IMMUNE CELL PHENOTYPE AND FUNCTION: IMPACT OF TYPE 2 DIABETES, OBESITY, AND EXERCISE

by

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Abstract

Chronic low-grade inflammation plays a key role in obesity and type 2 diabetes (T2D). Research on inflammation in obesity and T2D has tended to focus on inhibiting pro-inflammatory signaling with the function of anti-inflammatory cytokines being largely unexplored. Exercise is a cornerstone in the treatment of obesity and T2D and may confer some of its beneficial effects through anti-inflammatory mechanisms; however, the optimal anti-inflammatory exercise modality remains unclear. High-intensity interval training (HIIT) has become popular as a time efficient alternative to moderate intensity continuous training (MICT) but whether HIIT is anti-inflammatory like MICT, or pro-inflammatory, is unclear. This thesis examined the impact of T2D, obesity, and exercise on immune cell phenotype and function. In the first study, it was found that interleukin (IL) 10 was less effective at inhibiting inflammation in immune cells from individuals with T2D, indicating that "IL10 resistance" may impact chronic inflammation in T2D. The impact of exercise on immune cell phenotype and function were then examined in inactive adults with obesity who were randomized to short-term HIIT or MICT. HIIT and MICT reduced the ability of IL10 to inhibit inflammation in blood leukocytes, with a greater effect seen after HIIT. There were differential effects of HIIT and MICT on the anti-inflammatory functioning of IL6. During obesity and T2D, there is increased leukocyte migration/infiltration into adipose through a process mediated by chemokines and their respective chemokine receptors. HIIT and MICT resulted in differential effects on leukocyte chemokine receptors. MICT downregulated monocyte chemokine receptors, CCR2 and CXCR2, whereas HIIT upregulated monocyte, neutrophil and T cell chemokine receptor, CCR5. Exercise-induced changes in IL10 function and leukocyte chemokine receptors occurred in the absence of weight/fat loss, suggesting that these were direct immunomodulatory effects of exercise. All effects of exercise on immune cell phenotype and function occurred without changes in plasma

cytokines or chemokines. As levels of circulating cytokines/chemokines are often used to determine whether a treatment is pro- or anti-inflammatory the collective findings of these studies indicate that important immunomodulatory impacts of T2D and different types of exercise could be missed unless immune cell phenotype and function are also assessed.

Preface

I declare that this thesis is the result of my own work and that I have acknowledged all sources in the case of co-authored work with my contribution to each study outlined below.

Chapter 2: A version of Chapter 2 has been published. **Barry, JC**, Shakibakho, S, Durrer, C, Simtchouk, S, Jawanda, KK, Cheung, ST, Mui, AL & Little, JP. (2016). Hyporesponsiveness to the anti-inflammatory action of interleukin-10 in type 2 diabetes. Scientific Reports. 17;6:21244

The University Clinical Research Ethics Board (CREB number H13-00595).

Candidate Contribution

Planning: The candidate was responsible for the cross-sectional study involving human participants with or without type 2 diabetes and as such was involved in all recruitment and organization of the participants and participant testing.

Data Collection: The candidate supervised all human participant testing done within the lab and performed tasks including recruitment, screening, manual blood pressure, anthropometric measurements, and performed all DXA body composition scans. The candidate helped with whole blood culture supernatant collection but did not perform the flow cytometry experiments, which were performed by co-author C. Durrer. As this manuscript was a collaborative project with Dr. A. Mui, the candidate was not involved in the experiments performed in the Mui Lab, which included macrophage cell culture models (RAW264.7 and BMDMs), protein analysis of IL10 signaling components (IL10R1, STAT3, AMPKα), and AQX-MN100 experiments.

Manuscript: The candidate prepared all figures and tables (except for Figures 2.4, 2.5, and 2.6), prepared the first version of the manuscript, and coordinated edits from co-authors. The

candidate submitted the manuscript, prepared the response to the reviewer comments, and revised the manuscript accordingly.

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Candidate Contribution

Planning: This represented a sub-study of a larger randomized controlled trial (RCT). The candidate was involved in the recruitment and organization of participants for their testing days before (pre) and after (post) exercise training. The candidate was responsible for developing the research question to examine anti-inflammatory cytokine function with the primary supervisor.

Data Collection: The candidate measured manual blood pressure, anthropometrics, and performed all DXA body composition scans. The candidate performed and analyzed all measure outcomes with a few exceptions. The candidate did not perform the flow cytometry experiments, but was involved in the analysis, and the candidate helped with whole blood culture supernatant collection but did was not directly involved in the culture and treatment of whole blood cultures because she was interacting with participants and collecting the physiological measures at the same time.

Manuscript (*in preparation):* The candidate prepared all figures and tables and drafted the first version. The candidate will be in charge of all revisions, manuscript submission, and the response to reviewers/revisions.

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Candidate Contribution

Planning: This represented a sub-study of a larger RCT. The candidate was involved in the recruitment and organization of participants for their testing days before (pre) and after (post) exercise training. The candidate worked with the supervisor to develop the research question relating to chemokine receptor expression on different leukocytes.

Data Collection: The candidate measured manual blood pressure, anthropometrics, and performed all DXA body composition scans. The candidate performed and analyzed all measure outcomes with a one exception. The candidate did not perform the flow cytometry experiments, but was involved in the analysis.

