patel and Abate, 2013). Thus, the negative reputation of this cytokine may not be entirely justified, with the precise role of IL-6 in the pathogenesis of insulin resistance and T2D remaining to be clarified (Pedersen, 2011).

IL-6 plays an important role in orchestrating immunity; it is produced by a range of immune cells, including monocytes, macrophages, dendritic cells, T and B lymphocytes and endothelial cells. It also acts upon these cells to promote immune cell differentiation, whilst influencing immune cell migration and recruitment into
tissues (Romano et al., 1997, Diehl and Rincon, 2002, Kaplanski et al., 2003, Jagannathan-Bogdan et al., 2011). More specifically, IL-6 has been reported to influence T cell differentiation (Diehl and Rincon, 2002, Kimura and Kishimoto, 2010). Some have shown that IL-6 skews T cell differentiation in a way which is detrimental to health, by promoting Th17 cell production and inhibiting Treg development. Th17 cells promote tissue inflammation and have implications in autoimmune diseases, whereas Treg cells regulate inflammatory responses. Thus, the potential disruption in the balance of these T cell subsets due to IL-6 is thought to promote inflammatory disease (Kimura and Kishimoto, 2010). Others, however, demonstrate that despite inducing Th17 differentiation, IL-6 (in combination with TGF-β) alters the Th17 response by inhibiting pathogenic cytokine and chemokine production by these cells, whilst inducing their synthesis of the anti-inflammatory cytokine, IL-10 (McGeachy et al., 2007). Furthermore, IL-6 has been reported to inhibit T cell differentiation into the Th1 phenotype, instead promoting Th2 differentiation (Rincon et al., 1997, Diehl and Rincon, 2002). Like Th17 lymphocytes, Th1 cells induce the synthesis of proinflammatory mediators, some of which have been shown to impair insulin signalling and promote insulin resistance and T2D (Damsker et al., 2010, Jagannathan-Bogdan et al., 2011, Lumeng and Saltiel, 2011). Th2 lymphocytes, on the other hand, are ‘anti-inflammatory’ T cell subsets which produce a plethora of cytokines, some of which have Th1 inhibitory effects (Rincon et al., 1997). Hence, an IL-6-induced Th2 response may beneficially impact upon insulin signalling and T2D risk (Lumeng and Saltiel, 2011). Due to conflicting findings, however, the exact impact of IL-6 on T cell differentiation requires further investigation.
Like T cells, macrophages also exist in pro- and anti-inflammatory phenotypes, termed ‘M1’ and ‘M2’ respectively (Sica and Mantovani, 2012). M1 macrophages have been demonstrated to majorly contribute to the inflammation which drives insulin resistance, T2D and its associated cardiovascular co-morbidities, whilst M2 macrophages are regulatory cells which inhibit M1 macrophage function (Olefsky and Glass, 2010, Lumeng and Saltiel, 2011, Oh et al., 2012). Importantly, the Th2-associated cytokines, IL-4 and IL-13, induce alternative activation of macrophages into the M2 phenotype (Sica and Mantovani, 2012). Thus, it is possible that IL-6 driven Th2 activation may promote M2 macrophage polarisation so as to beneficially impact upon inflammation and prevent inflammation-induced insulin resistance and T2D onset and progression. However, the exact link between IL-6, Th2 activation and M2 macrophage polarisation is yet to be established.

In macrophages, IL-6 has been shown to enhance IL-4/IL-13-induced M2 polarisation in murine and human cells (Fernando et al., 2014). Similarly, blockade of IL-6 signalling in murine myeloid cells was found to prevent the alternative activation of macrophages, suggesting an important role for IL-6 in M2 macrophage polarisation (Mauer et al., 2014). However, although evidence is available to suggest that IL-6 may beneficially skew macrophage phenotype into the M2 phenotype, little research has been carried out to elucidate the effects of IL-6 on monocyte phenotype (Fernando et al., 2014, Mauer et al., 2014). Monocytes are macrophage precursors which circulate in the blood prior to differentiation upon tissue infiltration (Ziegler-Heitbrock et al., 2010). Aside from maintaining the tissue macrophage pool, monocytes have been shown to be functional cells within their own rights, contributing to homeostasis, immunity and tissue repair (Robbins and Swirski, 2010, Ziegler-Heitbrock et al., 2010). Like macrophages, monocytes are a heterogeneous cell type which may be
‘primed’ for the M1 or M2 macrophage phenotype, depending on the stimuli to which they are exposed (Bouhlel et al., 2009, Ziegler-Heitbrock et al., 2010, Appleby et al., 2013). Since it has been suggested that M2 macrophage polarisation may be promoted by elevated levels of IL-6 in the circulation (where monocytes reside) it may be important to determine the effects of IL-6 on monocytes to establish its ability to prime these cells for M2 macrophage polarisation prior to tissue infiltration (Fernando et al., 2014, Mauer et al., 2014).

Interestingly, it has been demonstrated that the ability for exercise to improve insulin sensitivity and glucose homeostasis may, in part, be attributed to IL-6 synthesis (in the absence of other pro-inflammatory mediators which would typically accompany IL-6 in an inflammatory episode) during exercise (Febbraio and Pedersen, 2002, Scheller et al., 2011, Pan et al., 2012). The acute response to exercise includes an increase in myocyte-derived IL-6 by contracting muscle (Febbraio and Pedersen, 2002, Pan et al., 2012). IL-6 may contribute to the hormetic response to exercise; where an initial stress triggers a beneficial compensatory response (Radak et al., 2008). For example, exercise-associated IL-6 is thought to inhibit TNFα, whilst inducing secretion of the anti-inflammatory cytokines, IL-4, IL-10 and IL-1Ra which also downregulate TNFα and other pro-inflammatory mediators (Febbraio and Pedersen, 2002, Petersen and Pedersen, 2005). However, whether the impact of IL-6 on monocyte/macrophage polarisation contributes to its effect on inflammatory mediator synthesis/action is not yet known. Nonetheless, although it is likely that IL-6 is not the sole player in the exercise hormesis theory, it appears to act as an initial inflammatory stressor which induces a compensatory biological response including regulatory cytokine synthesis and inhibition of pro-inflammatory cytokines, including TNFα. Since inflammatory mediators, such as TNFα, may disrupt insulin signalling, the ability for exercise-
associated IL-6 to regulate inflammation may contribute to the insulin-sensitising effects of exercise (Febbraio and Pedersen, 2002, Boura-Halfon and Zick, 2009, Olefsky and Glass, 2010).

Thus, this research aims to investigate the effect of IL-6 on monocyte phenotype by analysing the expression of M1 and M2 markers in THP-1 monocytes and in primary human monocytes following IL-6 treatment. Co-cultured human mononuclear cells, consisting of monocytes and lymphocytes, will also be used to investigate the link between IL-6, T cell activation and monocyte phenotype. The findings from this research may help to elucidate the process by which IL-6 may induce M2 macrophage polarisation. They may also provide insight into the mechanisms by which exercise, via IL-6 synthesis, might alter inflammation in a way which may beneficially impact upon insulin signalling and T2D risk.
4.2. Methods

4.2.1. Cell Culture

All cell culture consumables were manufactured by Corning® (Sigma Aldrich, Dorset, UK) and all reagents were obtained from Invitrogen (Invitrogen Ltd, Paisley, UK), unless otherwise stated.

4.2.1.1. THP-1 Cell Culture

The monocytic cell line, THP-1 (ATCC, Middlesex, UK), were grown in complete RPMI-1640 medium, supplemented with 10% US defined, irradiated and heat inactivated FBS (Thermo Fisher Scientific, Leicester, UK), 1% v/v minimum essential medium (MEM) non-essential amino acids, 1mM sodium pyruvate and 100U/ml penicillin/100μg/ml streptomycin mix.

Briefly, vials containing THP-1 cells were removed from liquid nitrogen and thawed rapidly by briefly immersing in a water bath set at 37°C, with constant agitation. Vial contents were transferred into a 25 cm² cell culture flask containing pre-warmed complete RPMI with 20% FBS. The culture was then placed in a 37°C, 5% CO2, humidified incubator for 2-3 days.

Cells were split every 2-3 days, when cells had reached a density of approximately 0.9x10⁶ cells/ml. Briefly, cells were centrifuged at 300xg for 10 minutes and resuspended in fresh RPMI. Cells were counted using a haemocytometer, checking for cell viability using the trypan blue exclusion method, as described in Chapter 3, Section 3.2.9.1. The desired number of viable cells was then transferred to a new 25 cm² culture flask and an appropriate volume of DMEM was added (0.25 ml/cm²) to obtain a cell density of 0.3x10⁶ cells/ml. Subsequently, cells were incubated in a 37°C, 5% CO2, humidified incubator. THP-1 cells were cultured up to passage 25.
In order to maintain the THP-1 stock, frozen cultures were prepared frequently. Cells were counted as explained above and cell suspensions were dispensed at 6x10^6 cells in a cryotube and centrifuged at 300xg for 10 minutes. On ice, the cell pellet was resuspended in 500μl of cold FBS. Subsequently, 500μl of FCS + 20% DMSO was slowly added to cells, bringing the freezing medium to a final concentration of 10% DMSO in FBS. Cells were slowly frozen (1°C per minute) in a ‘Mr Frosty’ Cryo Freezing Container (Sigma Aldrich, Dorset, UK), at -80°C, overnight, following which cells were transferred to liquid nitrogen for storage.

4.2.1.2. THP-1 Differentiation and Treatment

THP-1 were plated at 0.5x10^6 cells/ml and either used undifferentiated (‘THP-1 monocytes’, suspended cells) or were differentiated into adherent macrophage-like cells (‘THP-1 macrophages’) To differentiate, THP-1 were incubated with 5ng/ml Phorbol 12-myristate 13-acetate (PMA) for 48 hours in a 37°C, 5% CO2, humidified incubator.

To establish dose response to IL-6 and determine optimum concentration for treatment, THP-1 monocytes were treated with recombinant human IL-6 (Peprotech, London, UK) at concentrations between 1pg/ml to 10ng/ml for 24 hours. Based on SOCS-3 expression (see Fig. 4.1), an IL-6 concentration of 10ng/ml was deemed optimal for treatment and, subsequently, THP-1 monocytes and THP-1 macrophages were treated with 10ng/ml recombinant human IL-6 for 3, 6, 12, 24 and 48 hours in a 37°C, 5% CO2, humidified incubator. Untreated cells were used as negative controls.

To harvest, THP-1 monocytes were collected and centrifuged at 300xg for 10 minutes. Cells were washed twice in RPMI, centrifuging at 300xg for 10 minutes to pellet after washes. Alternatively, media was removed from THP-1 macrophages and adherent
cells were washed twice in pre-warmed PBS. Cells were then incubated with 0.25% trypsin-EDTA for 1-2 minutes at 37°C. RPMI was added at thrice the volume of trypsin used to stop the reaction and cells were pelleted by centrifugation at 300xg for 10 minutes. Cell pellets were used in subsequent PCR experiments (see section 4.2.2).

4.2.1.3. Primary Cell Procurement

Human blood was collected by venepuncture of the antecubital vein in EDTA. All phlebotomy equipment was provided by Greiner VACUETTE (Gloucester, UK). Samples were stored according to the University of Wales Institute’s HTA guidelines. Blood was fractionated using Histopaque®-1077 (Sigma-Aldrich, Dorset, UK), as described in Chapter 3, section 3.2.2 (including platelet wash), to obtain peripheral mononuclear cells (PMNCs). PMNCs were either cultured as they were (see section 4.2.1.4) or used in monocyte isolation procedures. Isolated monocytes were obtained using MACS MicroBeads (Miltenyi Biotec, Germany), as described in Chapter 3, section 3.2.3.

4.2.1.4. Primary Cell Culture and Treatment

PMNCs or isolated primary monocytes were cultured in complete RPMI-1640 medium, supplemented with 10% US defined, irradiated and heat inactivated FBS (Thermo Fisher Scientific, Leicester, UK), 1% v/v MEM non-essential amino acids, 1mM sodium pyruvate and 100U/ml penicillin/100μg/ml streptomycin mix.

Following procurement, PMNCs or isolated monocytes were plated at 4x10⁶ cells/ml in 12 well plates and treated with recombinant human IL-6 (Peprotech, London, UK) either at an exercise-associated physiological concentration of 100pg/ml (Starkie et al., 2003) or at a supraphysiological concentration of 1ng/ml. Alternatively, cells were left untreated as a negative control or treated with 15ng/ml recombinant human IL-4.
(Peprotech, London, UK) as a positive control for M2 polarisation. Cells were incubated for 3 hours in a 37°C, 5% CO2, humidified incubator.

Following treatment, where PMNCs were used, suspended cells (lymphocytes) were collected and centrifuged at 300xg for 10 minutes. The supernatants were carefully removed and retained for use in subsequent ELISA (see section 4.2.3) and the cell pellets were used for PCR (see section 4.2.2). Adherent cells (monocytes) were washed twice in PBS before adding 1ml of TRI Reagent® (Applied Biosystems, Warrington, UK) to harvest. Lysates were also used in subsequent PCR experiments. Similarly, after treatment, isolated monocytes were washed twice with PBS before harvesting in 1ml of TRI Reagent®, for use in subsequent PCR experiments.

4.2.2. PCR

Cell pellets were resuspended in 1ml of TRI Reagent® (Applied Biosystems, Warrington, UK) and RNA was extracted as described in Chapter 3, section 3.2.4. RNA was converted to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Warrington, UK), as described in Chapter 3, section 3.2.5. Gene expression was analysed via RT-PCR, either using Fast SYBR® Green or TaqMan® Fast Universal, No AmpErase® UNG assays in combination with an Applied Biosystems Fast 7500 Real-Time PCR System (Applied Biosystems, Warrington, UK), as described in Chapter 3, section 3.2.6. More specifically, Fast SYBR® Green RT-PCR was used to analyse gene expression of Dectin-1, IL-10, IL-1Ra and TNFα, using GAPDH as a housekeeping gene. β-actin was also used to normalise data, giving similar results as GAPDH (data not shown) (see Table 4.1 for primer sequences). Taqman® RT-PCR was used to analyse gene expression of MCP-1, SOCS-3 and IL-4, using GAPDH and GUSB as housekeeping genes. The comparative Ct method was employed to calculate relative gene expression in samples (for details, see Chapter 3,
section 3.2.6.3). In all cases, untreated cells were used as comparators for cytokine stimulated cells.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CATTGACCTCAACTACATG</td>
<td>TCTCCATGGTGGAAGAC</td>
</tr>
<tr>
<td>β-actin</td>
<td>TCCTGTGACATCCAGAAA</td>
<td>GAAGCATTGCGGTGGAC</td>
</tr>
<tr>
<td>Dectin-1</td>
<td>GGAAGCAACACATTGGAGAAATGG</td>
<td>CTTGGTAGGAGTCACACGTGC</td>
</tr>
<tr>
<td>IL-10</td>
<td>ACGGCCTGTCATCGATT</td>
<td>TGGGAGTTATATCAAGGATTCTTC</td>
</tr>
<tr>
<td>IL1Ra</td>
<td>GCCTCGCAGTGACCTAATC</td>
<td>GACAGGCAGATTCTTCTCCAT</td>
</tr>
<tr>
<td>TNFα</td>
<td>TGCGCTGACACTTGGAGTA</td>
<td>CTGGGCAAGGGCTTAGAGA</td>
</tr>
</tbody>
</table>

Table 4.1: Primer sequences used in SYBR® Green RT-PCR assay.

4.2.3. ELISA

ELISA was used to quantify IL-4 and IL-10 in samples. Assays were carried out according to manufacturer’s instructions, as described in Chapter 3, section 3.2.7.

4.2.4. Statistical Analysis

All data were expressed as mean ± SEM, unless otherwise stated. Where comparisons were made between the means of two samples, the t-tests analysis was used for data assumed to be normally distributed. Alternatively, one-way ANOVA with Tukey’s post-hoc analysis was used for multiple comparisons within groups of normally distributed data. Statistical analysis was performed using GraphPad Prism®5 software and results were deemed significant at p<0.05.
4.3. Results

4.3.1. IL-6 Dose Response
As shown in Fig. 4.1, THP-1 monocytes were found to express SOCS-3 at higher levels in THP-1 monocytes treated with 10ng/ml IL-6 when compared to untreated control THP-1 monocytes (1.66 ± 0.22). In comparison, treatments at <10ng/ml IL-6 did not induce SOCS-3 expression in THP-1 monocytes relative to untreated control cells (1ng/ml: 1.13 ± 0.28; 100pg/ml: 0.92 ± 0.17; 50pg/ml: 0.84 ± 0.18; 10pg/ml: 0.91 ± 0.10; 5pg/ml: 0.63 ± 0.22; 1pg/ml: 0.64 ± 0.18; 0.5pg/ml: 1.07 ± 0.34).

4.3.2. Gene Expression of M2 markers in THP-1 Monocytes Treated with IL-6
The gene expression of the M2 markers, Dectin-1 and IL-1Ra, did not alter significantly in THP-1 monocytes upon 10ng/ml IL-6 treatment. However, a trend towards reduced Dectin-1 expression (3h: 0.59 ± 0.22; 12h: 0.73 ± 0.44; 24h: 0.66 ± 0.33; 48h: 0.93 ± 0.78) and, a near-significant reduction in IL-1Ra expression (3h: 0.49 ± 0.01; 12h: 0.17 ± 0.23; 24h: 0.33 ± 0.11; 48h: 0.41 ± 0.25, p=0.07) was observed in cells treated with IL-6, relative to untreated control cells (see Fig. 4.2).

4.3.3. Gene Expression of M1 markers in THP-1 Monocytes Treated with IL-6
A non-significant trend towards increased gene expression of the M1 marker, MCP-1, was observed in THP-1 monocytes treated with 10 ng/ml IL-6 for (3h: 1.13 ± 0.21; 12h: 1.62 ± 0.50; 24h: 1.52 ± 0.27; 48h: 2.29 ± 0.50), relative to control cells (see Fig. 4.3). However, the gene expression of another M1 marker, TNFα, was found to decrease significantly with 10 ng/ml IL-6 treatment at all incubation time points investigated (3h: 0.52 ± 0.05 (p<0.05); 12h: 0.35 ± 0.16 (p<0.05); 24h: 0.23 ± 0.07 (p<0.01); 48h: 0.22 ± 0.03 (p<0.01), see Fig. 4.3), with greater significance being met at 24h and 48h.
Figure 4.1. SOCS-3 expression in response to IL-6 treatment of THP-1 monocytes. THP-1 monocytes were treated with varying concentrations of IL-6 for 24 hours in a 37°C, 5% CO2, humidified incubator. Untreated cells were used as controls. SOCS-3 expression was analysed using RT-PCR (n=3; values expressed as gene expression relative to GAPDH ± SEM, one-way ANOVA).
Figure 4.2. M2 marker gene expression in IL-6 treated THP-1 monocytes. THP-1 monocytes were incubated with 10ng/ml IL-6 for 3-24h. Untreated cells were used as controls. The expression of the M2 markers, Dectin-1 and IL-1Ra was analysed via RT-PCR (n=2; values expressed as gene expression relative to GAPDH ± SEM, one-way ANOVA).
Figure 4.3. M1 marker gene expression in IL-6 treated THP-1 monocytes. THP-1 monocytes were incubated with 10ng/ml IL-6 for 3-24h and M1 marker gene expression (MCP-1 and TNFα) was analysed via RT-PCR. Untreated cells were used as controls (n=2; values expressed as gene expression relative to GAPDH ± SEM; *p<0.05, **p<0.01, one-way ANOVA).
4.3.4. M2 marker Gene Expression in THP-1 Macrophages Treated with IL-6

Upon treatment with 10ng/ml IL-6, neither Dectin-1 nor IL-1Ra gene expression altered significantly in THP-1 macrophages. Both of these M2 markers, however, did show a trend towards reduced expression at all time points analysed (Dectin-1: 3h: \(0.67 \pm 0.05\); 12h: \(0.69 \pm 0.12\); 24h: \(0.81 \pm 0.21\); 48h: \(0.72 \pm 0.29\). IL-1Ra: 3h: \(0.81 \pm 0.08\); 12h: \(0.61 \pm 0.06\); 24h: \(0.51 \pm 0.07\); 48h: \(0.54 \pm 0.21\)), in comparison to untreated THP-1 cells (see Fig. 4.4). Moreover, the reduction in IL-1Ra gene expression following cell treatment with IL-6 neared significance \(p=0.07\).

4.3.5. M1 marker Gene Expression in THP-1 Macrophages Treated with IL-6

The M1 marker, MCP-1, was non-significantly elevated at a genetic level in THP-1 macrophages treated with 10ng/ml IL-6 (3h: \(1.40 \pm 0.35\); 12h: \(1.23 \pm 0.25\); 24h: \(1.32 \pm 0.19\); 48h: \(2.11 \pm 0.05\) see Fig. 4.5). In addition, TNF\(\alpha\) gene expression did not alter following treatment with IL-6 for 3h \((1.07 \pm 0.01)\) but was non-significantly reduced at 12h \((0.71 \pm 0.02)\) and 24h \((0.70 \pm 0.21)\), returning to baseline at 48h \((1.01 \pm 0.35)\) (see Fig. 4.5).
Figure 4.4. M2 marker gene expression in IL-6 treated THP-1 Macrophages.

PMA-differentiated THP-1 macrophages were treated with 10ng/ml IL-6 for 3-24h and gene expression of the M2 markers, Dectin-1 and IL-1Ra, was analysed via RT-PCR. Untreated THP-1 macrophages were used as controls. (n=2; values expressed as gene expression relative to GAPDH ± SEM, one-way ANOVA).
Figure 4.5. M1 marker gene expression in IL-6 treated THP-1 macrophages.

Expression of the M1 markers, MCP-1 and TNFα were analysed via RT-PCR in PMA-differentiated THP-1 macrophages treated with 10ng/ml IL-6 for 3-12h. Untreated THP-1 macrophages were used as controls (n=2; values expressed as gene expression relative to GAPDH ± SEM, one-way ANOVA).
Since the expected alterations in M1/M2 marker genes (as seen \textit{in-vivo} during the exercise intervention described in Chapters 2 and 3) could not be reproduced in an \textit{in-vitro} monocyte/macrophage model, a series of co-culture experiments were performed, involving primary human monocytes and lymphocytes, to more closely mimic the \textit{in vivo} setting, whereby T lymphocytes may impact upon monocyte/macrophage phenotype, potentially via synthesis of M2 stimuli, such as IL-4, in response to IL-6 (Rincon \textit{et al.}, 1997, Diehl and Rincon, 2002, Sica and Mantovani, 2012).

\textbf{4.3.6. IL-4 Gene Expression Primary Human Lymphocytes and Monocytes Treated with IL-6 in Co-Culture}

Following treatment of peripheral blood mononuclear cells (PBMCs) with 1ng/ml IL-6 for 3h, IL-4 gene expression was analysed in suspended cells (lymphocytes) and also in adherent cells (monocytes). When compared to untreated control cells, IL-4 expression was non-significantly elevated in IL-6 treated lymphocytes (17.35 ± 14.20), whilst IL-4 treated positive controls had significantly elevated levels of IL-4 expression, relative to untreated controls (1.25 ± 0.06, \(p<0.01\), see Fig. 4.6).

In monocytes, IL-4 expression in IL-6 treated monocytes was found to be significantly reduced (0.52 ± 0.04, \(p<0.01\)), whilst IL-4 treated positive controls had non-significantly elevated levels of IL-4 expression (1.68 ± 0.64), relative to controls (see Fig. 4.6).

\textbf{4.3.7. IL-4 Production by PBMCs Following IL-6 Treatment}

IL-4 was non-detectable in the supernatant of PBMCs incubated with 1ng/ml IL-6 for 3h or in untreated control samples (minimum detection levels of the ELISA assay was 0.25 pg/ml; data not shown).
Figure 4.6. IL-4 gene expression in primary lymphocytes and monocytes, treated with IL-6 whilst in co-culture. Human PBMCs were treated 1ng/ml IL-6 or 15ng/ml IL-4 for 3h, following which IL-4 gene expression was analysed in (A) suspended cells (lymphocytes) and in (B) adherent cells (monocytes) using RT-PCR (Lymphocytes, n=5; monocytes, n=3; values expressed as gene expression relative to GAPDH ± SEM, **p<0.01, t-test).
4.3.8. M2 marker gene expression in primary monocytes, treated with IL-6 whilst in co-culture with human lymphocytes

When compared to untreated controls, both Dectin-1 and IL-10 gene expression were non-significantly elevated in primary monocytes following treatment of monocyte-lymphocyte co-cultures with 100pg/ml IL-6 for 3h (Dectin 1: 1.98 ± 1.08; IL-10: 1.35 ± 0.5). Similarly, Dectin-1 and IL-10 expression did not alter significantly in monocytes following treatment of monocyte-lymphocyte co-cultures with 1ng/ml IL-6 for 3h (Dectin 1: 1.07 ± 0.14; IL-10: 1.25 ± 0.27). In contrast, a significant elevation in Dectin-1 gene expression levels (1.79 ± 0.19, \( p < 0.01 \)), but not in IL-10 (1.18 ± 0.08) was observed in IL-4 treated positive controls (see Fig. 4.7).

4.3.9. M1 marker gene expression in primary monocytes, treated with IL-6 whilst in co-culture with human lymphocytes

MCP-1 gene expression was non-significantly elevated in monocytes (3.30 ± 1.40) following 100pg/ml IL-6 treatment of monocyte-lymphocyte co-cultures, whereas it was significantly reduced (0.24 ± 0.16, \( p < 0.01 \)) in co-cultured cells treated with 1ng/ml IL-6, when compared to untreated controls. In contrast, TNFα gene expression did not alter significantly following cell treatment with 100pg/ml IL-6 (0.96 ± 0.32) or 1ng/ml IL-6 (0.88 ± 0.17), when compared to untreated controls. In contrast, both MCP-1 and TNFα gene expression were significantly reduced in IL-4 treated positive controls (MCP-1: 0.20 ± 0.11; TNFα: 0.49 ± 0.10, \( p < 0.01 \) for both, see Fig. 4.8).
Figure 4.7. M2 marker gene expression in primary monocytes, treated with IL-6 whilst in co-culture with human lymphocytes. Human PMNCs were treated with 100pg/ml or 1ng/ml IL-6 (or 15ng/ml IL-4 as a positive control) for 3h prior to separating adherent cells (monocytes) from suspension cells (lymphocytes) and analysing M2 marker (Dectin-1 and IL-10) gene expression in monocytes via RT-PCR. Comparisons were made with gene expression in untreated controls monocytes (n=5; values expressed as gene expression relative to GAPDH ± SEM, **p<0.01, t-test).
Figure 4.8. M1 marker gene expression in primary monocytes, treated with IL-6 whilst in co-culture with human lymphocytes. Human PMNCs were treated with 100pg/ml or 1ng/ml IL-6 (or 15ng/ml IL-4 as a positive control) for 3h prior to separating adherent cells (monocytes) from suspension cells (lymphocytes) and analysing gene expression of M1 markers (MCP-1 and TNFα) in monocytes via RT-PCR. Untreated monocytes were used as controls (TNFα, n=5; MCP-1, n=3; values expressed as gene expression relative to GAPDH ± SEM, **p<0.01, t-test).
4.3.10. MCP-1 Gene Expression in IL-6 Treated Purified Primary Human Monocytes

In order to investigate the impact of lymphocytes on the previously observed IL-6-induced downregulation of MCP-1 in monocytes co-cultured with lymphocytes (see Fig. 4.8), primary human monocytes were also treated with IL-6 in monoculture and MCP-1 gene expression was analysed.

Treatment of purified monocytes with 1ng/ml IL-6 for 3h did not alter MCP-1 expression when compared to controls (1.00 ± 0.13). IL-4 treated cells, however, showed a significant reduction in MCP-1 gene expression (0.13 ± 0.04, see Fig. 4.9), relative to controls.
Figure 4.9. MCP-1 gene expression in IL-6 treated purified primary monocytes. Monocytes were isolated from human PBMCs using magnetic immunoseparation. Purified monocytes were treated with 1ng/ml IL-6 or 15ng/ml IL-4 for 3h and MCP-1 expression analysed via RT-PCR (n=3; values expressed as gene expression relative to GAPDH ± SEM; p<0.01, t-test).
4.4. Discussion

IL-6 is often thought of as a pro-inflammatory cytokine with insulin-resistance inducing properties (Ouchi et al., 2011). In contrast, however, there is evidence to suggest that IL-6 may improve insulin sensitivity and glucose utilisation in insulin sensitive tissues, including the muscle and adipose tissue, and in whole body systems (Lyngso et al., 2002, van Hall et al., 2003, Bruce and Dyck, 2004, Febbraio et al., 2004, Carey et al., 2006, Pedersen, 2007, Awazawa et al., 2011). The findings presented in this chapter provide potential mechanisms by which IL-6 might induce its positive effects with regards to insulin signalling and glucose management. They focus on IL-6’s ability to alter monocyte phenotype, potentially priming them away from the M1 macrophage phenotype which has been shown to play a prominent role in the pathogenesis of insulin resistance, T2D and its associated cardiovascular co-morbidities (Olefsky and Glass, 2010, Pradhan Nabzdyk et al., 2013, You et al., 2013). These data suggest that IL-6 may inhibit monocyte synthesis of M1 associated, insulin signalling–disruptive cytokines, namely TNFα and MCP-1 (Cai et al., 2005, Steinberg et al., 2006, Gregor and Hotamisligil, 2011). Intriguingly, the data suggest that IL-6 may impact upon monocyte phenotype by targeting lymphocytes to evoke an anti-inflammatory response in juxtaposed monocytes.

Due to their similarity with primary monocytes and their ability to be differentiated into macrophage-like cells, THP-1 cells were chosen for use in initial investigations of the effect of IL-6 on monocytes/macrophages (Qin, 2012). THP-1 monocytes and THP-1 macrophages were treated with 10ng/ml IL-6 for 3 to 48h, the concentration of IL-6 being selected based on its ability to upregulate the gene expression of SOCS-3 (a molecule known to be upregulated upon IL-6 signalling) in THP-1 monocytes (see Fig. 4.1) and incubation times chosen to reflect the in vivo environment where
monocytes circulate in the blood for 1-2 days (Yasukawa et al., 2003, Italiani and Boraschi, 2014). Dectin-1, a major β-glucan receptor for the recognition and clearance of fungal species, and IL-1Ra, an inhibitor of IL-1-induced inflammation, were adopted as M2 markers, whereas the pro-inflammatory cytokines, MCP-1 and TNFα, were used as M1 markers (Petersen and Pedersen, 2005, Gales et al., 2010, Olefsky and Glass, 2010, Martinez and Gordon, 2014).

In THP-1 monocytes, no significant changes in M2 marker expression were detected following IL-6 treatment when compared to controls (see Fig. 4.2), although a trend towards reduced expression was found for both markers at all time points, with results nearing significance for IL-1Ra expression ($p=0.07$). In contrast, the gene expression of the M1 marker, MCP-1, increased non-significantly in THP-1 monocytes, with expression being most elevated (~2 fold) following 48h treatment with IL-6 (see Fig. 4.3). Conversely, however, TNFα, was significantly decreased in THP-1 monocytes following IL-6 treatment, with the reduction in expression being more pronounced and more significant following longer exposure (>24h) to IL-6 (24h and 48h, ~ 0.2 fold expression relative to untreated control, $p<0.01$), see Fig. 4.3). Together, these results suggest that IL-6 does not induce an M2-like phenotype in monocytic cells, but that it may reduce expression of the pro-inflammatory M1-associated cytokine, TNFα.

Although these findings do not completely reflect the literature, which states that IL-6 promotes an M2 phenotype in macrophages, they are not entirely confounding (Fernando et al., 2014, Mauer et al., 2014). To date, little research has been carried out in monocytes but IL-6 has previously been shown to reduce LPS-induced TNFα production by murine and human macrophages (Riley et al., 1999, Niemand et al., 2003). Moreover, IL-6 infusion has been found to reduce endotoxemia-induced TNFα production in humans (Starkie et al., 2003). It is thought that IL-6-induced STAT3
activation and subsequent SOCS3 induction are partly responsible for the modest reduction often seen in TNFα levels upon IL-6 exposure (Niemand et al., 2003). However, others have demonstrated that SOCS3 impairs the anti-inflammatory actions of IL-6 in macrophages since, although upregulated by IL-6, this cytokine suppressor molecule also inhibits IL-6 signalling (Yasukawa et al., 2003). Nonetheless, the IL-6-associated reduction in monocyctic TNFα gene expression observed in this study may further support a beneficial role for IL-6 in insulin-resistance and T2D, since TNFα has been thought to negatively impact upon insulin signalling and promote T2D onset and progression (Donath and Shoelson, 2011). Furthermore, TNFα has also been associated with accelerated atherosclerosis (McKellar et al., 2009). Thus, IL-6-associated reductions in TNFα in an inflammatory milieu may aid in the prevention of CVD, which is often a major complication in T2D (Barlovic et al., 2011).

As with THP-1 monocytes, no significant changes were observed in M2 marker expression in THP-1 macrophages following IL-6 treatment for 3-48h (see Fig. 4.4), when compared to untreated THP-1 macrophages. However, a trend towards reduced expression was found for both M2 markers, with the reduction in IL-1Ra expression nearing significance following IL-6 treatment ($p<0.07$). Also, like THP-1 monocytes, MCP-1 expression increased non-significantly in THP-1 macrophages, with expression being most elevated upon 48h treatment with IL-6, following which a ~2 fold increase was observed when compared to untreated THP-1 macrophages (see Fig. 4.5). In contrast to THP-1 monocytes, no significant changes in TNFα gene expression were observed in THP-1 macrophages following IL-6 treatment (see Fig. 4.5). Thus, in macrophage-like, THP-1 macrophages, direct IL-6 signalling does not appear to have an effect on cell phenotype. Again, these data do not correlate with previous findings which suggest that IL-6 is capable of promoting M2 macrophage polarisation,
with a particular emphasis on its ability to upregulate IL-1Ra, both on a cellular (inclusive of monocytes/macrophages) and systemic level (Tilg et al., 1994, Jordan et al., 1995, Steensberg et al., 2003, Petersen and Pedersen, 2005, Fernando et al., 2014, Mauer et al., 2014). It is possible, however, that an additional stimuli is required for IL-6 to have an impact on macrophage phenotype, since several previous investigations in this area have either been carried out using co-treatment of cells (IL-6 in addition to M2 stimuli, such as IL-4/IL-13) or in whole organisms, whereby additional systemic factors may contribute to IL-6’s ability to alter macrophage polarity and associated cytokine synthesis (Fernando et al., 2014, Mauer et al., 2014).

Interestingly, not only has IL-6 has been found to enhance IL-4/IL-13 induced M2 polarisation but it has also been shown to enhance IFNγ-induced M1 polarisation in murine macrophages. Thus, it has been suggested that IL-6 may enhance the phenotype to which a macrophage is already committed rather than acting as a lone stimulus for macrophage polarisation (Fernando et al., 2014). This is in line with a recent review by Kristiansen and Mandrup-Poulsen (2005) which argues that, “The cellular response to IL-6...may further depend on the combination of other external stimuli”, and may explain the conflicting findings with regards to the role of IL-6 in inflammation and associated insulin resistance. For example, in a classic immune response, IL-6 may be accompanied by a plethora of inflammatory mediators and may, therefore, work in combination with these to adopt a more pro-inflammatory role. In contrast, in an exercise setting, IL-6 may work alongside other exercise-induced mediators, such as adrenaline, to inhibit TNF-α synthesis and induce the production of anti-inflammatory mediators such as IL-10 and IL-1Ra (Febbraio and Pedersen, 2002, Pedersen et al., 2004). Thus, the monocyte response to IL-6 may depend on the milieu in which they exist and, hence, following exercise IL-6 may work in
combination with anti-inflammatory mediators to skew monocytes away from the M1 phenotype. Unfortunately, co-treatment of monocytes with IL-6 and M1/M2 stimuli was not carried out in the present study and, therefore, it is not possible to say from the current data whether the same could be said for the cells used in these investigations.