Manuscript: The candidate prepared all figures (except for Figure 3.1) and tables for the manuscript. The candidate drafted the first version of the manuscript and was involved in all revisions. The candidate submitted the manuscript to the journal.

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Barry, JC, Durrer, C, Simtchouk, S, Jung, M, & Little, JP. Short-term high-intensity interval training and moderate-intensity continuous training alter chemokine receptor expression in overweight/obese adults. *American Physiological Society. Intersociety Meeting: The Integrative Biology of Exercise VII*. November 2-4, 2016. Phoenix, AZ, USA.

Barry, JC, Durrer, C, & Little. JP. (2015) Resistance to the anti-inflammatory action of the interleukin-10 in type 2 diabetes. *International Diabetes Federation Congress* 2015, November 30 – December 4, 2015. Vancouver, BC, Canada

Beam, J, Broadbent, C, Ohlhauser, L, MacPherson, J, Kluftinger, J, Miller, H, & Little, JP. Relationships between hyperglycemia and cognitive dysfunction in type 2 diabetes. Exercise Physiologists of Western Canada (EPOWC), July 16-18, 2014. Kelowna, BC, Canada

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ADA	American Diabetes Association
AKT/PKB	Protein kinase B
AMPK	5’ adenosine monophosphate-activated protein kinase
BF	Body fat
BMDM	Bone-marrow derived macrophages
BMI	Body mass index
CCL	C-C motif ligand
CCR	C-C motif receptor
CDA	Canadian Diabetes Association
CGM	Continuous glucose monitoring
CSEP	Canadian Society for Exercise Physiology
CV	Coefficient of variation
CVD	Cardiovascular disease
CXCL	C-X-C motif ligand
CXCR	C-X-C motif receptor
DXA	Dual energy X-ray absorptiometry
EDTA	Ethylenediaminetetraacetic acid-coated
FFA	Free fatty acid

FPG	Fasting plasma glucose
FMO	Fluorescence minus one
GDP	Guanosine diphosphate
GLUT	Glucose transporter
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
HFD	High fat diet
HIIT	High-intensity interval training
HR	Heart rate
ICAM	Intercellular adhesion molecule
IGT	Impaired glucose tolerance
IκB	Inhibitor of kappa beta
IKK	IκB kinase
IL	Interleukin
IQR	Interquartile range
IL”X”R	IL”X” receptor
IL1RA	IL1 receptor antagonist
JAK	Janus kinase
LPS	Lipopolysaccharide

MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MFI	Median fluorescence intensity
MICT	Moderate-intensity continuous training
MIP	Macrophage inflammatory protein
NF-κB	Nuclear factor kappa beta
NGT	Normal glucose tolerance
PBMC	Peripheral blood mononuclear cell
PI	Propidium iodide
PI3K	Phosphatidylinositol-3 kinase
RCT	Randomized controlled trial
RIP	Receptor interacting protein
SH2	Src Homology 2
SHIP	SH2 domain-containing inositol 5'-phosphatase 1
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
T2D	Type 2 diabetes
TNF	Tumor necrosis factor

TNFR	Tumor necrosis factor receptor
TLR	Toll-like receptor
TRADD	TNFR-associated death-domain protein
TRAF	TNFR-associated factor
Tyk	Tyrosine kinase
VAT	Visceral adipose tissue
VCAM	Vascular cell-adhesion molecule
VO2	Oxygen uptake
WC	Waist circumference

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“I have not failed. I've just found 10,000 ways that won't work.” - Thomas A. Edison

To my grandparents, Reggie and Joe.

Chapter 1 Introduction

1.1 Obesity and type 2 diabetes (T2D)

In Canada, over half of the adult population are overweight or obese with 26% of adults classified as obese (body mass index [BMI] >30 kg/m^2) and 34% as overweight (BMI of 25 - 29.9 kg/m^2) [1]. Elevated BMI contributes to the rising incidence of T2D as adults who are obese are 2 – 4 times more likely to have T2D [2]. In 2015, 3.4 million Canadians were reported to have T2D and by 2025, this number is expected to increase to 5.0 million [3]. Obesity and T2D are growing health concerns in Canada and worldwide.

T2D is a metabolic condition that is characterized by chronic hyperglycemia, resulting from insufficient insulin secretion and/or action. T2D is associated with both micro- and macro-vascular complications that affect several organs such as blood vessels, heart, eyes, kidneys, and nerves [4]. The current diagnostic criteria used for T2D (see Table 1.1) are derived from studies on glycemic levels that are associated with increased risk of diabetes-specific microvascular complications, with particular focus on retinopathy [5, 6]. However, the main cause of mortality in T2D is cardiovascular disease (CVD), which accounts for 80% of all T2D-related deaths [7].

Table 1.1. Diagnostic criteria for type 2 diabetes and prediabetes.