It is also possible, however, that the THP-1 differentiation protocol used in this study may have inhibited IL-6 signalling, thus explaining the non-significant effect of this cytokine on macrophage phenotype. PMA has been shown to inhibit STAT-3-dependent IL-6 signalling in myeloid cells, either completely (100ng/ml) or partially (50ng/ml and 20ng/ml) (Sengupta et al., 1998). Thus, although every effort was made to avoid this by using very low PMA concentrations (5ng/ml) for differentiation, partial disruption of IL-6 signalling may have still occurred as a result of THP-1 differentiation. Thus, to avoid in vitro, THP-1-associated artefacts, primary human cells were also used in the present study to measure the effects of IL-6 on M1/M2 marker expression in human monocytes ex vivo. Since T cell-associated cytokines are said to impact upon macrophage polarisation, monocytes were treated with IL-6 in the presence of lymphocytes so as to more closely mimic the in vivo environment (Sica and Mantovani, 2012); PMNCs (primarily consisting of primary human monocytes and lymphocytes) were treated with 100pg/ml and 1ng/ml IL-6 for 3h, prior to separation of monocytes and lymphocytes via adherence techniques (as described by Fuss et al. (2009)), and then gene expression in each cell type was analysed. In primary monocytes, Dectin-1 was used as an M2 marker alongside the potent anti-inflammatory cytokine, IL-10, whereas MCP-1 and TNFα were adopted as M1 markers (Martinez and Gordon, 2014).
Following treatment of monocyte-lymphocyte co-cultures with 100pg/ml IL-6, the gene expression of the M1 and M2 markers (see Fig. 4.8 and Fig. 4.7, respectively) were not significantly altered in monocytes, when compared to untreated control cells. These data suggest that at 100pg/ml, IL-6 alone is not sufficient to induce phenotypic changes in co-cultured primary monocytes. The lack of effect observed may be due to the low concentration of IL-6 used; a concentration of 100pg/ml IL-6 was selected as this may be representative of an exercise setting, since strenuous exercise has previously been shown to induce circulating IL-6 levels of >100pg/ml (Starkie et al., 2003). However, where previous studies have shown that IL-6 induces M2 macrophage polarisation, cells have been treated with much higher concentrations of this cytokine (Roca et al., 2009, Fernando et al., 2014, Mauer et al., 2014). Therefore, to compare with the literature, cells were also treated with supraphysiological concentrations (1ng/ml) of IL-6. However, a similar pattern was observed, in that no significant changes in the expression of either M2 markers, Dectin-1 or IL-10 (see Fig. 4.7), or the M1 marker, TNFα (see Fig. 4.8), was detected in monocytes following incubation with 1ng/ml IL-6 for 3h, relative to untreated controls. Surprisingly, however, MCP-1 was found to be significantly reduced in these cells by approximately 0.2 fold (see Fig. 4.8). In fact, IL-6 induced a similar reduction in monocyte MCP-1 levels when compared to IL-4 (see Fig. 4.8), the latter being a well-known inducer of M2 polarisation and an inhibitor of the M1 macrophage phenotype (Mantovani et al., 2004). In promoting immune cell recruitment into tissues, this M1-associated chemokine (mainly produced by monocytes/macrophages) has been shown to play a role in insulin-resistant diabetes and atherosclerosis (Deshmane et al., 2009). Thus, these results suggest that IL-6 may work to prevent the onset and progression of these diseases by reducing monocyte production of MCP-1. Bearing in mind the potential
for monocytes to be primed into specific macrophage phenotypes (as suggested by Bouhlel et al. (2007)), the effects of IL-6 on MCP-1 synthesis by monocytes may also carry through to the tissue environment in which MCP-1 has been found to induce many of its disease-inducing activities (Panee, 2012). However, the concept of monocyte priming for macrophage polarisation requires validation since little research has been carried out into this area. Additionally, some believe that stimuli within the tissue environment may have the ability to induce a ‘switch’ in macrophage phenotype (Lee et al., 2013). Therefore, it is likely that the pre-exposure of monocytes to polarisation stimuli may play only be one of several influences on macrophage phenotype (Biswas and Mantovani, 2010, Lee et al., 2013). However, there is some debate regarding the actuality of macrophage switching in vivo and, thus, this area of research still requires further investigation (Dalmas et al., 2011).

In order to elucidate potential mechanisms by which IL-6 might downregulate M1 marker gene expression, the relationship between IL-6, T lymphocytes and monocytes was investigated. IL-6 has been shown to induce T cell differentiation into the Th2 phenotype via induction of the IL-4 gene. Not only does IL-4 stimulate further Th2 differentiation but it also induces the M2 macrophage phenotype, thus inhibiting M1 macrophage polarisation (Rincon et al., 1997, Diehl and Rincon, 2002, Sica and Mantovani, 2012). Hence, in the present study, there was reason to believe that T lymphocytes (which make up the majority of PMNCs) may have indirectly contributed to the effects of IL-6 on monocyte MCP-1-expression, via secretion of a secondary signal, such as IL-4 (Okita et al., 2013). To investigate this, IL-4 gene expression was analysed in the lymphocyte fraction of IL-6 treated co-cultured cells and secreted IL-4 was quantified in supernatants. While IL-4 gene expression in the monocyte fraction was significantly downregulated by ~0.5 fold following IL-6 treatment, IL-4 gene
expression levels were found to be non-significantly elevated in the lymphocyte fraction (see Fig. 4.6). However, IL-4 was not detectable in the supernatant of IL-6 treated co-cultured cells, using an assay with a minimum detection level of 0.25 pg/ml (data not shown). Therefore, the data obtained in the current study are equivocal with regard to whether lymphocyte-derived IL-4 may be accountable for the effect of IL-6 (and – by extension – exercise) on priming of monocytes for a predominantly M2 rather than M1 macrophage phenotype.

It should be noted that others have shown that IL-6 actually inhibits the synthesis of T cell-associated cytokines, including IL-4, whilst in co-culture with alternatively-activated macrophages (Fernando et al., 2014). Furthermore, some argue against the relevance of T cells in macrophage polarisation altogether, stating that it is the macrophages which influence T cell phenotype as opposed to the other way around (Italiani and Boraschi, 2014). However, the data appears to suggest – albeit with the reservations expressed above – that a lymphocyte associated-factor may have contributed to the IL-6-induced downregulation of MCP-1 expression in monocytes observed in the present study. This is demonstrated by the lack of effect on MCP-1 expression when monocytes were cultured with 1ng/ml IL-6 independent of lymphocytes (see Fig. 4.9), whilst IL-4 treatment significantly reduce MCP-1 levels whether monocytes were treated with IL-6 in the presence or absence of lymphocytes (see Fig. 4.8 and Fig. 4.9, respectively). Others have shown that IL-6 may induce expression of the receptor for IL-4, IL-4Rα, in macrophages and myeloid cells, making them more responsive to IL-4 induced alternative activation (Fernando et al., 2014, Mauer et al., 2014). Similarly, IL-6 receptor-deficient murine myeloid cells were found to be non-responsive to IL-4 induced alternative activation, suggesting a close relationship between these two cytokines with regards to their inter-linked role in
macrophage polarisation (Mauer et al., 2014). Thus, it is possible that, although IL-4 expression and synthesis was not found to be upregulated by IL-6 in the present study, the expression of IL-4Rα may have been induced in monocytes following IL-6 treatment, making them more sensitive and responsive to existing background levels of IL-4, such as that produced by T lymphocytes (Lawrence and Natoli, 2011, Sica and Mantovani, 2012). However, IL-4Rα expression was not analysed in the present study and, thus, further investigations may help to elucidate whether its upregulation also occurs in monocytes following IL-6 treatment.

Together with the previous literature, these findings may play particular relevance in the context of exercise, whereby M2 polarisation has been shown to be induced, without alterations in systemic levels of IL-4/IL-13 but with increased IL-6 synthesis by working muscle (Suzuki et al., 2000, Malm et al., 2004, Peake et al., 2005, Pedersen and Febbraio, 2008, Yakeu et al., 2010, Ikeda et al., 2013, Oliveira et al., 2013, Mauer et al., 2014). Moreover, the data displayed in Chapter 3 closely relate to those in this study, whereby participation in a moderate-intensity, 8 week walking intervention significantly increased circulating levels of IL-6, acutely after each exercise bout (see Fig. 3.3), whilst also increasing the expression of the M2 markers, Dectin-1 and IL-10 (see Fig. 3.2) and decreasing expression of the M1 marker, MCP-1 (see Fig. 3.1) in peripheral monocytes. Thus, it is possible that, by priming monocytes away from the M1 macrophage phenotype, exercise-derived IL-6 may be partly responsible for the insulin-sensitising effects of exercise, providing a further mechanisms for the health benefits of exercise with regards to T2D and associated CVD (Starkie et al., 2003, Sica and Mantovani, 2012). However, it must be noted that, compared to the concentrations of IL-6 used in this study, much lower concentrations of IL-6 were detected following exercise participation (1.63pg/ml ± 1.32 pg/ml, see
Fig. 3.3) and, thus, it is likely that, alongside IL-6, other exercise-induced factors may have contributed to the effects of exercise on monocyte phenotype previously observed.

In summary, the data displayed in the present chapter demonstrate the ability for IL-6 to alter monocyte phenotype by downregulating the gene expression of the pro-inflammatory mediators, TNFα and MCP-1. Since these cytokines are associated with the M1 macrophage phenotype, it is suggested that IL-6 may prime monocytes away from the M1 phenotype which has been implicated in the insulin resistant and T2D state (Olefsky and Glass, 2010, Lumeng and Saltiel, 2011, Oh et al., 2012). Lymphocytes, presumably T cells, were found to influence the ability for IL-6 to inhibit M1-marker gene expression, but the data were equivocal as to whether this occurred in association with, or independent of alterations in IL-4 synthesis and secretion. Nevertheless, the effects demonstrated in this study ascribe a role for IL-6 in altering monocyte phenotype in a way which may beneficially impact upon insulin signalling, T2D risk and the onset of T2D-associated cardiovascular co-morbidities.
CHAPTER 5

The Role of PPARγ in Monocyte Priming for M1/M2 Macrophage Polarisation

5.0. Introduction

The beneficial impact of exercise participation on insulin resistance and T2D risk is well publicised, with several key studies, such as the Diabetes Prevention Study in Finland, the Diabetes Prevention Program and the Da Qing IGT and Diabetes Study, finding significant reductions in diabetes incidence following lifestyle interventions which included increased physical activity (Pan et al., 1997, Eriksson et al., 1999, Diabetes Prevention Program Research, 2002, Colberg et al., 2010). The mechanisms by which exercise exerts its beneficial actions with regards to insulin signalling and T2D risk are currently under investigation (Colberg et al., 2010). However, exercise is known to alter inflammation in a way which reduces systemic levels of inflammatory mediators that disrupt insulin signalling whilst also elevating levels of regulatory cytokines (Petersen and Pedersen, 2005, Golbidi et al., 2012).

One way in which exercise may potentially alter systemic inflammation is by altering macrophage phenotype (Yakeu et al., 2010, Ikeda et al., 2013, Oliveira et al., 2013). Macrophages are a heterogeneous group of cells which may be broadly classified as ‘M1’ or ‘M2’ cells. Although both play important roles in health, M1 macrophages are thought to greatly contribute to inflammation by producing pro-inflammatory mediators, such as MCP-1 and TNFα, both of which are thought to impair insulin signalling and promote the development of T2D (Hotamisligil et al., 1993, Mantovani et al., 2004, Gustafson, 2010, Olefsky and Glass, 2010). Furthermore, M1
Macrophages have been shown to play specific roles in the development of CVD and atherosclerosis, the latter of which is often accelerated and more severe in individuals with diabetes (Buono et al., 2003, Barlovic et al., 2011, Oh et al., 2012). In contrast, M2 macrophages have been shown to inhibit M1 macrophage-derived inflammation via production of regulatory cytokines, such as IL-1Ra, and are, hence, deemed beneficial in the prevention and management of insulin resistance, T2D and associated cardiovascular complications (Mantovani et al., 2004, Odegaard et al., 2007, Goldfine et al., 2013).

Interestingly, exercise has been demonstrated to increase systemic levels of M2-inducing stimuli (IL-10 and IL-4) (Balducci et al., 2010). However, many others have shown no effect on systemic levels of IL-4 in response to physical activity (Suzuki et al., 2000, Malm et al., 2004, Peake et al., 2005). Nonetheless, exercise has been found to induce an increase in M2 macrophage marker expression in tissue (Ikeda et al., 2013, Oliveira et al., 2013). Moreover, macrophage depletion during exercise was found to inhibit insulin sensitivity in muscle (Ikeda et al., 2013). Hence, it is possible that exercise-induced M2 macrophage polarisation may beneficially impact upon insulin sensitivity in order to prevent the onset and progression of T2D and its cardiovascular co-morbidities. Therefore, much research focus has been placed on elucidating mechanisms by which exercise might drive macrophages towards the M2 phenotype, including from the current author’s research group (Yakeu et al., 2010).

Although several molecular mechanisms have been associated with M2 macrophage polarisation, the current study will focus on PPARγ, which may have particular relevance in the context of exercise. PPARγ is an important regulator of macrophage-derived inflammation which has been shown to be upregulated during exercise (Butcher et al., 2008, Rigamonti et al., 2008, Thomas et al., 2012, Ahmadian et al.,
2013). Its activation in macrophages is thought to indirectly repress pro-inflammatory pathway activation, such as that of NF-κB and AP-1, and also to directly upregulate many PPARγ target genes that exert anti-inflammatory effects (Rigamonti et al., 2008, Ahmadian et al., 2013). Moreover, PPARγ has been shown to be supportive of M2 activation; not only is its expression and activity elevated by the well-established M2 inducers, IL-4 and IL-13, but it has also been shown to be necessary for the full induction of certain M2-associated molecules, such as CD36 and Dectin-1, in response to these stimuli (Feng et al., 2000, Coste et al., 2003, Paintlia et al., 2006, Berry et al., 2007, Gallardo-Soler et al., 2008, Van Ginderachter et al., 2008). To this end, PPARγ is said to be a key regulator of the M2 macrophage phenotype (Charo, 2007). In support of this, macrophage-specific PPARγ deletion has been found to inhibit M2 macrophage polarisation, increase levels of inflammatory mediators and induce systemic insulin resistance and glucose intolerance (Hevener et al., 2007, Odegaard et al., 2007, Oliver et al., 2010, Ahmadian et al., 2013). Thus, macrophage-specific PPARγ activation following exercise may provide a link to increased M2 marker expression and improved glucose management.

The nuclear transcription factor, STAT-6, is thought to be largely responsible for the ability for PPARγ to enhance IL-13/IL-4-induced M2 macrophage polarisation (Szanto et al., 2010). The response of monocytes/macrophages to either IL-4 or IL-13 is said to be very similar since these cytokines both signal via a common receptor, IL-4Rα, to induce and activate STAT-6, which is proposed to be largely accountable for M2 macrophage polarisation in response to IL-13/IL-4 (Hart et al., 1999, Szanto et al., 2010). Furthermore, STAT-6 has been found to act as a PPARγ co-factor in macrophages due to the close proximity of PPARγ and STAT-6 response elements on PPARγ target genes, allowing for their regulation by activation of either pathway.
Hence STAT-6 activation has been found to majorly impact upon PPARγ-induced gene expression, including those genes which dictate macrophage phenotype (Szanto et al., 2010). Moreover, independent of STAT-6, IL-4 and IL-13 are also said to promote the generation of endogenous PPARγ ligands (Huang et al., 1999, Coste et al., 2003, Berry et al., 2007, Harmon et al., 2011, Czimmerer et al., 2012). Accordingly, IL-4 and IL-13 have been found to increase PPARγ activity in macrophages and, likewise, PPARγ activation has been shown to enhance IL-13/IL-4-induced M2 macrophage polarisation (Huang et al., 1999, Berry et al., 2007, Bouhlel et al., 2007, Van Ginderachter et al., 2008, Szanto et al., 2010). Thus, there is evidence that on some level, there may exist a synergistic role for PPARγ and IL-13/IL-4 (potentially via STAT-6), in M2 macrophage polarisation. However, to date, little research has been carried out with regards to the effects of these two signalling pathways within macrophage precursors, monocytes.

Interestingly, some have indicated that PPARγ activation may be important in inducing M2 marker expression in IL-4/IL-13 treated monocytes (Bouhlel et al., 2007, Yakeu et al., 2010). Accordingly, the data presented in Chapter 3 indicate a role for exercise-induced PPARγ activation in the priming of monocytes for M2 polarisation (see Fig. 3.2), possibly via an increase in the PPARγ-activating properties of serum post-exercise (see Fig. 3.6). Others have shown similar effects with regards to the PPARγ-activating properties of serum following exercise participation (Thomas et al., 2012). Thus, the ability for PPARγ to prime monocytes into the M2 macrophage phenotype may be important in an exercise setting since, in contrast to macrophages which reside in tissues, monocytes circulate in the blood and are, hence, more likely to be exposed to the circulating PPARγ-activating factors which appear to be generated following exercise (Ziegler-Heitbrock et al., 2010). Therefore, it is
hypothesised that M2 macrophage polarisation as a result of exercise may be instigated via PPARγ activation in circulating monocytes prior to macrophage differentiation. However, there are several limitations associated with the research on which this hypothesis is based. For instance, Bouhlel and colleagues treated primary human monocytes with PPARγ agonists, GW1929 (600nM) and rosiglitazone (100nM) during cell maturation, measuring M2 marker expression after cells had differentiated (Bouhlel et al., 2007). Therefore, it is uncertain at which point in the differentiation process PPARγ may have exerted its M2-inducing effects. Furthermore, Yakeu et al. (2010) used mixed polymorphonuclear cells to conduct their research and, thus, it is possible that the reported PPARγ-induced changes in M1/M2 marker expression were not monocyte-specific.

Hence, the current research aims to investigate the effect of monocyte-specific PPARγ activation on the priming of cells for the M1 or M2 phenotype. In doing this, it is hoped that further evidence will be provided for the role of PPARγ in the priming of monocytes for M2 macrophage polarisation, a process which may beneficially improve insulin sensitivity and the onset and progression of T2D and its cardiovascular co-morbidities.
5.2. Methods

5.2.1. Cell Culture
All cell culture consumables were manufactured by Corning® (Sigma Aldrich, Dorset, UK) and all reagents were obtained from Invitrogen (Invitrogen Ltd, Paisley, UK), unless otherwise stated

5.2.1.1. THP-1 Cell Culture
THP-1 monocytes (ATCC, Middlesex, UK), were cultured and maintained as described in Chapter 4, section 4.2.1.1.