	Fasting plasma glucose (FPG)	2 hour oral glucose tolerance test (OGTT) plasma glucose (2hPG)	Hemoglobin A1C (HbA1c)
Type 2 Diabetes	≥ 7 mmol/L	≥ 11.1 mmol/L	≥ 6.5%
Prediabetes	5.6 - 6.9 mmol/L (ADA) 6.1 - 6.9 mmol/L (CDA)	7.8 - 11.0 mmol/L	5.7 - 6.4% (ADA) 6.0 - 6.4% (CDA)

ADA, American Diabetes Association; CDA, Canadian Diabetes Association

1.2 Prediabetes

Prediabetes is diagnosed when glucose concentrations are higher than normal but still below diagnostic thresholds of T2D (see Table 1.1). Prediabetes is associated with a high risk of T2D with annual incidence rates of progression to T2D estimated at between 6 - 11% [8-10] and a lifetime risk of progression into T2D estimated at 74% [11]. Prediabetes is a growing concern in Canada with ~6 million adults living with prediabetes [3].

The Diabetes Prevention Program followed the progression of prediabetes to T2D in a group of non-diabetic individuals with impaired glucose tolerance (IGT). Those individuals who progressed into T2D had a 12.6% incidence of background diabetic retinopathy and more strikingly, individuals who did not progress into T2D but remained with IGT had a 7.9% incidence of background diabetic retinopathy [12]. Other studies also demonstrate diabetic complications in individuals with IGT [13, 14], further indicating that complications occur prior to T2D onset.

The relationship of glucose levels and 20-year CVD mortality was studied in three large European studies [15]: the Whitehall Study, the Paris Prospective Study and the Helsinki Policeman Study. Non-diabetic adult males who were in the upper 2.5% of the 2h plasma glucose and fasting blood glucose had an increased risk of CVD mortality, with age-adjusted hazard ratios of 1.8 and 2.7 respectively, compared to male adults in the lower 97.5%. In addition, The Diabetes Epidemiology: Collaborative analysis Of Diagnostic criteria in Europe (DECODE) study demonstrated a relationship between 2h plasma glucose and CVD mortality [16]. Several meta-analyses further support this concept of elevated, but non-diabetic, glucose levels being a risk factor for CVD [17, 18]. Increased risk of CVD and micro- and macro-

vascular complications in individuals with prediabetes highlights the need for preventative interventions prior to the onset of overt T2D.

1.3 Pathophysiology of T2D

Although T2D is characterized by hyperglycemia, the two main pathological features of T2D are insulin resistance and beta cell dysfunction [19]. When hyperglycemia manifests, insulin resistance and beta-cell dysfunction are already well-established. Estimates indicate that early metabolic defects leading to T2D occur ~10 years prior to diagnosis [20, 21]. This indicates that individuals likely live with various degrees of prediabetes for years before clinical diagnosis.

The pathology of insulin resistance and beta cell dysfunction are complex. It is thought that insulin resistance may be caused by a combination of factors including body fat distribution [22-24], lipotoxicity in liver and skeletal muscle [25, 26], and inflammation in adipose tissue [27-30]. In addition, during insulin resistance there is increased production of pro-inflammatory cytokines [27, 31-33] and reduced production of insulin-sensitizing adipokines such as adiponectin [34]. Some of the factors involved in insulin resistance are also involved in beta cell dysfunction. Key players in beta cell dysfunction include insulin resistance [35, 36], lipotoxicity [37-39], glucotoxicity, glucolipotoxicity [39, 40], deposition of islet amyloid polypeptide, oxidative stress [41-43], and islet inflammation [44-47]. There are many possible mechanistic causes of insulin resistance and beta cell dysfunction; however, both pathologies are complex with many factors playing a role.

1.4 Insulin resistance

A main pathological feature of T2D is insulin resistance, which is a condition that is characterized by a reduced response of peripheral tissues to insulin. The main organs affected by insulin resistance are the liver, skeletal muscle and adipose tissue.

1.4.1 Liver insulin resistance

In the fasting state, the high demand for glucose is met by hepatic glucose production, consisting of glycogen breakdown (glycogenolysis) and glucose synthesis (gluconeogenesis). In the postprandial state, insulin is released to suppress hepatic glucose output; however, in T2D, there is resistance to the suppressive effects of insulin on hepatic glucose output [48]. Fasting hyperglycemia is the major pathophysiology of liver insulin resistance but insulin resistance in the liver (combined with insufficient insulin production) also contributes to postprandial hyperglycemia due to the failure of insulin to appropriately suppress hepatic glucose output following food/carbohydrate consumption [49].

1.4.2 Skeletal muscle insulin resistance

Another key organ affected by insulin resistance is skeletal muscle, which is the principal site for insulin-mediated glucose disposal [19]. When insulin resistance occurs in skeletal muscle, insulin-mediated glucose uptake is impaired. These defects in insulin action appear to be at the level of intracellular insulin signaling, which impairs insulin-dependent glucose transporter (GLUT)-4 translocation. This contributes to reduced glucose oxidation and impaired glycogen

synthesis [50, 51]. Insulin resistance in the muscle is believed to be the primary defect in T2D [52] contributing to postprandial hyperglycemia.

The mechanism of insulin resistance in skeletal muscle is not fully known, but increased levels of intramyocellular lipids may play a role. In skeletal muscle, chronically elevated levels of plasma free fatty acids (FFA) impacts insulin resistance [53, 54]. By artificially elevating plasma FFAs in insulin-sensitive individuals, insulin signaling is impaired, resulting in insulin resistance [55]. Conversely, when plasma FFAs are reduced by acipimox (long-acting anti-lipolytic drug) in insulin-resistant individuals, there is increased insulin-stimulated glucose disposal in skeletal muscle [56-58]. The mechanisms underlying lipid-induced insulin resistance in skeletal muscle have not been fully elucidated but likely involve ceramide synthesis, diacylglycerol accumulation, inflammation, and oxidative stress [59, 60]. Taken together, it appears that an oversupply of lipids contributes to impaired insulin signaling and results in insulin resistance in skeletal muscle.

1.4.3 Adipose tissue insulin resistance

Adipose tissue is the third major organ affected by insulin resistance. The major function of adipose tissue is for energy storage and the main cells of adipose tissue, adipocytes, have a large capacity to store excess energy as triglycerides [61]. During fasting, triglycerides are broken down into FFAs via lipolysis. In the postprandial state, insulin suppresses lipolysis to limit FFA release. However, during insulin resistance, the suppressive impact of insulin is reduced in adipose tissue, resulting in elevated basal rates of lipolysis and FFA release from adipose [62]. Elevations in circulating FFAs may contribute to insulin resistance within adipose tissue and in other organs (e.g., skeletal muscle).

Adipose is also an endocrine organ and secretes adipokines (which may be insulin-sensitizing or insulin-desensitizing) [63]. In obesity and T2D there is an imbalance of adipokine secretion favouring an increase in pro-inflammatory adipokines that promote insulin resistance (e.g., tumor necrosis factor [TNF] α, interleukin [IL] 6, and monocyte chemoattractant protein [MCP] 1) and a decrease in adipokines that improve insulin sensitivity (e.g., adiponectin) [63, 64]. Mechanistically, insulin resistance in adipose may be linked to elevated FFAs. In obesity, the function of adipocytes is altered due to hypertrophy. Hypertrophied adipocytes develop insulin resistance [65], become more lipolytic, and lose their storage capacity [66, 67]. In addition, inflammation is induced through increased production of pro-inflammatory cytokines including TNFα, and reduced secretion of insulin-sensitizing adipokines such as adiponectin [68, 69]. This cycle is reinforced as these increased levels of pro-inflammatory cytokines and FFAs promote further lipolysis [70, 71] by impairing insulin signaling in adipocytes [72]. This "spill over" of excess FFAs into major tissues may be play a large role in tissue-specific insulin resistance [26].

1.5 Beta cell dysfunction

Beta cell dysfunction is the second pathological feature of T2D. Pancreatic beta cells are responsible for secreting insulin when levels of blood glucose increase. As progression into T2D occurs, the response of beta cells, and therefore insulin secretion, become dysfunctional.

1.5.1 Changes in the beta cell insulin secretion in the progression into T2D

As the progression into T2D occurs, the insulin response becomes altered. In order to maintain normal glycemic control, insulin secretion is increased (hyperinsulinemia) as a compensatory mechanism (see Figure 1.1). While beta cells can maintain this compensatory increase in insulin secretion, glycemic control can be maintained. However, this hyperinsulinemic response is believed to place extra stress on the beta cell and eventually the compensatory mechanisms fail and glycemic control is no longer maintained resulting in hyperglycemia. Therefore, the onset and degree of beta cell failure or dysfunction is a major determinant of hyperglycemia and the clinical diagnoses of prediabetes or T2D. In T2D, hyperinsulinemia can no longer fully compensate and therefore hyperglycemia becomes pronounced. The pathophysiology of beta cell dysfunction highlights the value of intervening early in the natural progression of T2D (i.e., at the prediabetes stage) in order to prevent progression to severe beta cell decompensation or failure, at which point reversal of hyperglycemia and associated complications is hypothesized to be much more difficult.

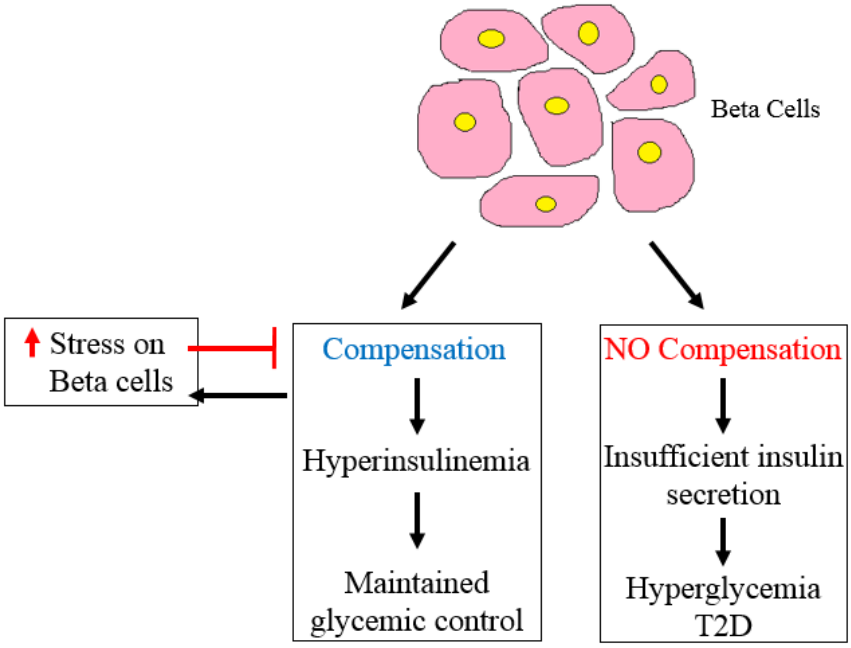

Figure 1.1. Insulin response during the progression of T2D. During compensation, insulin secretion increases (hyperinsulinemia) and glycemic control is maintained; however, when compensation fails, insulin secretion is insufficient and results in hyperglycemia and T2D.