5.2.1.2. THP-1 Treatment
THP-1 cells were plated at 0.3-0.5x10^6 cells/ml. Cells were treated with 1µM rosiglitazone (GlaxoSmithKline, Uxbridge, UK) and/or 15ng/ml recombinant human IL-13 (R&D Systems, Abingdon, UK) and incubated for 24h in a 37°C, 5% CO2, humidified incubator.

For priming experiments, THP-1 monocytes were pre-treated for 24h with 1µM rosiglitazone and/or combined IL-13 and IL-4 (10ng/ml of each). Following treatment, cells were washed twice in RPMI, centrifuging at 300xg for 10 minutes to pellet after washes. Subsequently cells were resuspended in supplemented RPMI and differentiated into THP-1 macrophages using 5ng/ml PMA for 48h. At this stage, cells were either harvested as described below or, alternatively, cells were treated with 100ng/ml of lipopolysaccharide (LPS; Ultrapure LPS from Escherichia coli K12, Invitrogen Ltd, Paisley, UK) for 4h prior to harvesting.

To investigate the effects of maturation, THP-1 were treated with 5ng/ml PMA for 2h, 4h, 6h, 8h, 12h and 24h, with suspended and adherent cells being harvested at each
time point. Additionally, for protein analysis THP-1 were treated with 1µM rosiglitazone and/or 15ng/ml recombinant human IL-4 (Peprotech, London, UK) for 12h in a 37°C, 5% CO2, humidified incubator before harvesting for protein analysis.

To harvest cells, undifferentiated (suspended) THP-1 monocytes were collected and centrifuged at 300xg for 10 minutes. Cells were washed twice in RPMI twice, centrifuging at 300xg for 10 minutes to pellet after washes. Alternatively, media was removed from PMA-differentiated (adherent) THP-1 macrophages and cells were washed twice in pre-warmed PBS. Cells were then incubated with 0.25% trypsin-EDTA for 1-2 minutes (or until cells detach) at 37°C. RPMI was added at thrice the volume of trypsin used to stop the reaction and cells were pelleted by centrifugation at 300xg for 10 minutes. Cell pellets were used in subsequent PCR (see section 5.2.2) and Western Blot assays (see section 5.2.3).

5.2.2. PCR

Cell pellets were resuspended in 1ml of TRI Reagent® (Applied Biosystems, Warrington, UK) and RNA was extracted as described in Chapter 3, section 3.2.4. RNA was converted to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Warrington, UK), as described in Chapter 3, section 3.2.5. Gene expression was analysed via RT-PCR, using the Fast SYBR® Green assay in combination with an Applied Biosystems Fast 7500 Real-Time PCR System (Applied Biosystems, Warrington, UK), as described in Chapter 3, section 3.2.6 (see Table 5.1 for primer sequences). GAPDH as a housekeeping gene. β-actin was also used to normalise data, giving similar results as GAPDH (data not shown). The comparative C_T method was employed to calculate relative gene expression in samples (for details, see Chapter 3, section 3.2.6.3). Untreated cells were obtained alongside all treatments and time points for use as comparators.
Table 5.1: Primer sequences used in SYBR® Green RT-PCR assay

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CATGACCTCAACTACATG</td>
<td>TCTCCATGGTGGAAGGAC</td>
</tr>
<tr>
<td>β-actin</td>
<td>TCCTGTGGGATCCACGAA</td>
<td>GAAGCATTTGCCGTTGAC</td>
</tr>
<tr>
<td>PPARγ</td>
<td>CGTGGGCCAGATTGAA</td>
<td>CCTGTTACGAGAGATCCA</td>
</tr>
<tr>
<td>CD36</td>
<td>GGAAGTGATGGAGGAGAGC</td>
<td>GAGACTGTTGTTGCCTCAGCT</td>
</tr>
<tr>
<td>Dectin-1</td>
<td>GGAAGCAGACACATGAGAATGG</td>
<td>CTTGGTAGGAGTCACACTGTC</td>
</tr>
<tr>
<td>IL1Ra</td>
<td>GGCCTCCGACATACCTACAC</td>
<td>GGACAGGACATCTCCCTCCAT</td>
</tr>
<tr>
<td>MCP-1</td>
<td>TGCTCATACGAGCCTACTCC</td>
<td>GGGACACTTGCTGTGGATTCT</td>
</tr>
<tr>
<td>TNFα</td>
<td>TGCCCTGCTGACTTTGGAGTG</td>
<td>CTGGGCCAGAGGCTTGATTAGA</td>
</tr>
</tbody>
</table>

5.2.3. Western Blotting

The expression of PPARγ, STAT-6 and phospho-STAT-6 (Tyr641) were analysed in THP-1 monocytes and THP-1 macrophages, using actin as an internal control. All antibodies were obtained from New England Biolabs (Herts, UK), other than the actin antibody which was obtained from Sigma Aldrich (Dorset, UK).

5.2.3.1. Protein Extraction and Quantification

Following cell treatment and harvest, cell pellets were resuspended in 112μl RIPA Buffer (with phosphatase and protease inhibitors), for 30 minutes, mixing every 10 minutes, to extract cellular proteins. Samples were sonicated for 10 seconds at an amplitude of 35% using a VCX 500 Ultrasonic Processor (Sonics & Metrials (UK) Ltd., Suffolk, UK) to shear DNA and particulate material was removed by centrifugation. Protein lysates were stored at -80°C prior to quantification. Proteins
were quantified in samples using the Bio-Rad DC Protein Assay (Bio-Rad, Hertfordshire, UK), as described in Chapter 3, section 3.2.8.2.

5.2.3.2. Protein Separation by SDS-PAGE Electrophoresis

Samples were normalised to 15μg/μl protein, proteins denatured and samples loaded onto NuPAGE® 12-well, 10% Bis-Tris, 1.0mm Gels (Invitrogen Ltd, Paisley, UK), as described in Chapter 3, section 3.2.8.3. Additionally, a molecular weight standard marker, MagicMark™ (Invitrogen Ltd, Paisley, UK), was loaded on all gels. Electrophoresis was carried out at 200V for 1h and 10 minutes at 4°C, as described in Chapter 3, section 3.2.8.3

5.2.3.3. Electrophoretic Transfer of Proteins

The iBlot® Dry Blotting System (Invitrogen Ltd, Paisley, UK) was used to transfer protein from gels to nitrocellulose membranes, as described in Chapter 3, section 3.2.8.4.

5.2.3.4. Immunoblotting of Proteins

The PPARγ (81B8) and STAT-6 (D3H4) rabbit monoclonal antibodies were used to investigate PPARγ and STAT-6 expression, respectively, in samples. Similarly, the phospho-stat6 (Tyr641) (C11A12) rabbit monoclonal antibody was used to analyse STAT-6 phosphorylation at tyrosine 641 in samples. As per manufacturer’s instruction, antibodies were diluted 1:1000 in 5% BSA diluted in TBS-T. Molecular bands at 53 kDa and 57 kDa corresponded to PPARγ isoforms 1 and 2, respectively, whereas molecular bands corresponding with STAT-6 and phosph-STAT-6 had a molecular weight of 110kDa and 100kDa, respectively. The actin (20-33) rabbit polyclonal antibody was used to control for potential loading variations. The actin antibody was diluted 1:5000 in 5% BSA diluted in TBS-T and bands were visualised
at 42kDa. A goat anti-rabbit IgG, HRP-linked antibody was used as a secondary antibody for all antibodies. For PPARγ, STAT-6 and phosphor-STAT-6 analysis, the secondary antibody was diluted 1:1000 in 0.25% non-fat dried milk in TBS-T. For actin visualisation, the secondary antibody was diluted 1:3000 in 0.25% non-fat dried milk in TBS-T.

Immunoblotting was carried out as described in Chapter 3, section 3.2.8.5. Amersham™ ECL Select™ (GE Healthcare Life Sciences, Buckinghamshire, UK) was used as per manufacturer’s instructions and immunoreactive complexes were detected by exposing membranes to X-Ray film (Kodak® BioMax® light film, Sigma-Aldrich (Dorset, UK)) in an autoradiography hypercassette (GE Healthcare Life Sciences, Buckinghamshire, UK), as described in Chapter 3, section 3.2.8.5. Relative band intensity was determined by scanning densitometry using Image J software (available at http://rsb.info.nih.gov/).

5.2.4. Statistical Analysis

All data were expressed as mean ± standard deviation (SD), unless otherwise stated. Where comparisons were made between the means of two samples, t-tests were used when data was assumed to be normally distributed. Alternatively, one-way ANOVA with Tukey’s post-hoc analysis was used for multiple comparisons within groups of normally distributed data. Statistical analysis was performed using GraphPad Prism®5 software and results were deemed significant at $p<0.05$. 
5.3. Results

5.3.1. Gene Expression of PPARγ and Downstream CD36 in THP-1 Monocytes Treated with IL-13 and/or Rosiglitazone

The expression of PPARγ in rosiglitazone only treated cells was not significantly different from controls (0.94 ± 0.12 fold). In THP-1 monocytes treated with IL-13 and IL-13 + rosiglitazone, however, the expression of PPARγ decreased significantly (0.69 ± 0.11 fold and 0.71 ± 0.05 fold, respectively, \( p < 0.05 \) for both) when compared to untreated control cells. Furthermore, IL-13 and IL-13 + rosiglitazone treated THP-1 monocytes expressed significantly less PPARγ mRNA than rosiglitazone only treated cells (see Fig. 5.1).

Fig. 5.1 also demonstrates that CD36 expression was significantly increased in rosiglitazone treated cells (1.52 ± 0.06 fold, \( p < 0.01 \)) when compared to untreated control cells. CD36 expression in IL-13 (0.89 ± 0.06 fold) and IL-13 + rosiglitazone (1.21 ± 0.13 fold) treated cells did not change when compared to the control, whilst rosiglitazone treated cells also had significantly higher levels of CD36 expression when compared to IL-13 and IL-13 + rosiglitazone treated THP-1 monocytes (\( p < 0.01 \) and \( p < 0.05 \), respectively).
Figure 5.1. PPARγ and CD36 expression in IL-13 and/or rosiglitazone treated THP-1 monocytes. THP-1 monocytes were treated with 15ng/ml IL-13, 1µM rosiglitazone (RSG) or a combination of the both for 24h. Untreated cells were used as controls and gene expression was measured via RT-PCR (n=3; values expressed as gene expression relative to GAPDH ± SD; * denotes significance compared to control (*p<0.05, **p<0.01), † denotes significance compared to rosiglitazone (†p<0.05; ††p<0.01), one-way ANOVA).
5.3.2. Gene Expression of M1 and M2 Markers in THP-1 Monocytes Treated with IL-13 and/or Rosiglitazone

As shown in Fig 5.2, cells treated with rosiglitazone alone did not alter gene expression of the M2 marker, IL-1Ra, when compared to controls (0.85 ± 0.34 fold). IL-1Ra expression was, however, elevated in IL-13 treated cells, when compared to controls (2.16 ± 0.09 fold, \(p<0.01\)). IL-13 + rosiglitazone treated cells, on the other hand, expressed significantly less IL-1Ra compared to control cells (0.03 ± 0.001 fold), cells treated with rosiglitazone or IL-13 alone (\(p<0.01\) for all).

Fig. 5.3 demonstrates that the gene expression of the M1 marker, MCP-1, did not alter in cells treated with rosiglitazone alone (0.81 ± 0.27 fold) when compared to control cells. Expression did, however, increase significantly with IL-13 treatment of cells (11.59 ± 3.84 fold; \(p<0.01\) for both) and non-significant elevations in MCP-1 were observed with IL-13 + rosiglitazone treatment (4.78 ± 2.51 fold), relative to controls. Additionally, when compared to rosiglitazone only treated cells and rosiglitazone + IL-13 treated cells, IL-13 treatment alone induced a significant increase in THP-1 monocyte MCP-1 expression (\(p<0.01\) and \(p<0.05\), respectively).
**Figure 5.2. IL-1Ra expression in IL-13 and/or rosiglitazone treated THP-1 monocytes.** THP-1 monocytes were treated with 15ng/ml IL-13, 1µM rosiglitazone (RSG) or a combination of the both for 24h. Untreated cells were used as controls and gene expression was measured via RT-PCR (n=3; values expressed as gene expression relative to GAPDH ± SD; * denotes significance compared to control (**p<0.01), † denotes significance compared to rosiglitazone (††p<0.01), ‡ denotes significance compared to IL-13 (‡‡p<0.01), one-way ANOVA).
Figure 5.3. MCP-1 expression in IL-13 and/or rosiglitazone treated THP-1 monocytes. THP-1 monocytes were treated with 15ng/ml IL-13, 1µM rosiglitazone (RSG) or a combination of both for 24h. Untreated cells were used as controls and gene expression was measured via RT-PCR (n=4; values expressed as gene expression relative to GAPDH ± SD; * denotes significance compared to control (**p<0.01), one-way ANOVA).
5.3.3. The Effect of THP-1 Monocyte Priming with Rosiglitazone and/or IL-13/IL-4 on M1 and M2 Marker Gene Expression in THP-1 Macrophages

THP-1 monocytes were primed via pre-treatment with 1µM rosiglitazone and/or combined IL-13 and IL-4 (10ng/ml of each) for 24h, following which cells were washed and differentiated into THP-1 macrophages using 5ng/ml PMA for 48h.

The expression of the M2 marker, IL-1Ra, was non-significantly reduced in rosiglitazone primed cells when compared to controls (0.73 ± 0.16 fold), whereas priming with IL-13 + IL-4 (0.12 ± 0.07 fold) or rosiglitazone + IL-13 + IL-4 (0.11 ± 0.12 fold) significantly reduced IL-1Ra expression, when compared to untreated controls (see Fig. 5.4, p<0.01 for all). Further, rosiglitazone primed cells had significantly higher levels of IL-1Ra expression when compared IL-13 + IL-4 and rosiglitazone + IL-13 + IL-4 (p<0.05 for both).

Fig. 5.5 demonstrates that expression of the M1 marker, TNFα, was non-significantly reduced in THP-1 macrophages primed with rosiglitazone (0.72 ± 0.07 fold). TNFα expression was significantly reduced, however, in cells primed IL-13 + IL-4 (0.26 ± 0.15 fold, p<0.01) and rosiglitazone + IL-13 + IL-4 (0.22 ± 0.11 fold, p<0.05), when compared to controls. Additionally, rosiglitazone + IL-13 + IL-4 priming significantly reduced TNFα expression when compared to rosiglitazone treatment only.
Figure 5.4. The effect of rosiglitazone and/or IL-13/IL-4 priming of THP-1 monocytes on IL-1Ra expression in THP-1 macrophages. THP-1 monocytes were primed for 24h with 1µM rosiglitazone (RSG) and/or 15ng/ml IL-4 or IL-13 (or 10ng/ml IL-4/IL-13 when used in combination) prior to differentiation and gene expression analysis via RT-PCR. Untreated THP-1 macrophages were used as controls (n=3; values expressed as gene expression relative to GAPDH ± SEM; * denotes significance compared to control (**p<0.01), † denotes significance compared to rosiglitazone (†p<0.05), one-way ANOVA).
Figure 5.5. The effect of rosiglitazone and/or IL-13/IL-4 priming of THP-1 monocytes on TNFα expression in THP-1 macrophages. THP-1 monocytes were primed for 24h with 1µM rosiglitazone (RSG) and/or 15ng/ml IL-4 or IL-13 (or 10ng/ml IL-4/IL-13 when used in combination) prior to differentiation and gene expression analysis via RT-PCR. Untreated THP-1 macrophages were used as controls (n=3; values expressed as gene expression relative to GAPDH ± SEM; * denotes significance compared to control (**p<0.01), † denotes significance compared to rosiglitazone (†p<0.05), one-way ANOVA).
5.3.4. The Effect of LPS Challenge on M1 and M2 Marker Gene Expression in Rosiglitazone and/or IL-13/IL-4 Primed THP-1 Macrophages,

Subsequent to cell priming, THP-1 macrophages were challenged with 100ng/ml LPS. Alternatively, ‘non-primed’ THP-1 macrophages (i.e. cells not pre-treated with rosiglitazone and/or IL-4/IL-13) were challenged with 100ng/ml LPS for use as controls. Relative to untreated THP-1 macrophages, LPS challenged THP-1 macrophages were found to have significantly elevated levels of the M2 marker, IL-1Ra (14.29 ± 0.12 fold, p<0.01, data not shown).

Subsequent to priming with IL-4 + IL-13, cells challenged with LPS significantly increased gene expression of the M2 marker, IL-1Ra, when compared to unprimed controls treated with LPS (2.06 ± 0.12 fold, p<0.01). IL-1Ra gene expression in cells challenged with LPS subsequent to being primed with rosiglitazone (1.63 ± 0.19 fold and rosiglitazone + IL-13 + IL-4 (1.49 ± 0.33 fold) also appeared to be elevated when compared to control cells but this increase was non-significant (see Fig. 5.6).