1.6 Inflammation

An emerging theme in both insulin resistance and beta cell dysfunction is the presence of inflammation, which is recognized to play a major role in both the progression and pathology of obesity and T2D.

Acute inflammation is the process to resolve infection or tissue injury and is comprised of (1) vascular events, such as increased blood flow and vascular permeability, (2) cellular events, including increased activation and recruitment of granulocytes (i.e. neutrophils), monocytes and lymphocytes, and (3) increased production of inflammatory protein and other mediators [73]. Cytokines are polypeptides that are mainly produced by activated leukocytes such as lymphocytes and macrophages and are the main inflammatory proteins involved in mediating both local and systemic inflammatory processes [74]. More specifically, chemoattractant

cytokines known as chemokines are the main mediators of leukocyte migration. Acute inflammation is a protective response to infection or tissue injury and is mediated by a various vascular and cellular events along with increased production of cytokines.

Neutrophils are the first leukocytes to infiltrate the site of inflammation, where they produce toxic species such as reactive oxygen species to enable neutrophil-mediated killing to enhance the immune response [73, 75]. In order to prevent excessive tissue damage due to an accumulation of these neutrophil-derived toxic species, there is a switch from neutrophil to monocyte/macrophage infiltration [76], which acts in pathogen clearance and wound healing [73]. Under normal conditions inflammation is self-limiting; however, when inflammation is not resolved appropriately, chronic inflammation occurs. Increased infiltration of monocytes/macrophages plays an important role in perpetuating chronic inflammation in conditions such as obesity and T2D [28, 29, 44]. The majority of research on chronic inflammation associated with obesity and T2D has focused on studying macrophages, which become activated and migrate into the site of inflammation (in particular adipose tissue), where they contribute to sustaining an increased level of pro-inflammatory cytokines such as TNFα, IL1β, and IL6 [77]. The regulation of leukocyte recruitment to tissues is clearly important for understanding inflammation in obesity and T2D.

1.7 Inflammation in obesity and T2D

1.7.1 Inflammation in adipose tissue

Most individuals with T2D and prediabetes are overweight or obese. Although excess body weight is a major risk factor for developing T2D, not all overweight/obese individuals develop T2D. Some studies suggest that it is not the excess weight *per se* but dysfunctional

cellular processes within adipose tissue that link obesity and T2D risk. Adipose tissue is in an inflamed state in both obesity and T2D [30] due to an increased recruitment of immune cells such as monocytes/macrophages [28, 29] and T cells [27]. This leads to a subsequent increase in the production of pro-inflammatory cytokines (particularly TNFα) within adipose tissue, which is mechanistically linked to the development of insulin resistance [27]. Mechanistically, the pro-inflammatory cytokines released from adipose tissue are believed to perpetuate a state of chronic low-grade inflammation, which directly impairs insulin signaling in adipose itself (contributing to elevated rates of lipolysis) and other organs, including skeletal muscle and liver (which contributes to postprandial and fasting hyperglycemia) [30].

In addition to cytokine spillover from inflamed adipose tissue, circulating immune cells may also contribute to chronic inflammation in prediabetes and T2D. For example, high glucose activates inflammatory signaling and increases the secretion of pro-inflammatory cytokines from circulating monocytes and cultured macrophages [31-33]. This has been linked to elevated toll-like receptor (TLR) expression on monocytes, suggesting that hyper-activation of the innate immune system is detectable in blood monocytes in patients with T2D. If circulating leukocytes are more pro-inflammatory in the presence of insulin resistance and/or T2D they could perpetuate systemic low-grade inflammation through increased secretion of cytokines, increased oxidative stress, and/or promote tissue inflammation once they leave the bloodstream.

1.7.2 Inflammation in the pancreas

Similarly to the process occurring in adipose tissue, hyperglycemia can induce pro-inflammatory cytokines [46, 47] and immune cell infiltration in the pancreas [78], therefore contributing to beta cell dysfunction. Hyperglycemia-induced inflammation is hypothesized to

promote a cycle of chronic inflammation in the presence of insulin resistance and beta cell dysfunction.

1.8 Mediators of inflammation

1.8.1 Tumor necrosis factor alpha (TNFα)

TNFα is a major mediator of the immune response and is essential during host defense [79, 80]. This pro-inflammatory cytokine, which is part of the TNF family, is produced by several cell types including neutrophils and activated T cells, but is predominantly produced by activated monocytes/macrophages [73, 81] and its effects are mediated by the downstream effects of TNFα signaling.

1.8.1.1 TNFα signaling

TNFα binds to TNF receptor (TNFR) 1 or TNFR2, although TNFR1 is described as the main receptor in mediating the signaling cascade [81]. Once TNFα binds the TNFR1, there is a trimerization of the receptor and a conformational change resulting in recruitment of TNFR-associated death-domain protein (TRADD). The serine/threonine kinase, receptor interacting protein (RIP) 1, and subsequently TNFR-associated factor (TRAF) 2 form a complex with TRADD (TRADD-RIP1-TRAF2), which can then dissociate from TNFR1. At this stage of signaling, TNFα can result in either cell survival or cell death. In the TNFα-mediated survival pathway, the TRADD-RIP1-TRAF2 complex recruits various signaling molecules including ubiquitin ligases, and LUBAC proteins, which further associate with IκB kinase (IKK) and transforming growth factor beta kinase (TAK) 1. The resultant downstream effect of this

pathway is the phosphorylation and degradation of inhibitor of kappa beta (IκB), which under normal conditions is complexed with and inhibits nuclear factor kappa beta (NF-κB), resulting in activation of NF-κB. Once dissociated from IκB, NF-κB can translocate into the nucleus where it regulates gene transcription of cell survival genes, including cFLIP, which inhibits pro-apoptotic caspase-dependent pathways. In the TNFα-mediated cell death pathway, the TRADD-RIP1-TRAF2 complex dissociates from TNFR1, where it then binds to Fas-associated death domain-containing protein FADD within the cytoplasm, resulting in initiation of apoptosis through a caspase-dependent pathway [73, 82]. TNFα is an essential pro-inflammatory cytokine responsible for both cell survival and cell death signaling.

1.8.1.2 TNFα in obesity and T2D

Spiegelman and colleagues were the first to report elevated levels of TNFα mRNA and circulating TNFα in rodent models of obesity and T2D [83]. Furthermore, when TNFα was neutralized using a recombinant soluble TNFα receptor antibody, these rodent models demonstrated a greater response to insulin. Tracer studies using glucose clamps suggested this response was due to a greater insulin-stimulated peripheral glucose utilization rate and therefore greater peripheral glucose uptake (i.e., improved insulin sensitivity when TNFα signaling was reduced) [83]. A follow-up study observed that TNFα inhibited insulin signaling and insulin-stimulated glucose uptake in adipocytes [84]. TNFα secretion from adipose during obesity and T2D is widely regarded as a key mediator of insulin resistance.

Similar to mouse models, early reports demonstrated that TNFα mRNA and protein were higher in adipose tissue samples from humans with obesity [72, 85]. Further evidence supported a role for TNFα in obesity-related insulin resistance in studies showing that weight reduction

improved insulin sensitivity with a concurrent decrease in TNFα mRNA expression [72]. However, human studies failed to show that TNFα neutralizing antibodies could improve insulin sensitivity in obesity [86]. These studies suggested that TNFα is important but that there is a greater complex interplay among inflammatory mediators that are involved in the progression of insulin resistance and T2D.

1.8.2 Interleukin 6 (IL6)

IL6 is part of the hematopoietin or Class I cytokine family and is produced by leukocytes including T and B lymphocytes, and monocytes/macrophages [87], as well as endothelial cells [73], adipocytes [88] and skeletal muscle cells [89]. Initially described as a growth factor in the differentiation of B cells to plasma cells [90], IL6 also plays important roles in the acute phase response [91], in promotion of differentiation and activation of T lymphocytes [92], and in mediating neutrophil trafficking [93]. IL6 is a pleiotropic cytokine that plays a major role in various processes during inflammation.

IL6 is important in the switch from acute to chronic inflammation as characterized by a switch from neutrophil to monocyte/macrophage migration and infiltration and within this process plays several important roles. Initially, IL6 promotes neutrophil trafficking through an increase of intercellular adhesion molecule (ICAM), vascular cell-adhesion molecule (VCAM) [94], and L-selectin [95], all of which are required in leukocyte trafficking. To avoid excessive damage from accumulating neutrophil-induced toxic species, there is a switch from neutrophil to monocyte/macrophage recruitment. When neutrophils undergo apoptosis, the membrane bound IL6 receptor (IL6R) is shed to create the soluble IL6R, which then activates endothelial cells via trans-signaling (described in detail below). The activated endothelial cells then produce less

neutrophil-attracting chemokines and more monocyte-attracting chemokines such as MCP1 [96, 97]. Furthermore, IL6 induces factors such as granulocyte colony stimulator factor and granulocyte macrophage colony stimulating factor, which then further promote macrophage maturation and activation [98]. IL6 plays a major role in neutrophil-trafficking and the switch from neutrophil to monocyte-mediated indicating an important role of IL6 from acute to chronic inflammatory responses.

1.8.2.1 IL6 signaling

The IL6R complex consists of the IL6R, also known as CD126 (ligand-binding glycoprotein) and gp130, also known as CD130 (signal-transducing component) [87]. IL6R is expressed on hepatocytes and leukocytes, including monocytes, macrophages, neutrophils, and T and B lymphocytes [99], whereas gp130 is ubiquitously expressed [100]. IL6 can initiate "classical signaling" via a cell membrane-bound IL6R (IL6Ra) or "trans-signaling" via a soluble IL6R (sIL6R) [101]. The sIL6R is generated through either shedding of the membrane bound IL6R via proteolysis of the A Disintegrin and Metalloproteinases or through alternative splicing of IL6R mRNA [97, 102]. As gp130 is ubiquitously expressed, sIL6R can initiate IL6 trans-signaling on cells that lack the membrane bound IL6R. The downstream effects of IL6 occur through either a classical or trans-signaling pathway via a membrane-bound or soluble receptor, respectively.