Relative to untreated THP-1 macrophages, LPS-challenged THP-1 macrophages were found to have significantly elevated expression of the M1 marker, TNFα (31.91 ± 7.22 fold, p=0.01, data not shown). Gene expression of the M1 marker, TNFα, did not alter significantly following LPS challenge of rosiglitazone (2.06 ± 0.47 fold), IL-13 + IL-4 (0.60 ± 0.22 fold) or rosiglitazone + IL-13 + IL-4 (1.02 ± 0.33 fold) primed THP-1 macrophages, when compared to the response evoked by LPS in control cells (see Fig. 5.7). However, following LPS challenge, TNFα expression was significantly reduced in IL-13 + IL-4 primed THP-1 macrophages when compared to cells primed with rosiglitazone only (p<0.05).
Figure 5.6. IL-1Ra expression in IL-13, IL-4 and/or rosiglitazone primed THP-1 macrophages, challenged with LPS. Rosiglitazone (RSG) and/or IL-13/IL-4 primed cells were challenged with 100ng/ml LPS. Untreated THP-1 cells were also challenged with LPS and used as controls. Gene expression was measured via RT-PCR (n=3; values expressed as gene expression relative to GAPDH ± SEM; **p<0.01, one-way ANOVA).
Figure 5.7. TNFα expression in IL-13, IL-4 and/or rosiglitazone primed THP-1 macrophages, challenged with LPS. Rosiglitazone (RSG) and/or IL-13/IL-4 primed cells were challenged with 100ng/ml LPS. Untreated THP-1 cells were also challenged with LPS and used as controls. Gene expression was measured via RT-PCR (n=3; values expressed as gene expression relative to GAPDH ± SEM, † denotes significance compared to rosiglitazone (†p<0.05), one-way ANOVA).
5.3.5. Analysis of PPARγ and Target Gene (CD36) Expression and STAT-6 Activation upon THP-1 Differentiation

Fig. 5.8 shows a time dependent increase in PPARγ expression in THP-1 following incubation with the inducer of differentiation, PMA. When compared to undifferentiated controls (THP-1 monocytes), PPARγ expression was elevated at all time points following PMA treatment (2h: 3.44 ± 0.14 fold; 4h: 4.00 ± 0.58 fold; 6h: 7.34 ± 0.32 fold; 8h: 9.21 ± 0.45 fold; 12h: 9.39 ± 0.45 fold; 24h: 8.11 ± 0.21 fold). Additionally, as also shown in Fig. 5.8, PPARγ protein expression was significantly elevated in PMA-differentiated THP-1 macrophages, relative to undifferentiated THP-1 monocytes (1.27 ± 0.14 fold, p<0.05).

When compared to undifferentiated controls, the gene expression of a PPARγ downstream gene, CD36, was also notably elevated in PMA treated cells but only after 6h of treatment. CD36 expression levels remained elevated when compared to controls but began to return to baseline following 12h of treatment (2h: 1.29 ± 0.18 fold; 4h: 2.28 ± 0.22 fold; 6h: 43.20 ± 1.33 fold; 8h: 62.25 ± 2.38 fold; 12h: 35.67 ± 1.63 fold; 24h: 8.65 ± 0.18 fold, see Fig. 5.8).

Fig. 5.9 demonstrates that STAT-6 phosphorylation was significantly elevated in PMA-differentiated THP-1 macrophages, relative to undifferentiated THP-1 monocytes (2.4 ± 0.13 fold, p<0.01).
Figure 5.8. The effect of THP-1 differentiation on PPARγ and CD36 expression.

THP-1 monocytes (THP-1 mon) were used as controls or treated with 5ng/ml PMA to generate THP-1 macrophages (THP-1 mac). A) PPARγ protein expression was measured (in bands outlined) via Western blot prior to (THP-1 mon) and after (THP-1 mac) 48h PMA treatment. B) PPARγ and C) CD36 gene expression was analysed via RT-PCR following incubation with PMA over various time points (n=3, protein analysis, protein values expressed as intensity normalised to actin ± SD, *p<0.05, t-test; mRNA analysis, n=1, mRNA values expressed as gene expression relative to GAPDH ± SD).
Figure 5.9. The effect of THP-1 differentiation on phospho-STAT-6 protein expression. THP-1 monocytes (THP-1 mon) and THP-1 macrophages (THP-1 mac) were treated with IL-4 for 24h. Phospho-STAT-6 (P-STAT-6) protein expression was measured (in bands outlined) via Western blot and densitometry analysis was carried out (n=3; values expressed as intensity normalised to actin ± SD; **p<0.01, t-test).
5.3.6. Analysis of PPARγ, STAT-6 and Phospho-STAT-6 (Tyr641) Protein Expression in Rosiglitazone and/or IL-4 Treated THP-1 Monocytes

Fig. 5.10 demonstrates that PPARγ protein expression was significantly increased with rosiglitazone treatment (2.01 ± 0.05 fold), IL-4 treatment (2.02 ± 0.25 fold) and combined rosiglitazone + IL-4 treatment (2.25 ± 0.16 fold) of THP-1 monocytes, relative to untreated controls (p<0.01 for all).

In contrast, Fig. 5.11 demonstrates that STAT-6 protein expression was unaltered in THP-1 monocytes treated with rosiglitazone (1.03 ± 0.14 fold), IL-4 (1.12 ± 0.04 fold) or rosiglitazone + IL-4 (1.14 ± 0.03 fold), when compared to control cells. Fig. 5.11 also shows that phospho-STAT-6 (P-STAT-6) was non-detectable at a protein level in untreated or rosiglitazone treated THP-1 monocytes but that P-STAT-6 levels in cells treated with IL-4, either alone or combination with rosiglitazone were expressed at 0.35 ± 0.03 fold in IL-4 treated cells and 0.50 ± 0.05 fold in IL-4 + rosiglitazone treated cells when compared to total STAT-6 expression. Additionally, Fig. 5.11 shows that P-STAT-6 expression was significantly elevated in IL-4 + rosiglitazone treated cells when compared to IL-4 only treated cells (p<0.05).
Figure 5.10. Representative immunoblots and densitometric analysis of PPARγ protein expression in rosiglitazone and/or IL-4 treated THP-1 monocytes. THP-1 monocytes were treated with 1μM rosiglitazone (RSG) and/or 15ng/ml IL-4 for 24h. Untreated THP-1 monocytes were used as controls and protein expression analysed via Western blot (n=3; values expressed as intensity normalised to actin ± SD; **p<0.01, one-way ANOVA).
Figure 5.11. Representative immunoblots and densitometric analysis of STAT-6 and phospho-STAT-6 protein expression in rosiglitazone and/or IL-4 treated THP-1 monocytes. THP-1 monocytes were treated with 1µM rosiglitazone (RSG) and/or 15ng/ml IL-4 for 24h. Untreated THP-1 monocytes were used as controls and A) STAT-6 protein expression and B) P-STAT-6 protein expression were analysed via Western blot (p<0.05). (n=3; values expressed as intensity normalised to actin ± SD; (A) one-way ANOVA, (B) *p<0.05, t-test).
5.4. Discussion

It has been proposed that the exercise-induced activation of PPARγ may be partly responsible for associated improvements in insulin sensitivity and reduced T2D risk, potentially due to the effects of PPARγ on macrophage phenotype (Butcher et al., 2008, Yakeu et al., 2010, Ikeda et al., 2013). PPARγ is considered a key regulator of M2 macrophage polarisation and has been implied in the priming of monocytes for the M2 macrophage phenotype. M2 macrophages are thought to be a key factor in the prevention of insulin resistance and, hence, beneficial in a T2D setting (Mantovani et al., 2004, Bouhlel et al., 2007, Charo, 2007, Odegaard et al., 2007). Importantly, however, the in vitro data presented in this chapter do not support this hypothesis but rather suggest that PPARγ activation alone does not alter monocyte phenotype and may, in fact, impede upon IL-13/IL-4-mediated priming of monocytes for differentiation into M2 macrophages. To this end, it is proposed that cell maturity may impact upon PPARγ’s role in M2 polarisation; whilst it may promote the M2 phenotype in macrophages, it may impede M2 marker expression in monocytes.

PPARγ may be activated by various ligands, both synthetic and natural (Berger and Moller, 2002). To date, the mechanisms underlying PPARγ activation during exercise are unknown, however, it is thought that exercise may generate PPARγ activating factor(s) which are secreted into the serum (Thomas et al., 2012, see Chapter 3, Fig. 3.6). In vitro, PPARγ ligands may be used to mimic the effects of exercise on PPARγ activity. Rosiglitazone, for example, is an antidiabetic drug which binds with high affinity to PPARγ, acting as a synthetic PPARγ ligand (Lehmann et al., 1995, Floyd and Stephens, 2012). Hence, due to its potent PPARγ activating ability, rosiglitazone was used in the present study to investigate the effect of PPARγ activation on monocyte phenotype and its role in monocyte priming. Additionally, as well
established inducers of the M2 macrophage phenotype, IL-4 and/or IL-13 were also used in this research to promote the M2 state (Murray and Wynn, 2011b). THP-1 monocytes were chosen for use in *in vitro* investigations since these cells are said to closely resemble monocytes in the vasculature (Qin, 2012).

As expected, when THP-1 monocytes were stimulated with the PPARγ agonist, rosiglitazone, mRNA expression of the PPARγ regulated gene, CD36, was significantly increased by ~1.5 fold, despite unaltered expression of PPARγ itself (see Fig. 5.1) (Berry *et al.*, 2007, Oh *et al.*, 2012). On the other hand, IL-13 treatment significantly decreased PPARγ gene expression when compared to untreated control cells (~0.7 fold) and rosiglitazone only treated cells. Additionally, IL-13 significantly reduced CD36 expression when compared to rosiglitazone only treated cells. Importantly, the IL-13-induced reductions in PPARγ and CD36 expression were maintained even when cells were co-treated with IL-13 and rosiglitazone (see Fig. 5.1). Similar effects have previously been reported following treatment of murine bone marrow-derived monocytes with IL-4 and rosiglitazone, whereby a non-significant decrease in PPARγ gene expression was found with combined IL-4 and rosiglitazone treatment when compared to treatment with rosiglitazone alone (Szanto *et al.*, 2010).

Nonetheless, it is suggested that IL-13 (or IL-4) does not upregulate or activate PPARγ in monocytes as it is thought to do in macrophages, but instead it seems to downregulate PPARγ expression and activity to the point of having an inhibitory effect on PPARγ activation by a potent ligand, such as rosiglitazone.

Similarly, in THP-1 monocytes, IL-13 and rosiglitazone appeared to act in an antagonistic manner with regards to the expression of markers of M1 and M2 polarisation, MCP-1 and IL-1Ra, respectively, whereas stimulation with IL-13 alone, significantly increased the expression of both markers of polarisation (IL-1Ra, ~2.2
fold; MCP-1, ~11.6 fold; see Fig. 5.2 and Fig. 5.3, respectively). In contrast, cells treated with rosiglitazone and IL-13 in combination had significantly reduced expression of IL-1Ra relative to controls (~0.03 fold) and cells treated with rosiglitazone/IL-13 alone (see Fig. 5.2). Likewise, MCP-1 expression was significantly decreased in IL-13 + rosiglitazone treated cells when compared to cells treated with IL-13 alone (see Fig. 5.3). Of note, rosiglitazone only treatment had no effect on the expression of markers of the M1 or M2 phenotype (see Fig. 5.3 and Fig. 5.2, respectively).

The ability for IL-13 (and IL-4) to induce MCP-1 and IL-1Ra expression in monocytic cells has previously been shown by others (Vannier et al., 1996, Szczepanik et al., 2001). These effects appear conflicting since IL-1Ra is an immunoregulatory cytokine said to be preventative of inflammatory-linked diseases, such as insulin-resistance (Hao et al., 2012, Liu and Yang, 2013, Martinez and Gordon, 2014). However MCP-1 is thought to impair insulin signalling and contribute to insulin-resistance and associated diseases (Gustafson, 2010, Olefsky and Glass, 2010). Moreover, MCP-1 has also been shown to promote atherosclerosis, a co-morbidity commonly associated with T2D (Aiello et al., 1999, McKellar et al., 2009). Some, however, have shown that MCP-1 might actually beneficially alter inflammation by inducing IL-4 synthesis by T cells (Gu et al., 2000, Olefsky and Glass, 2010, Sica and Mantovani, 2012). Thus, there is a potential for IL-13-induced MCP-1 to contribute to IL-4-driven M2 macrophage polarisation. This is yet to be clarified, however, with the general consensus pointing towards elevations in MCP-1 (as an M1 marker and pro-inflammatory mediator) being detrimental to health (Gustafson, 2010, Olefsky and Glass, 2010).
In a similar way, the inhibitory effects of rosiglitazone on the monocytic response to IL-13 with regards to M1/M2 marker expression seem contradictory; on one hand rosiglitazone was shown to thwart upregulation of the M2 marker, IL-1Ra in response to IL-13, whereas on the other hand it was also shown to inhibit IL-13-induced expression of the M1 marker, MCP-1. Thus, it is difficult to distinguish the effects of rosiglitazone (and/or IL-13) on the priming of monocytes for M1/M2 macrophage polarisation from these data – hence, it is suggested that additional markers of polarisation should be investigated in response to these stimuli so as to better interpret these findings. Regardless, it is evident from this data that rosiglitazone and IL-13 act in an antagonistic manner in THP-1 monocytes in relation to M1/M2 marker expression.

Interestingly, the opposite pattern has been shown in macrophage-like cells, whereby rosiglitazone and IL-13 treatment has been shown to significantly upregulate the expression of the M2 markers, mannose receptor (MR) and IL-1Ra in THP-1 macrophages and primary human macrophages (Isa et al., 2011). In contrast, Szanto et al. (2010) suggest a ‘dispensable role’ for PPARγ in IL-4-mediated M2 polarisation, demonstrating that the absence of PPARγ does not alter the ability for IL-4 to induce markers of M2 polarisation. Nonetheless, the aptitude for PPARγ to act antagonistically with IL-13 and inhibit M2 marker expression appears to be a monocyte-specific phenomenon which has not been observed in macrophages. However, it is presently unknown as to whether the M1/M2 characteristics induced at a monocytic level may be ‘carried over’ following cell maturation into macrophages. Thus, to investigate this, a similar method to that used by Bouhlel et al. (2007) was adopted to ‘prime’ monocytes for M2 macrophage polarisation. However, instead of differentiating cells in the presence of polarisation stimuli, a two-step protocol was
used whereby THP-1 monocytes were initially polarised by pre-treatment with IL-4/IL-13 and/or rosiglitazone, following which cells were differentiated into THP-1 macrophages in the absence of these stimuli. In this way, it was hoped that the results obtained would be specific to the effects of monocytic priming on macrophage phenotype, eliminating the possibility that observed effects were as a result of macrophage exposure to polarising stimuli.

Rosiglitazone priming, either alone or in combination with IL-4/IL-13, did not alter the expression of the M2 markers (IL-1Ra) or the M1 marker (TNFα) (see Fig. 5.4 and Fig. 5.5, respectively). Once again, these findings provide further evidence to support the theory that PPARγ has a ‘dispensable’ role in STAT-6-associated M2 macrophage polarisation, even in primed cells (Szanto et al., 2010). On the other hand, surprisingly, when cells were primed with IL-13 and IL-4, IL-1Ra was significantly reduced in comparison to control (~0.12 fold) and rosiglitazone primed cells (see Fig. 5.4). These results do not reflect those previously published which demonstrate an elevation in the expression of M2 markers with IL-13 and/or IL-4 priming of human monocytes (Bouhlel et al., 2007). They are also conflicting of the data displayed in Fig. 5.2 which show an IL-13-induced upregulation of IL-1Ra in THP-1 monocytes, and the findings of others who have shown an increase in IL-1Ra with combined IL-4 and IL-13 treatment of peripheral blood mononuclear cells (PBMCs; a group of cells containing monocytes) (Vannier et al., 1996). A possible reason for the disparity in findings may involve the use of PMA as a differentiation stimuli, since PMA is thought to induce the M1 macrophage phenotype. However, the PMA concentrations used within this study were deliberately kept within the range previously shown not to induce M1 polarisation (Maeß et al., 2014).
Importantly, expression of the M1 marker, TNFα, was found not to be elevated but, in fact, significantly reduced by ~0.22 fold in IL-4/IL-13 primed cells, relative to controls (see Fig. 5.5). Similar to MCP-1, TNFα is a pro-inflammatory mediator with direct implications in insulin resistance (Boura-Halfon and Zick, 2009, Olefsky and Glass, 2010). Furthermore, TNFα has been implied in the progression of atherosclerosis, which is often accelerated and amplified in T2D (McKellar et al., 2009). Thus, it is possible that the ability for IL-4 and IL-13 to prime monocytes for reduced TNFα expression upon cell differentiation may beneficially impact upon the development of insulin resistance-associated T2D and its cardiovascular comorbidities.

Importantly, the concept of macrophage ‘switching’, whereby M2 cells might switch to M1 cells and vice versa, via exposure to polarisation stimuli within the tissue microenvironment was also investigated by challenging primed THP-1 macrophages with LPS, a pro-inflammatory mediator and inducer of the M1 phenotype (Bouhlel et al., 2007, Biswas and Mantovani, 2010, Lee et al., 2013). Following cell priming with IL-13 and IL-4, cells challenged with LPS were shown to express IL-1Ra at significantly higher levels (~2 fold), relative to control cells (non-primed THP-1 macrophages challenged with LPS) (see Fig. 5.6). LPS has previously been demonstrated to induce IL-1Ra synthesis in monocytes (Arend et al., 1991, Jenkins et al., 1994). Interestingly, the same does not appear to occur in mature human macrophages, in which IL-1Ra is thought to be secreted constitutively rather than induced. Therefore, it has been proposed that the requirement for a stimulus to induce IL-1Ra synthesis in myeloid cells is dependent on their maturation state (Janson et al., 1991). Even so, the capacity for IL-4 and IL-13 to further induce IL-1Ra expression in response to LPS challenge, supports their ability to promote the M2 phenotype in monocyte/macrophages. In contrast, in cells primed with IL-4 and IL-13, LPS
challenge was found to non-significantly reduce TNFα expression by ~0.6 fold, when compared to unprimed control macrophages (Fig. 5.7). Moreover, TNFα expression in IL-4 and IL-13 primed macrophages was found to be significantly reduced in comparison to expression in rosiglitazone only primed cells, following LPS challenge. Interestingly, TNFα expression following LPS challenge of rosiglitazone primed cells appeared to be non-significantly elevated. However, when IL-4 and IL-13 were used in combination with rosiglitazone to prime cells prior to LPS challenge, this non-significant increase in TNFα expression was notably reduced so that TNFα expression levels were similar to those in unprimed, LPS-challenged control cells (see Fig. 5.7). Therefore, taken together, these results suggest that following LPS challenge, IL-4 and IL-13 primed cells do not undergo macrophage switching to the M1 phenotype but may actually adopt more of an anti-inflammatory M2 phenotype, at least with regard to alterations in IL-1Ra. However, it is important to explore additional markers of M1/M2 macrophage polarisation to clarify whether this phenomenon is evident for other M2 markers.