There are three main signaling pathways that are responsible for the downstream effects of IL6, which include the "Phosphatidylinositol-3 kinase (PI3K)/Protein kinase B (AKT)", "Ras/Mitogen-activated protein kinase (MAPK)", and "Janus kinase (JAK)/Signal transducer and activator of transcription (STAT)" pathways [87, 101]. Of these three pathways, the best

studied is the JAK/STAT pathway. Once IL6 binds to the IL6R, the IL6-IL6R associates with gp130 resulting in gp130 dimerization that can then initiate activation of the JAK family, comprised of Jak1, Jak2, and tyrosine kinase 2 (Tyk2). JAKs then phosphorylate tyrosine residues within the gp130; these phosphorylated tyrosine residues serve as docking sites for the Src Homology 2 (SH2) domains of STAT3. After STAT3 becomes phosphorylated by JAK and Tyk, phosphorylated STAT3 molecules can from a dimer at the point of the SH2/phosphotyrosine interactions. Additionally, phosphorylated STAT3 dimers undergo a conformational change revealing a nuclear localization signal, which results in translocation of the STAT3 dimer into the nucleus to initiate transcription of STAT3-dependent genes [73, 103]. IL6 signaling induces STAT3-target genes that not only play a role in inflammation but also in cell survival and proliferation, angiogenesis and metastasis [73, 103-105]. Furthermore, IL6/STAT3 induces the production of suppressor of cytokine signaling (SOCS) 3, which when bound to the JAK2-gp130 of the IL6R complex negatively regulates IL6 signaling [106]. Many of the pleiotropic biological effects of IL6 are mediated by the JAK/STAT signal cascade.

1.8.2.2 IL6 is both pro- and anti-inflammatory

IL6 is typically categorized as a pro-inflammatory cytokine given its above mentioned role in the acute phase response, neutrophil trafficking, and monocyte maturation. However, there is evidence that IL6 may also possess anti-inflammatory actions. Aderka et al. [107] have shown that treatment of lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells (PBMC) with IL6 results in a dose-dependent reduction in TNFα secretion suggesting an anti-inflammatory action. Several reviews present evidence that IL6 trans-signaling is pro-inflammatory, whereas IL6 classical signaling is regenerative and anti-

inflammatory [97, 102]. The production of IL6 from contracting skeletal muscle during exercise is also hypothesized to elicit anti-inflammatory actions [89, 108]. These anti-inflammatory actions have been demonstrated in human studies showing reduced TNFα production following exercise or IL6 infusion in an experimental model of LPS-induced sepsis [109] and through IL6-mediated upregulation of "classic" anti-inflammatory cytokines such as IL1 receptor antagonist (IL1RA), soluble TNFα receptor, and IL10 [109, 110]. The differential pro- and anti-inflammatory effects of IL6 may be mediated by the type of signaling (classical vs. trans-signaling) or by the source of IL6 (i.e. muscle vs. leukocyte).

1.8.2.3 IL6 in obesity and T2D

IL6 levels are increased during inflammatory conditions such as obesity [111, 112], IGT [113], and T2D [114, 115]. Furthermore, IL6 is strongly correlated to measures of obesity/adiposity, such as subcutaneous and visceral adipose tissue (VAT), BMI, waist circumference (WC) [116, 117], and measures of insulin resistance/T2D [112, 118]. Elevated levels of IL6 may even predict the likelihood of developing T2D [119]. IL6 is a major marker of chronic-low grade inflammation in obesity and T2D.

During obesity, IL6 is released from inflamed adipose tissue [120], and in particular VAT; however, only a small amount of this is released by adipocytes [121]. Adipose tissue also contains leukocytes, including macrophages, which not only play a major role in the chronic-low grade inflammation of obesity, but are also a significant source of IL6 suggesting a role of IL6 in adipose inflammation [28]. Administration of recombinant human IL6 in healthy males resulted in hyperglycemia and insulin resistance, suggested to occur through the increased release of glucagon [122]. Mechanistically, IL6 may also impair hepatic insulin signaling [123, 124], which

may have occurred due to impaired activation of the insulin signaling due to an interaction of IL6-induced SOCS3 and the insulin receptor [125]. Furthermore, the depletion of IL6 improved hepatic insulin signaling during obesity [126]. Several mechanisms implicate IL6 in the adverse effects of inflammation during obesity and T2D.