Surprisingly, rosiglitazone was found to inhibit IL-13/IL-4-induced elevations in IL-1Ra expression in LPS-challenged primed cells (see Fig. 5.6). A similar effect has been shown with rosiglitazone treatment of adipose explants from subcutaneous white adipose tissue (which contains macrophages), whereby rosiglitazone was found to induce reductions in IL-1Ra secretion, when compared to PMA treated explants (similar to LPS, PMA has also been shown to induce IL-1Ra synthesis) (Juge-Aubry et al., 2003, Dayer et al., 2006). Thus, in the context of LPS-induced IL-1Ra synthesis, rosiglitazone appears to have a negative effect on IL-13 and IL-4-induced M2 macrophage polarisation. In contrast, rosiglitazone had no effect on the expression of the M1 associated marker, TNFα, in response to LPS challenge of IL-4 and IL-13.
primed cells (see Fig. 5.7). These findings are in disparity with those described by Bouhlel et al. (2007), who showed that IL-13/IL-4 priming of monocytes inhibited the synthesis of TNFα (alongside two other markers of M1 macrophage phenotype, MCP-1 and CCL-3) in response to LPS challenge, with reductions enhance upon combined rosiglitazone treatment. However, since Bouhlel et al. differentiated cells in the presence of polarising stimuli, it is not possible to determine whether the changes observed occurred at a monocyte level or perhaps later on in the differentiation process (Bouhlel et al., 2007). Thus, it is possible that this disparity in findings is as a result of differential responses to the cell’s maturation state. Regardless, taken together, these data suggest that monocyte-specific PPARγ activation may have a contradictory outcome compared to that observed within macrophages: i.e. paradoxically, it appears to actually impair the ability for IL-4/IL-13 to prime monocytes for the M2 macrophage phenotype (Bouhlel et al., 2007, Szanto et al., 2010).

Hence, the question is posed – why do monocyctic cells respond so differently when compared to macrophages with regards to PPARγ-induced M2 polarisation? One possibility is that PPARγ expression is limited within monocytes and, as Szanto et al. (2010) state, “expression of the [PPARγ] receptor...[is] usually not sufficient to elicit optimal or maximal responses”. In support of this, it has been demonstrated that elevations in PPARγ expression occur during differentiation of primary human monocytes to macrophages (Moore et al., 2001, Szanto and Nagy, 2005). In a similar manner, Fig. 5.8 shows that the same can be said for THP-1 cells since PPARγ expression was also shown to increase in response to PMA-induced THP-1 differentiation; PPARγ mRNA expression was shown to be elevated in a time-dependent manner during PMA-induced differentiation, peaking at approximately 9.5 fold increased PPARγ gene expression relative to undifferentiated THP-1 monocytes.
Additionally, THP-1 macrophages were shown to have approximately 1.2 fold higher expression of PPARγ protein, relative to undifferentiated THP-1 monocytes (see Fig. 5.8). Furthermore, PPARγ activity, as demonstrated by CD36 expression, was also shown to be elevated in a time-dependent manner upon PMA-induced differentiation. However, CD36 expression was notably elevated after 6 hours of PMA treatment (see Fig. 5.8). Despite being based on preliminary findings, these data perhaps suggest that, although PPARγ gene expression was shown to be elevated at lower time points, the expression ‘threshold’ required to induce gene expression changes associated with PPARγ activation (e.g. elevated CD36 expression) was only met >6h. In addition to repeating these experiments, it may also be interesting to investigate the effect of monocyte-macrophage differentiation on the expression of markers of PPARγ activation (including CD36), at both a gene and protein level.

Further, investigations were carried out to determine the effect of rosiglitazone and/or IL-4 on PPARγ protein expression in THP-1 monocytes. As shown in Fig. 5.10, rosiglitazone was found to significantly upregulate PPARγ protein expression in THP-1 monocytes by ~2 fold, when compared to untreated controls. These findings suggest that, although expressed at lower levels when compared to differentiated cells, PPARγ is still inducible within monocytes. Similarly, IL-4 treatment was also able to upregulate PPARγ by ~2 fold, with the addition of rosiglitazone non-significantly increasing expression levels to ~2.3 fold (see Fig. 5.10). Once again, these results are supportive of those by Szanto and colleagues who demonstrated that IL-4 directly influenced PPARγ expression in human monocytes at an mRNA level (via STAT-6 activation) and that rosiglitazone did not significantly alter this effect (Szanto et al., 2010).
Following on, STAT-6 activation was investigated upon rosiglitazone and/or IL-4 treatment of THP-1 monocytes via analysis of tyrosine 641 phosphorylation which has been deemed critical for IL-4-induced STAT-6 activation (Mikita et al., 1996). Total STAT6 protein levels were also analysed for comparison. As expected, STAT-6 phosphorylation depended on the presence of IL-4 and was, thus, not detectable in untreated cells or cells treated with rosiglitazone alone. Interestingly, however, STAT-6 phosphorylation in rosiglitazone and IL-4 treated cells was significantly elevated when compared to IL-4 only treated cells (see Fig. 5.11). Surprisingly, this observation may indicate a role for PPARγ in STAT-6 activation in monocytes, and hence a potential for PPARγ to contribute to STAT-6-induced M2 polarisation. However, this relationship was not always apparent via use of rosiglitazone in the present study.

Instead, the data presented suggest that the PPARγ-induced STAT-6 activation observed in THP-1 monocytes was not sufficient to induce M2 marker expression in monocytes or to prime monocytes into the M2 macrophage phenotype. As with PPARγ, this may be due to insufficient levels of activated STAT-6 within monocytes, which (following IL-4 treatment) was found to be approximately 2.4 fold less than that in macrophages when compared to total STAT-6 levels (see Fig. 5.9). This may explain the lack of an additive effect of PPARγ activation on IL-4/IL-13-induced M2 priming of THP-1, since STAT-6 has been shown to be necessary for many PPARγ-associated functions, including the induction of markers of M2 activation (Szanto et al., 2010). However, if insufficient levels of STAT-6 phosphorylation were wholly responsible for the effects observed in this study, it would be assumed that, alongside rosiglitazone, IL-4 and IL-13 would also have been unable to induce the M2 markers in THP-1, when this was not the case (see Fig. 5.2 and Fig. 5.6). It also does not explain the inhibitory effects of PPARγ on IL-13/IL-4-induced M1/M2 marker expression (see
Fig. 5.2, Fig. 5.3 and Fig. 5.6). Therefore, it is possible that STAT-6, and maybe even PPARγ-independent mechanisms may be responsible for the antagonistic relationship between rosiglitazone and IL-13/IL-4 in monocytes. Some have demonstrated that rosiglitazone may function in a ‘non-genomic’ manner, independent of PPARγ, potentially via crosstalk with other signalling pathways (Singh et al., 2005). For example, rosiglitazone has been shown to up-regulate the suppressor of cytokine signalling-1 (SOCS-1) in glial cells independent of PPARγ activation (Park et al., 2003). Since SOCS-1 is suppressive of IL-4 signalling, it is possible that rosiglitazone may also induce SOCS-1 in monocytes, hence inhibiting the actions of IL-4 in these cells (Jiang et al., 2000). Of course, non-genomic effects might also occur within macrophages, however the significance of these effects might be greater in monocytes where PPARγ is expressed at low levels when compared to macrophages, which express PPARγ to a greater extent and, hence, the balance may be such that the net effect of rosiglitazone will be via its genomic PPARγ-dependent effects in macrophages. In support of this, an antagonistic relationship between rosiglitazone and IL-4 signalling was not observed in THP-1 macrophages (data not shown). However, this suggestion is merely speculative and further investigations are required to determine the precise mechanisms by which PPARγ activation may alter IL-4/IL-13-induced M1/M2 marker expression in monocytes.

In conclusion, in the present study PPARγ was shown not to prime THP-1 monocytes for the M2 macrophage phenotype and evidence was obtained suggesting that, in contrast, it may potentially inhibit the M2-polarising activities of IL-4 and/or IL-13. Thus, it appears that the previously reported ability for exercise to induce the expression of M2 markers in circulating human monocytes (see Chapter 3) may occur independently of exercise-associated elevations in PPARγ activity and should
therefore be attributed to other exercise-induced factors. Despite this, PPARγ activation has been demonstrated to promote M2 polarisation in differentiated macrophages, whilst inhibiting M1-associated inflammatory mediators (Feng et al., 2000, Coste et al., 2003, Paintlia et al., 2006, Berry et al., 2007, Gallardo-Soler et al., 2008, Rigamonti et al., 2008, Van Ginderachter et al., 2008, Ahmadian et al., 2013). Therefore, the generation of PPARγ-inducing factors in serum following exercise may still be important for intra-tissue M2 macrophage polarisation and associated improvement in insulin resistance-related T2D (Mantovani et al., 2004, Odegaard et al., 2007, Goldfine et al., 2013, Ikeda et al., 2013). However, further research is necessary to determine the ability of these factors to traverse into tissues so as to alter macrophage phenotype. Furthermore, although macrophage-specific PPARγ may have major implications on insulin sensitivity and glucose management (potentially via its effects on inflammation), the impact of monocyte-specific PPARγ on these homeostatic processes may be of less importance. However, future studies may aid to fully elucidate the impact of monocyte-specific PPARγ activation on inflammation, insulin resistance and T2D.
CHAPTER 6

General Discussion

6.1. Overview

The research presented in this thesis aimed to investigate the mechanisms by which exercise participation may improve insulin resistance and T2D risk (Pan et al., 1997, Eriksson et al., 1999, Diabetes Prevention Program Research, 2002, Colberg et al., 2010). In order to achieve this, an 8 week, moderate-intensity exercise study was carried out involving 19 sedentary, high risk females and the effects of exercise participation on T2D and associated CVD risk factors were analysed (Chapter 2).

Mechanistic investigations focussed on the effects of exercise-induced factors on markers of macrophage polarisation. A link between exercise-induced PPARγ activation and elevated expression of markers of M2 macrophage polarisation, alongside decreased M1 marker expression, has previously been reported in mixed mononuclear cells (PMNCs) (Butcher et al., 2008, Yakeu et al., 2010). Since individuals with T2D are said to have an increased M1:M2 macrophage ratio, with M1 macrophages being said to promote T2D and its cardiovascular co-morbidities and M2 macrophages inhibiting the effects of M1 cells, it is believed that exercise-induced promotion of M2 polarisation is particularly relevant in a T2D setting (Odegaard et al., 2007, Olefsky and Glass, 2010, Pradhan Nabzdyk et al., 2013, You et al., 2013). Since PPARγ activation in monocytes has been shown to enhance M2 macrophage polarisation, the work of this thesis aimed to determine whether similar changes occurred in isolated primary monocytes subsequent to exercise participation (Bouhlel et al., 2007). So as to achieve this, the expression of markers of PPARγ and M1/M2
macrophage activation were analysed in primary monocytes following participation in an 8 week, moderate-intensity walking intervention (Chapter 3). Additionally, levels of M2-polarising and PPARγ activating stimuli were measured in serum to elucidate the mechanisms by which any observed changes in monocyte phenotype may have occurred (Chapter 3).

Following on, investigations into the mechanisms by which exercise might have induced any observed changes in monocyte M1/M2 marker expression were also carried out using in vitro methods. The role of IL-6 in M2 polarisation is a fairly novel concept. However, since IL-6 serum levels were found to be increased following exercise participation (see Chapter 3), and IL-6 has been shown to enhance or necessitate IL-4/IL-13-induced M2 polarisation in murine macrophages, in vitro studies using THP-1 monocytic cells were carried out with the aim of elucidating whether this exercise-induced factor may have contributed to the elevations in M2 markers observed in monocytes following participation in the exercise programme (Chapter 4) (Fernando et al., 2014, Mauer et al., 2014). Similarly, since elevations were observed in PPARγ activity in monocytes and in the availability of PPARγ-activating factors in serum following participation in the exercise intervention (Chapter 3), the ability for PPARγ activation to prime monocytes for IL-4/IL-13-induced M2 macrophage polarisation was also investigated using in vitro methods (Chapter 5). The major findings of this thesis are outlined below.
6.2. Discussion of Major Findings

6.2.1. Exercise Participation Beneficially Alters T2D and CVD Risk Factors, including Insulin Sensitivity in Sedentary Females

The beneficial impact of physical activity on T2D and CVD risk is widely reported (Warburton et al., 2006, Colberg et al., 2010). Accordingly, Chapter 2 demonstrates that participation in the exercise intervention administered in this study beneficially altered T2D and CVD risk factors, including body weight, BMI, waist circumference, serum triglyceride levels, aerobic capacity and insulin sensitivity in high risk, sedentary females.

As the greatest predictor of T2D risk, reductions in excess body weight indicate improvements in T2D risk following participation in the exercise intervention (Hu et al., 2001, Goldstein and Mueller-Wieland, 2007). Similarly, as predictors of T2D risk and/or its cardiovascular co-morbidities, the reductions in waist circumference and triglyceride levels and improvements in aerobic capacity observed following participation in the exercise intervention were also indicative of beneficial improvements to health (Janssen et al., 2004, Poirier et al., 2005, Samuel et al., 2010, Chaput et al., 2011, Reusch et al., 2013, Tchernof and Despres, 2013). The exercise intervention was also shown to significantly improve insulin sensitivity, as measured by the McAuley’s ISI (McAuley et al., 2001). Since insulin resistance often precedes T2D, this finding is of major importance and further demonstrates the ability for participation in an 8 week, moderate-intensity programme, such as the one used in this study, to beneficially alter T2D risk factors (Tabak et al., 2009).

Overall, therefore, the data in Chapter 2 demonstrate that participation in an 8 week moderate-intensity exercise intervention sufficiently alters key predictors of T2D and
CVD risk in high risk, sedentary females and that prescription of a similar moderate-intensity brisk walking programme may be beneficial for the prevention and management of T2D and its cardiovascular complications. However, in order to achieve full validation of these findings, these investigations must be repeated in alternative cohorts, to include males and individuals with T2D, in order to elucidate the effects of exercise participation in T2D/CVD risk in these individuals.

6.2.2. Exercise Participation Induces Markers of M2 Polarisation in Human Monocytes

In Chapter 3, it is demonstrated that participation in the exercise intervention increased gene expression of M2 markers in isolated human monocytes; both IL-10 and Dectin-1 were significantly elevated, whereas IL-1Ra showed a trend towards increased expression. Others have previously shown similar results (Kawanishi et al., 2010, Yakeu et al., 2010, Ikeda et al., 2013, Oliveira et al., 2013). For example, comparable results were demonstrated in PBMCs following participation in a similar exercise intervention (Yakeu et al., 2010). However, these exercise-associated effects have not previously been reported in monocytes. In contrast, gene expression of the M1 marker, MCP-1, was found to be significantly decreased following exercise participation. Again, similar findings have previously been shown in PMNCs and insulin-sensitive tissues, but never in monocytes (Kawanishi et al., 2010, Yakeu et al., 2010, Oliveira et al., 2013). Thus, these findings are novel and might be used to contribute to the current literature base surrounding the effects of exercise on macrophage polarisation.

Another M1 marker, TNFα was also investigated and gene expression was found to be elevated in response to the exercise intervention. TNFα is thought to directly impair insulin signalling and, promote insulin resistance. Thus, elevations in the expression of this cytokine may be detrimental in a diabetic setting (Hotamisligil et al., 1993,
Donath and Shoelson, 2011). However, TNFα gene expression and not protein expression was measured and, hence, it is important to determine whether the elevation in TNFα gene expression following exercise is evident at a protein level. Additionally, it might also be worthwhile to measure the TNF-α inhibitor, sTNF-R, since this has also been shown to be increased following exercise participation and, thus, may neutralise the effects of exercise on this insulin resistance-inducing cytokine (Venkatasamy et al., 2013). However, sTNF-R levels were not measured in the present research.

In summary, therefore, it was shown that participation in the 8 week, moderate-intensity exercise intervention induced markers of M2 polarisation in monocytes in a way which may prime them for M2 macrophage polarisation.

6.2.3. Exercise Participation Induces PPARγ Activity in Monocytes, Potentially via Generation of PPARγ Activating Factors in Serum

In Chapter 3, exercise training was shown to have no effect on PPARγ gene expression in monocytes and detection of PPARγ at the protein level in monocytes was questionable. However, PPARγ activation, as estimated by the expression of associated/downstream genes (i.e. PGC-1α, CD36 and LXRα), appeared to increase in monocytes following participation in the exercise intervention. It was shown that exercise-induced PPARγ activation may have occurred due to the generation of PPARγ-activating factors, which were found to increase in serum following participation in the exercise programme. However, the availability of exogenous PPARγ ligands in serum was not found to be increased following exercise participation, indicating that, in exercise, PPARγ might be activated independently of exogenous ligand production. Interestingly, gene expression of the enzyme COX-2 was shown to be significantly elevated in monocytes following participation in the
exercise intervention. COX-2 is known to promote endogenous PPARγ ligand synthesis, thus, its upregulation indicates a potential for exercise to induce endogenous intracellular PPARγ ligand production as a means of activating PPARγ (Heusinkveld et al., 2011, Sica and Mantovani, 2012, Díaz-Gandarilla et al., 2013). Thus, it is possible that exercise participation may activate PPARγ by several different mechanisms.

Although exercise has previously been demonstrated to activate PPARγ in mixed mononuclear cells, this has never been shown before in isolated monocytes (Butcher et al., 2008, Yakeu et al., 2010). Since it has been suggested that PPARγ activation in monocytes might be important in priming them for the M2 macrophage phenotype, this result might indicate further mechanisms by which exercise may induce the expression of M2 macrophage markers and, consequently, beneficially impact upon inflammation and associated insulin resistance/T2D risk (Bouhlel et al., 2007, Odegaard et al., 2007).

Furthermore, not only might the activation of PPARγ in monocytes be important, but its activation in insulin sensitive tissues may also improve glucose metabolism, lipid management and inflammation (Harmon et al., 2011, Wahli and Michalik, 2012). In this way, some anti-diabetic drugs (namely TZDs) target PPARγ to exert their glucose lowering effects (Lehmann et al., 1995, Floyd and Stephens, 2012). However, detrimental side effects have been associated with these therapeutic agents and, thus, it is proposed that exercise may provide a safe alternative to these drugs due to its ability to increase the PPARγ activating properties of serum in a way which may potentially activate PPARγ systemically. Therefore, these findings may further support the use of exercise prescription as a means of preventing the onset and development of T2D.
6.2.4. PPARγ may have a ‘Dispensable’ Role in the Priming of Monocytes for the M2 Macrophage Phenotype

Despite a link being shown between PPARγ activity and M2 markers of polarisation in monocytes (Chapter 3), mechanistic in vitro studies demonstrated that PPARγ activation did not enhance the expression of M2 markers of polarisation in cultured THP-1 monocytes (Chapter 5). Rosiglitazone, a potent PPARγ ligand, was used to activate PPARγ in THP-1 monocytes, either alone or in combination with the M2 stimuli, IL-4 and/or IL-13. In undifferentiated THP-1 monocytes, treatment with rosiglitazone alone did not alter the expression of M2 (or M1) markers in THP-1 monocyte. However, an antagonistic response between rosiglitazone and IL-13 was observed in respect of M1 and M2 marker expression in THP-1 monocytes; where IL-13 significantly induced the expression of both M1 (MCP-1) and M2 (IL-1Ra) markers of polarisation, when cells were treated with combined IL-13 and rosiglitazone both M1 and M2 markers were significantly decreased. It was evident that rosiglitazone and IL-13 did not act in a synergistic manner, as has been previously shown in THP-1 and human macrophages (Isa et al., 2011). Similarly, when used in monocyte priming techniques (whereby THP-1 monocytes were pre-incubated with the M2 inducing stimuli, IL-4 and IL-13, and/or rosiglitazone prior to differentiation), rosiglitazone priming alone did not impact on THP-1 macrophage phenotype nor did it enhance or alter the response to IL-13/IL-4 priming with regards to M1/M2 marker expression in these cells. Furthermore, subsequent LPS challenge of primed cells was used to investigate the extent to which macrophages are able to “switch” phenotype following polarisation with IL-4/IL-13. M1/M2 marker expression was unaltered in rosiglitazone primed cells challenged with LPS, relative to non-primed, LPS-challenged cells. Also, rosiglitazone was shown to inhibit IL-4/IL-13-induced M2
marker (IL-1Ra) expression in primed cells challenged with LPS. Hence, overall, these results suggest that PPARγ activation (via rosiglitazone treatment) alone does not alter M1/M2 marker expression in THP-1 cells and a potentially antagonistic effect of PPARγ on IL-4/IL-13-induced monocyte/macrophage polarisation. However, the latter suggestion is based on experiments using only one maker of each of the M1 or M2 macrophage phenotypes and, thus, to truly clarify the effects of rosiglitazone (and IL-13/IL-4) on monocyte polarisation it is necessary to investigate their effect on other markers of polarisation.

Nonetheless, these findings are in disparity with those by Bouhlel et al. who placed importance on the role of PPARγ in the priming of monocytes for M2 macrophage polarisation (Bouhlel et al., 2007). However, the priming protocol used by Bouhlel et al. differentiated cells in the presence of M2 stimuli and/or rosiglitazone and, hence, the effects observed may have been attributable to PPARγ activation at a later maturation state in monocyte/macrophages than that investigated in the current research. Instead, the findings demonstrated in Chapter 5 more closely correlate with suggestions made by Szanto et al. who proposed a ‘dispensable role’ for PPARγ in IL-4-mediated M2 polarisation in macrophages and also show a (non-significant) antagonistic response to PPARγ expression in IL-4 and rosiglitazone treated human monocytes (Szanto et al., 2010).

In Chapter 5, attempts were also made to establish why a differential response to PPARγ activation with regards to IL-4/IL-13-induced M2 polarisation were observed in THP-1 monocytes when compared to those previously documented in macrophages (Bouhlel et al., 2007, Isa et al., 2011). PPARγ expression was shown to be more highly expressed in THP-1 macrophages when compared to THP-1 monocytes, perhaps demonstrating that PPARγ expression levels in monocytes might not be sufficient to
induce a response subsequent to activation. However, its expression following rosiglitazone and/or IL-4 treatment was shown to be significantly elevated at a protein level in THP-1 monocytes. Furthermore, PPARγ activation by rosiglitazone was shown to enhance the ability for IL-4 to upregulate STAT-6 phosphorylation and activity in THP-1 monocytes. Since STAT-6 is thought to be largely responsible for M2 polarisation in response to IL-4/IL-13 and can act as a PPARγ cofactor, this evidence suggested that, in enhancing IL-4-associated STAT-6 phosphorylation, rosiglitazone treatment should enhance IL-4-stimulated M2 polarisation (Szanto et al., 2010). However, this was not found to be the case in the present study and, hence, it was suggested that STAT-6-independent mechanisms may be responsible for the antagonistic relationship between PPARγ and IL-13/IL-4 observed in monocytes with regards to the expression of markers of polarisation. However, it was not possible to fully elucidate the precise mechanisms by which these events occurred and subsequent research should aim to investigate this further, for example by determining the impact of STAT-6 inhibition or elevated PPARγ expression (perhaps via gene transfection of monocytes with a PPARγ expression construct) on the ability for PPARγ to alter IL-13/IL-4-induced alterations in monocyte M1/M2 marker expression.

In summary, the results from Chapter 5 may be used to suggest that PPARγ activation may not be responsible for the exercise-induced elevation in monocyte M2 marker expression presented in Chapter 3. Hence, an alternative mechanism for the exercise-associated increase in monocyte M2 marker expression was sought.

6.2.5. Exercise-Induced Increases in Serum IL-6 May Prime Monocytes Away from the M1 Macrophage Phenotype

Serum IL-6 was found to be significantly elevated following participation in the 8 week, moderate-intensity exercise intervention outlined in Chapter 2. This cytokine is
known to be upregulated immediately after exercise, returning to baseline within 1-2 hours after cessation (Moldoveanu et al., 2000, Gleeson et al., 2011). Recent evidence has shown that IL-6 may enhance and might even necessitate IL-4-dependent M2 macrophage polarisation (Fernando et al., 2014, Mauer et al., 2014). Hence, the ability for IL-6 to alter M1 or M2 polarisation in THP-1 cells and human monocytes was assessed.

In THP-1 monocytes and macrophages, M2 marker gene expression was unaltered following IL-6 treatment. However, gene expression of the M1 marker, TNFα, was found to significantly decrease following acute (3h and 12h) and chronic (24h and 48h) treatment with 10ng/ml IL-6. In THP-1 macrophages, however, M1 marker expression was not significantly altered in response to IL-6 treatment. To ensure that these effects were not just artefacts of using cultured cells, the response of primary human monocytes to IL-6 was also investigated. To more closely mimic the in vivo environment, ex vivo experiments were carried out whereby primary human monocytes and lymphocytes (as PBMCs) were co-cultured. At exercise-associated levels (100pg/ml; see Starkie et al. (2003)), IL-6 did not induce alterations in M1 or M2 marker expression. However, IL-6 at a concentration of 1ng/ml was shown to significantly reduce expression of the M1 marker, MCP-1 (whilst having no effect on M2 marker expression). Thus, combined with the observations in THP-1 monocytes and in the exercise intervention, IL-6 treatment of monocytic cells appears to potentially prime monocytes away from the M1 macrophage phenotype.

It was hypothesised that IL-6 may downregulate M1 marker (MCP-1) expression in primary monocytes by inducing IL-4 synthesis by T lymphocytes. The ability for IL-6 to enhance T cell differentiation into IL-4 synthesising Th2 cells has previously been demonstrated (Rincon et al., 1997, Diehl and Rincon, 2002). In the present study,
however, IL-4 gene expression levels were found to be non-significantly altered (although elevated) in lymphocytes, while IL-4 secretion was not detectable in the supernatant of IL-6 treated co-cultured cells (an ELISA kit with a minimum detection level for IL-4 of 0.25 pg/ml was used). The equivocal nature of this data meant that it could not be confirmed whether IL-4 synthesis by T cells did play a role in IL-6-induced reductions in MCP-1 expression. However, an as yet unknown lymphocyte associated-factor appeared to have contributed to the effects of IL-6 on monocytes MCP-1 expression since its downregulation was obliterated in monocytes cultured with 1ng/ml IL-6 devoid of lymphocytes. It has recently been suggested that, instead of enhancing IL-4 synthesis, IL-6 may induce expression of the IL-4 receptor, IL-4Rα, in monocytes, allowing them to become more responsive to existing levels of IL-4. This has previously been demonstrated in macrophages and myeloid cells (Fernando et al., 2014, Mauer et al., 2014). However, IL-4Rα expression was not measured in the present study and, thus, further research is required before firm conclusions are made with regards to the mechanisms by which IL-6 may downregulate M1 marker expression in monocytes.

Nonetheless, since M1 macrophages are thought to majorly contribute to the inflammation which drives insulin resistance, IL-6’s role (as identified in this study) in priming monocytes away from the M1 macrophage phenotype may provide an alternative mechanism by which exercise may beneficially alter T2D risk (see Chapter 2) and prevent T2D onset (Pan et al., 1997, Eriksson et al., 1999, Diabetes Prevention Program Research, 2002, Colberg et al., 2010, Olefsky and Glass, 2010). In support of this, monocyte MCP-1 expression was found to be significantly reduced, whilst serum IL-6 was significantly increased following participation in the exercise intervention administered as part of this research (Chapter 3). However, it must be
noted that the IL-6-associated reductions in M1 marker expression were only observed with IL-6 treatment at supraphysiological levels, which were much higher than those measured in serum following participation in the 8 week, moderate intensity exercise intervention (Chapter 3). Hence, it is likely that other exercise-induced factors may also have contributed to the effects of exercise on monocyte phenotype (Chapter 3).

6.2.6. Summary of Research Findings

In summary, the main research findings demonstrate that:

- Exercise participation beneficially alters T2D and CVD risk factors, including insulin sensitivity in sedentary, high-risk females.
- Exercise-induced PPARγ activity in monocytes is not responsible for the increase in markers of M2 polarisation (or decrease in the M1 marker, MCP-1) observed in these cells, with the precise exercise-induced factor remaining to be elucidated.
- PPARγ activating factors are generated and measurable in serum following exercise participation, however, these factors are of unknown origin and yet to be identified.
- IL-6 is also generated following participation in exercise and, in a lymphocyte-dependent manner, IL-6 may downregulate M1 markers in monocytes \textit{ex vivo}. However, exercise-associated levels of IL-6 alone may not be sufficient to induce alterations in M1/M2 marker expression in monocytes.
Figure 6.1. Summary of research findings and potential implications.

Participation in a moderate-intensity exercise programme induced markers of M2 polarisation in monocytes, independent of PPARγ. Further, in vitro investigations demonstrated that the PPARγ ligand, rosiglitazone, inhibits M2 marker expression in monocytes. Nonetheless, since PPARγ activation is known to promote macrophage M2 polarisation and, following exercise the PPARγ activating potential of serum was enhanced, exercise may extend its M2 polarising potential to intra-tissue macrophages.

Ex vivo studies suggested that exercise-associated IL-6 generation (probably from active muscle cells) may induce synthesis of lymphocyte-associated factor(s) that downregulate M1 marker expression in monocytes, driving them away from the M1 macrophage phenotype which may contribute to the promotion of insulin resistance and ultimately T2D.
6.3. Limitations of Research

There are several limitations which have been mentioned in relevant chapters. However, some general limitations to this research will be discussed below.

6.3.1. *In Vitro* Investigations using THP-1 Cells

THP-1 cells are known to share similarities with primary monocytes in the vasculature and have been demonstrated to be suitable for investigations into macrophage polarisation (Qin, 2012, Chanput *et al.*, 2014). However, there are some differences between cultured THP-1 cells and PBMC-derived monocytes and macrophages, such as higher expression of inflammatory cytokines in PBMCs when compared to THP-1 cells (Schildberger *et al.*, 2013, Chanput *et al.*, 2014). Additionally, the use of monoculture does not allow for investigations into the cellular-to-cellular interactions which may occur *in vivo*. In the same way, despite co-treatment of cells, it is difficult to directly mimic the *in vivo* setting where cells may be exposed to multiple stimuli at any one time. Therefore, although the use of cultured cells, such as THP-1, may closely mimic the *in vivo* environment, *in vitro* studies should always be repeated *in vivo* to validate the results observed in culture and to definitively confirm findings.

6.3.2. Investigations into PPARγ Reliant on Ligand Activation

As a potent PPARγ ligand and an anti-diabetic agent, rosiglitazone was deemed suitable for use as an activator of PPARγ in *in vitro* investigations in the present study (Norris and Sigmund, 2012). However, this therapeutic agent was shown to be more potent than natural PPARγ ligands which might be synthesised following exercise participation (Chapter 3) and also to be responsible for ‘non-genomic’ PPARγ-independent effects (Singh *et al.*, 2005) which again would not necessarily be the case for exercise-associated natural PPARγ ligands. Thus, *in vitro* experiments into
rosiglitazone-induced PPARγ activation on monocyte phenotype may not be comparable to the \textit{in vivo} setting whereby the majority of individuals will not have rosiglitazone in their systems. Therefore, it is suggested that future research should make use of both natural and synthetic on monocyte/macrophage polarisation to avoid limitations associated with the use of synthetic agents.

\textbf{6.3.3. Gene Expression Analysis Independent of Protein Expression Analysis}

The findings from this thesis rely heavily on gene expression analysis. Since gene expression does not always correlate well with protein synthesis, investigations into gene expression may not always mirror the protein response (Maier \textit{et al.}, 2009). This limitation may be particularly relevant in research surrounding monocyte/macrophage polarisation, whereby surface-proteins may act as useful biomarkers for various phenotypic states (Mosser and Edwards, 2008). It should be noted that measurement of M1/M2 marker expression in primary monocytes via flow cytometry was attempted during this study, but this proved problematic, perhaps because expression levels in these cells are low when compared to macrophages which are more widely used in polarisation experiments (see Appendix, Fig. A4.1). Additionally, logistical restraints with regard to processing of samples for use in flow cytometry immediately after collection from exercising participants meant that the measurement of surface marker expression via flow cytometry during the \textit{in vivo} exercise study would have been difficult. Thus, in future studies, the findings presented in this thesis must be backed up by evidence of similar responses at a protein level.

\textbf{6.4. Future Directions}

Despite the limitations associated with this research, the findings presented in this thesis highlight novel insights into the effects of exercise on monocyte phenotype whilst suggesting mechanisms by which these effects might occur. To the best of the
author’s knowledge, this research is the first to demonstrate that PPARγ activity is increased specifically within human monocytes following exercise participation. Similarly, it has also never before been shown that exercise also induces markers of M2 polarisation and inhibits expression of the M1 marker, MCP-1 in human primary monocytes. Although others have demonstrated the generation of PPARγ-activating factors in serum following exercise participation, this research is the first to demonstrate that PPARγ activation in exercise may not occur in a ligand-dependent manner (Thomas et al., 2012).

This research also supports (and disputes) the findings of others. In demonstrating that PPARγ does not play a significant role in enhancing M2 marker expression in monocytes, the findings by Szanto et al. (2010), who propose a ‘dispensable’ role for PPARγ in macrophage polarisation, are affirmed. However, the findings from this thesis demonstrate that this is also apparent in monocytes. In showing this, the findings by Bouhlel et al. (2007), who demonstrated enhanced M2 polarisation with PPARγ activation, are disputed. In contrast, the findings presented in this thesis demonstrate a role for IL-6 in M2 polarisation, hence supporting the work of Mauer et al. (2014) and Fernando et al. (2014), who also show the importance of IL-6 in M2 macrophage polarisation. Once again, this is the first study to show that the same effects are observed in monocytes.

As is the case with most research, there are still questions which remain unanswered or results which remain to be clarified. Hence, potential future directions for this research, which it is to be hoped may provide such clarifications, will now be suggested.
It is proposed that the study outlined in Chapter 2 and Chapter 3 should be repeated in males, to make findings applicable to the general population. Similarly, since the overarching aim of this research was to elucidate mechanisms by which exercise may prevent insulin resistance and T2D onset/progression, it is also important to repeat this research in individuals with T2D to demonstrate how participation in an exercise programme similar to the one used in this study may aid in the management of T2D. Additionally, use of a larger cohort in a similar exercise study may allow for correlation/regression analysis to be carried out on research findings. Preliminary correlation analysis using the data obtained in Chapter 2 and Chapter 3 showed significant correlations between the observed exercise-induced acute increase in IL-6 (Fig. 3.3) and elevation in expression of the M2 marker, Dectin-1 (Fig. 3.2) ($r=0.601$, $p=0.039$). Furthermore, acute exercise-induced changes in IL-6 also significantly correlated with improvements in an alternative measure of insulin sensitivity, QUICKI, following participation in the exercise programme ($r=0.430$, $p=0.047$). Additionally, acute alterations in serum PPARγ-activating properties post exercise (baseline versus post initial exercise bout, Fig. 3.6) were found to negatively correlate with fasting plasma glucose concentration (Fig. 2.5) following participation in the exercise intervention ($r=-0.494$, $p=0.022$). However, although these analyses provide interesting insights into mechanisms that may potentially underpin the beneficial impact of exercise on T2D/CVD risk, it is necessary to repeat these findings using a larger cohort in order to confirm the validity of these correlations. Thus, in carrying out future research on larger and varied cohorts to that used in this study, it may be possible to provide further evidence for the benefits of exercise prescription in the prevention and management of T2D.
In Chapter 3, it was mentioned that monocytes expressed a profile similar to the M2b macrophage subset described by Mantovani et al. (2004); they produced high levels of IL-10 but also the M1-associated cytokine, TNFα. It was suggested that to further clarify this, the expression of other markers of the M2b phenotype (such as IL-6, IL-1 and CCL1, with the absence of IL-12) should be analysed at both a gene and protein level (Mantovani et al., 2004, Hao et al., 2012, Kharraz et al., 2013). Flow cytometry analysis may be important in facilitating identification of the type of M2 cells induced by exercise. Table 1.1 demonstrates potential markers for identifying macrophage subtypes, however, it first needs to be elucidated whether these markers are expressed by monocytes at a detectable level.

Despite demonstrating ways in which M1/M2 marker expression may be altered in monocytes, it was not possible to elucidate whether these phenotypic changes would be retained following tissue migration and differentiation of monocytes to macrophages. Hence, it would be interesting to determine whether tissue macrophages have more of an M2 phenotype subsequent to participation in the exercise intervention. It is suggested that adipose tissue macrophages would be most interesting for investigations since these cells are thought to largely contribute to the inflammation which drive insulin resistance (Abel et al., 2001, Lumeng et al., 2007a, Harford et al., 2011). Alternatively, migration chambers may be useful for in vitro/ex vivo investigations into the effect of migration and differentiation on monocyte/macrophage phenotype. In this way, it would also be possible to investigate the effects of exposure to M1/M2 stimuli post-differentiation on macrophage phenotype and, hence, the concept of phenotype ‘switching’ in a controlled manner. In carrying out these investigations, it may be possible to determine the true relevance of exercise-induced alterations in monocytes on macrophage phenotype. Since much
is known about how macrophage phenotype impacts on disease state but these cells are highly inaccessible when compared to monocytes, this may support the use of monocytes as a biomarker for disease.

Monocytes are often classified based on CD14/CD16 expression and a loose link has been made between monocyte CD14/CD16 expression and macrophage phenotype with regards to M1 and M2 polarisation, although more research is required in this area (Mehta and Reilly, 2012). However, to date, it has not been shown how M1/M2 marker expression correlates with CD14/CD16 expression in monocytes. This may be important since there is often confusion surrounding the classification of monocytes and that of macrophages (monocytes are classified as classical and non-classical, whereas as macrophages, are termed classically activated (M1) or alternatively activated). Hence, if a link can be made between classification of monocytes and that of macrophages, the literature may be more comprehensible and, once again, this may potentiate the use of monocytes (which are easily accessible cells) as biomarkers for macrophage-associated diseases.

In Chapter 4, it was shown that IL-6 may inhibit M1 macrophage polarisation in a lymphocyte-factor dependent manner. It has recently been suggested that IL-6 may induce IL-4Rα expression by monocytes, making them more responsive to already existing basal levels of IL-4, as demonstrated by others in macrophages (Fernando et al., 2014, Mauer et al., 2014). However, the expression of IL-4Rα in response to IL-6 and in response to exercise was not measured in this study. In measuring this, it may be possible to show for the first time that IL-6 induces IL-4Rα in monocytes, as it has been shown to do in macrophages. Additionally, research into the expression of IL-4Rα in monocytes following exercise participation may unveil further mechanisms by which exercise induces M2 markers/downregulates M1 markers in these cells.
Additionally, a microarray approach may be useful to identify potential lymphocyte-derived monocyte/macrophage polarising factors (other than IL-4) following IL-6 treatment in order to provide further insight into the mechanisms by which IL-6 may influence macrophage polarisation.

In Chapter 5, an antagonistic relationship between rosiglitazone-induced PPARγ activation and IL-4/IL-13-induced STAT-6 activation was investigated. Investigations into the impact of PPARγ and STAT-6 expression levels on the interaction between these two transcription factors may be useful in determining their role in M1/M2 marker expression in monocytes. Gene inhibition (via use of inhibitors/knockout models) and gene overexpression (via transfection with expression vectors) may indicate the importance of STAT-6 and PPARγ for monocyte M1/M2 marker expression and may provide an insight into why these transcription factors appear to interact differently in monocytes when compared to their macrophage derivatives.

### 6.5. Conclusion

In conclusion, despite showing that participation in an 8 week, moderate-intensity exercise intervention both induces PPARγ activation and increases gene expression of markers of M2 macrophage polarisation in monocytes, the findings from this thesis demonstrate that PPARγ may play a dispensable role in the priming of monocytes for either M1/M2 macrophage polarisation. Nonetheless, the exercise-associated increase in PPARγ activating properties of serum may still be important for intra-tissue M2 macrophage polarisation and associated improvements in insulin resistance-related T2D (Mantovani et al., 2004, Odegaard et al., 2007, Goldfine et al., 2013, Ikeda et al., 2013). Instead, in a lymphocyte-associated manner, IL-6 appears to play a role in priming monocytes away from the M1 macrophage phenotype, and hence to shifting the M1:M2 balance towards a predominantly M2-polarised macrophage population,
which is thought to be beneficial with regards to the prevention and management of insulin resistance, T2D and its cardiovascular co-morbidities (Olefsky and Glass, 2010). Since serum IL-6 was shown to be elevated following participation in the exercise intervention, and IL-6 has been shown to enhance M2 polarisation in response to M2 stimuli, there is a potential for IL-6 to have contributed to the beneficial changes observed in monocyte phenotype upon exercise and, accordingly improvements in T2D and CVD risk factors, including insulin sensitivity. However, the concentrations of IL-6 required to induce downregulation of the M1 marker, MCP-1, was substantially higher that those measured in serum following exercise participation and, thus, it is likely that other exercise-associated factors might also contribute to the observed effects of exercise. Additionally, although IL-6 was able to downregulate the expression of a marker of M1 polarisation, it had no effect on M2 markers and, hence, further research is required to elucidate the exact mechanisms by which exercise might induce markers of M2 polarisation in monocytes in a way which may prime them for the M2 macrophage phenotype. Nonetheless, this research provides evidence that participation in an exercise intervention, such as the one administered in this study, may sufficiently improve insulin sensitivity and, hence, T2D/CVD risk in a way which may be used to support the prescription of physical activity in the prevention and management of T2D.
APPENDIX

A1. Miltenyi MACS MicroBead Validation

A1.1. Evidence for the Rationale for Monocyte Isolation

Previous studies have investigated exercise-induced PPARγ activation in exercise and associated changes in M1/M2 marker expression in PBMCs, assuming that observed outcomes were attributable to monocytes (Butcher et al., 2008, Yakeu et al., 2010). However, it is not possible to definitively state that the alterations seen in PPARγ and/or certain M2 markers upon exercise solely occur within monocytes since other leukocytes may also express these, as shown in Fig. A1. Hence, the importance of monocyte isolation from PBMCs in the investigation of particular genes, specifically MCP-1 which is more highly expressed in the non-monocyte fraction of PBMCs, is evident.
Figure A1.1. Expression of genes of interest to this research in human monocytes and non-monocytes obtained from mixed PBMCs. Monocytes were isolated from mononuclear cells via direct magnetic labelling using CD14 MACS MicroBeads. The unlabelled cells, termed ‘non-monocytes’ were also collected. Gene expression of several genes of interest to this study were analysed via RT-PCR in isolated monocytes and non-monocytes. Whole PBMC fractions were used as comparator. This data demonstrates that, as well as being expressed in monocytes, all the genes of interest to this study were expressed in non-monocytes, with MCP-1 being expressed to a greater degree than in monocytes. These data, therefore, demonstrate the importance of isolating monocytes prior to use in investigations in this study in order to ensure that all results are attributable solely to monocytic cells (n=5 in all cases; values expressed as fold gene expression relative to GAPDH ± SEM;* p<0.05).
A1.2. Analysis of Monocyte Purity via Flow Cytometry

Monocyte purity was analysed using flow cytometry. Mixed mononuclear cells were used as a comparator to purified monocytes. To do this, monocytes were either isolated from mononuclear cells prior to flow cytometry, or mononuclear cells were used without further processing.

To isolate monocytes for flow cytometry, CD14-conjugated MicroBeads were used as previously described (Chapter 3, section 3.2.3). However, following the 15 minute incubation with CD14 MicroBeads at 2 – 8 ºC, cells were incubated with an anti-CD14-PC7 mouse monoclonal antibody or an equivalent amount of a PC7-labelled matched isotype control (Beckman Coulter, High Wycombe, UK) for 15 minutes, protected from light at RT. Additionally, both monocyte and non-monocyte fractions were obtained from the isolation procedure, following which they were washed via resuspension in 1-2mL of flow cytometry buffer (PBS, 0.5% BSA, 2mM EDTA) and centrifugation at 300xg for 10 minutes at RT. The supernatant was removed and the cell pellet resuspended in 300μl of flow cytometry buffer (PBS, 0.5% BSA, 2mM EDTA) per 10⁷ cells. Cells were transferred into suitable flow cytometry tubes.

Additionally, mononuclear cells (obtained as described in Chapter 3, section 3.2.2) were resuspended in 100μl of flow cytometry buffer per 10⁷ cells prior to staining with an anti-CD14-PC7 mouse monoclonal antibody or an equivalent amount of a PC7-labelled matched isotype control (Beckman Coulter, High Wycombe, UK) for 15 minutes, protected from light at RT. Cells were then washed with 1-2mL of flow cytometry buffer and centrifuged at 300xg for 10 minutes at RT. The supernatant was completely removed and the cells washed once more in 1-2mL of flow cytometry buffer prior to centrifugation at 300 x g for 10 minutes at RT. Cells were resuspended in 300μl flow cytometry buffer and transferred into suitable tubes for analysis via flow
Flow cytometry was carried using the BD Accuri C6 flow cytometer (BD Biosciences, Oxford, UK).

As shown in Fig A1.2, monocyte number was elevated in isolated monocytes when compared to the non-monocyte (‘leuk’) fraction. Additionally, CD14 expression was notably elevated in isolated monocytes when compared to mixed mononuclear cells. In contrast, CD14 expression by non-leucocytes was no different to that expressed by mixed mononuclear cells. The increase in CD14-associated fluorescence in the monocyte fraction was also notably different from background fluorescence, as determined by the isotype control. On the other hand, there was no difference between test and isotype control fluorescence in the non-monocyte fraction, suggesting that these cells do not express detectable levels of CD14. Overall, these results confirm that CD14 MACS MicroBeads may be used for the efficient isolation of monocytes from mixed mononuclear cells.
Figure A1.2. Assessment of monocyte purity following direct CD14-MicroBead isolation from PBMCs. Flow cytometry was used to determine monocyte purity following isolation via CD14 MACS MicroBeads. An anti-CD14-PC7 mouse monoclonal antibody and an IgG2a-PC7-labelled isotype control were used to obtain scatter plots of A) mononuclear cells and B) isolated monocytes. Histograms displaying the fluorescence intensity were also generated from C) anti-CD14 stained mononuclear cells, D) anti-CD14 stained isolated monocytes, E) isotype control stained mononuclear cells and F) isotype control stained mononuclear cells.
A2. RT-PCR

A2.1. Primer Optimisation

Primer optimisation was carried out as outlined in Chapter 3, section 3.2.6.2 to ensure optimal primer volumes were used in RT-PCR experiments. Tubes were set up (in triplicate) with varying volumes of F to R primers, 5µl of Fast SYBR® Green mastermix, 1µl of 5ng/µl cDNA and, where necessary, RNase free water was added to a total volume of 10µl, as shown in Table A2.1. RT-PCR was carried out as per manufacturer’s instructions, using the Applied Biosystems Fast 7500 Real-Time PCR System (Applied Biosystems, Warrington, UK), as described in Section 3.2.6.4. Mean fluorescence signal (ΔRn) and Ct values were obtained (exemplar data for IL-1Ra primer optimisation is shown in Table A2.1 and plotted in Fig A2.1 (where each point demonstrates a different F:R primer volume ratio). The primer volumes giving the highest fluorescence signal i.e. ΔRn, and greater amplicon levels, as indicated by a lower Ct value, were deemed optimal and selected for use.
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Table A2.1. Primer optimisation for Fast SYBR® Green RT-PCR. Varying volumes of F and R primers were added to an RT-PCR reaction mix before carrying out RT-PCR. Example Ct values and fluorescence (ΔRn) values (obtained during IL-1Ra primer optimisation) are displayed.
Figure A2.1. Example scatter plot to determine optimum primer volumes.

Varying volumes of F and R primers were used to amplify cDNA and Ct values were plotted against fluorescence intensity (ΔRn) in order to determine optimum primer volume ratios for subsequent RT-PCR. For IL-1Ra, the optimal F:R primer volume is circled (F:1.5µl, R:2µl) since this primer combination produced greater amplicon levels, as indicated by a lower Ct value, and gave a high fluorescence signal (ΔRn).
A2.2. Dissociation Curve

Dissociation curves were generated for each SYBR® Green RT-PCR reaction to ensure that gene amplification was specific to the gene of interest/housekeeping gene (see Fig A2.2).

**Figure A2.2. Example dissociation curve.** Dissociation curve of PGC1α showing one discrete PCR product and, hence, primer specificity in THP-1 cell cDNA samples.
A2.3. PCR Efficiency

As described in Chapter 3, section 3.2.6.3, for RT-PCR reactions PCR amplification efficiency was analysed using serial dilutions of cDNA over 3-log or 6-log dilution range (6-log dilutions in primary cells were not possible due to low levels of starting cDNA), to generate Ct values for the gene of interest/housekeeping gene. Ct values were plotted against log cDNA concentrations and the equation of the semi-log regression line was used to calculate PCR efficiency (E), using the following equation:

\[ E = (10^{1/slope} - 1) \times 100 \]

A slope of -3.32 indicates 100% efficiency; however values of 100 ±10% are generally accepted.

![Figure A2.3. Example PCR efficiency curve. CD36 efficiency in RT-PCR analysis in isolated human monocytes was calculated as 92% using the equation E = (10^{1/slope} - 1) \times 100.](image)
A3. THP-1 Gene Reporter Assay

It was attempted to use THP-1 in gene reporter assay, however, these cells proved difficult to transfec using a range of reagents (efficiency of transfection reagents was analysed via flow cytometry analysis subsequent to GFP transfection of cells).

Fig. A3.1 demonstrates the effects of THP-1 transfection (via the most efficient transfection reagent tested and that used for HEK-293T transfection in gene reporter assays, Lipofectamine® LTX Reagent) with 100ng of each of the PPRE-luciferase reporter construct and the PPARγ expression vector and 2ng of the Renilla plasmid, as described for HEK-293T cells (as described in Chapter 3, section 3.2.9.3). Following transfection, cells were treated with various concentrations of rosiglitazone for 24 hours. Additionally, cells were transfected with a positive luciferase control (QIAGEN, West Sussex, UK) for 24 hours. Subsequently, media was removed, cells were washed and lysed and luminescence was read, as described in Chapter 3, section 3.2.9.3. Luminescence values were normalised to transfection efficiency by use of a ratio of Luciferase-to-Renilla luminescence in each case, and reported as RLU.

As demonstrated in Fig. A3.1, transfection of THP-1 with Lipofectamine® LTX Reagent resulted in increased RLU in positive luciferase controls but there was little variation in RLU between different rosiglitazone treatments and, hence, the assay was not deemed sensitive enough for use in investigations and hence, HEK-293T cells were used instead.
Figure A3.1. PPRE-luciferase gene reporter assay to demonstrate THP-1 transfection efficiency. Gene reporter assay was used to demonstrate the efficiency of THP-1 transfection. Cells transfected with a positive luciferase control were shown to have elevated luciferase activity. However, a dose response was not observed in cells transfected with PPRE-luciferase reporter construct and the PPARγ expression vector and, therefore, when using THP-1 cells, the assay did not appear to be sensitive enough to detect quite large variation in concentrations of PPARγ activating factors. Thus, it was decided that THP-1 cells were not suitable for use in gene reporter assays in this research. (n=3 for all; values expressed as fold RLU (normalised to renilla) ± SD, one-way ANOVA).
A4. Supplementary Ex Vivo Data

Attempts were made to measure M1 and M2 surface marker expression in primary monocytes (gated from PBMCs based on CD14 expression) for use in this research. To achieve this, human PBMC were procured from whole blood using Histopaque® 1077, as described in Chapter 3, section 3.2.2. Subsequently, cells were plated at 4x10^6 cells/ml in supplemented RPMI in 12 well plates and treated with 15ng/ml recombinant human IL-4 (Peprotech, London, UK) or, alternatively, control cells were left untreated for 3h in a 37°C, 5% CO2, humidified incubator.

Flow cytometry was used to analyse expression of the M1 markers, CD86 and CD64, whilst CD163 was used as an M2 marker. Following treatment, cells were washed twice with PBS, centrifuging at 300 xg for 10 minutes between washes. Subsequently, cells were centrifuged at 300 xg for 10 minutes at RT to pellet and the supernatant was removed. Cell pellets were resuspended in 100µl of flow cytometry buffer (PBS, 0.5% BSA, 2mM EDTA) and incubated with anti-CD86-PE, anti-CD64-PC5 and anti-CD163-FITC antibodies or suitable isotype controls, as per manufacturer’s instructions (Beckman Coulter, High Wycombe, UK). Subsequently, cells were washed via resuspension in 1-2mL of flow cytometry buffer and centrifuged to pellet. The supernatant was removed and the cell pellet resuspended in 300µl of flow cytometry buffer (PBS, 0.5% BSA, 2mM EDTA) per 10^7 cells. Cells were transferred into suitable flow cytometry tubes and analysed in the FC500 MPL flow cytometer using MXP/CXP software (Beckman Coulter, High Wycombe, UK).

Surprisingly, the inducer of M2 polarisation, IL-4, was shown to significantly increase expression of the M1 marker, CD86 (control, 38.6 ± 0.5; IL-4, 42.8 ± 0.5, p<0.01), whilst having no effect on the expression of CD64 (control, 111.7 ± 1.5; IL-4, 108.7 ± 1.53), relative to controls. In contrast, the M2 marker, CD163, was shown to be
reduced following IL-4 treatment, relative to controls (control, 7.2 ± 0.8; IL-4, 6.8 ± 0.1, \(p<0.01\)) (see Fig. A4.1).

Thus, it did not appear that flow cytometry was suitable for the detection of changes in monocyte phenotype despite others publishing that these markers were suitable in monocytes and macrophages (Deszo et al., 2004). Unfortunately, time restraints meant that it was not possible to investigate suitable markers for analysis of M1/M2 marker expression in monocytes via flow cytometry. Hence, it was decided that RT-PCR analysis would solely be used for the analysis of markers of M1/M2 polarisation in monocytes as this method of analysis has been shown to be sufficient in previous research in this area and suitable markers in monocytes had been elucidated (Bouhlel et al., 2009, Yakeu et al., 2010).
Figure A4.1. Flow cytometry for the analysis of M1 and M2 marker expression in IL4 treated primary human monocytes. PBMCs were treated with IL-4 or left untreated for control. CD14 was used to gate monocytes and M1 (CD86 and CD64) and M2 (CD163) marker expression was analysed. Surprisingly, IL-4 upregulated the marker of M1 polarisation, CD86, and downregulated the M2 marker CD163. Hence, this method of analysis of markers of polarisation in monocytes was not deemed suitable for use in subsequent studies (n=3; values expressed as mean fluorescence intensity (X Mean) ± SD; **p<0.01, t-test).
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