Conversely, IL6 may have beneficial effects during obesity and T2D. Evidence showed that mice deficient in IL6 develop mature-onset obesity with IGT, but following treatment with IL6 have reduced obesity [127, 128]. In regards to T2D, Klover et al. [123] demonstrated impaired hepatic insulin signaling by IL6; however, there was no impairment in skeletal muscle insulin signaling. This effect may be due to other biological effects of IL6 such as increased lipolysis and fat oxidation [129], and increased glucose uptake [130]. These biological effects could be further explained by the association of IL6 and 5' adenosine monophosphate-activated protein (AMPK) in both muscle and adipose [131]. In addition to these other biological effects of IL6, the source of IL6 may also be important. Evidence demonstrated that the source of elevated IL6 release during exercise is from contracting muscle [132] and not immune cells such as monocytes [133, 134]. Although evidence indicates that IL6 is a mediator of inflammation in obesity and T2D, there is controversy with evidence also implicating beneficial effects of IL6 in these conditions.

1.8.3 Interleukin 10 (IL10)

IL10 is part of the interferon or Class II cytokine family [73] and is an important anti-inflammatory cytokine involved in resolving inflammation. IL10 was initially described as a cytokine synthesis inhibitor factor produced by Th2 cells [135] and is now shown to be produced by nearly all leukocytes including monocytes, macrophages, dendritic cells, neutrophils, and T

and B lymphocytes [136]. Some biological effects of IL10 include reduced pro-inflammatory cytokine/chemokine secretion, increased proliferation and differentiation of B lymphocytes, and reduced proliferation of CD4+ T cells but enhanced proliferation of CD8+ T cells [136]. Another major function of IL10 is to inhibit macrophage proliferation/activation [137] and the subsequent increase in pro-inflammatory cytokines (i.e. TNFα, IL1, and IL6) [138]. Furthermore, IL10 increases the release of anti-inflammatory mediators such as soluble TNFR [139] and IL1RA [140]. These pleiotropic effects of IL10 provide evidence that IL10 is one of the most potent anti-inflammatory cytokines.

1.8.3.1 IL10 signaling

The IL10 receptor (IL10R) is a heterotetramer comprised of two IL10R1 polypeptide chains and two IL10R2 chains [141]. IL10R1 is expressed only on leukocytes, with high levels observed on monocytes, macrophages, and lymphoid organs, whereas IL10R2 is widely expressed [142]. IL10 initiates two signaling cascades including the JAK/STAT and the PI3K/AKT pathways [143]. In the context of inflammation, the JAK/STAT pathway is thought to be of more importance as the PI3K/AKT pathway is suggested to promote cell viability and proliferation [143, 144]; however, contrary evidence exists indicating an anti-inflammatory role for the PI3K/AKT pathway [145]. IL10 may mediate its anti-inflammatory effects via the JAK/STAT or PI3K/AKT signaling pathways.

The JAK/STAT pathway is the best characterized signaling pathway of IL10. When IL10 binds to the IL10R complex, there is a transphosphorylation of the JAK family, including Jak1 and Tyk2, which are constitutively associated with the IL10R1 and IL10R2, respectively [141, 146]. Phosphorylation of JAK1 and Tyk2 results in the phosphorylation of tyrosine residues on

the IL10R1, which act as docking sites for the SH2 domains of STAT3 [147]. Activated STAT3 molecules form a dimer, which translocates into the nucleus to induce STAT3-target genes that play a role in immune cell proliferation/differentiation, and inhibition/termination of pro-inflammatory cytokines and inflammatory processes [143]. Some of these IL10/STAT3-target genes are important in inhibition/termination of pro-inflammatory cytokines or signaling. For example IL1RA and TNFR2 inhibit IL1β and TNFα activity respectively [82, 148], B-cell lymphoma 3-encoded protein, an IκB protein, inhibits LPS-induced TNFα production [149], and SOCS3, negatively regulates IL6 signaling [150]. IL10 can also activate PI3K and its effectors AKT, glycogen synthase kinase 3, and p70 S6 kinase [144, 145, 151]. Although there is evidence that IL10 mediates anti-inflammatory effects through PI3K, most of the research indicates that the anti-inflammatory effects of IL10 are mediated through the JAK/STAT signaling pathway.

1.8.3.2 IL10 suppression of pro-inflammatory cytokine synthesis

LPS is an outer membrane component of Gram-negative bacteria that induces the release of pro-inflammatory cytokines and chemokines in monocytes/macrophages [152, 153] and other cell types [154-156]. LPS is commonly used in immunology experiments to induce a predictable and potent pro-inflammatory response. In particular, LPS leads to the rapid induction of TNFα [157], which as mentioned above is a key cytokine involved in the propagation of obesity and T2D-related chronic inflammation. IL10 can potently inhibit production of LPS-induced TNFα [158] and the secretion of TNFα from macrophages and other leukocytes [149, 159, 160].

IL10 has been shown to inhibit ~20% of LPS-induced genes [161]; however, although many STAT3-target genes are implicated in this inhibitory action they cannot account for the full anti-inflammatory effect of IL10 [142]. Evidence exists that the anti-inflammatory action of IL10

may occur independent of the JAK/STAT and PI3K/AKT pathways. For example, the activation of the SH2 domain-containing inositol 5'-phosphatase 1 (SHIP) 1 pathway has been shown to be crucial for IL10 action [162, 163]. SHIP1, expressed predominantly in hemopoietic cells, inhibits pro-inflammatory signaling by degrading PIP3 [164]. Chan et al. have shown that SHIP1-mediated IL10 signaling functions to inhibit translation of TNFα mRNA, contributing to its anti-inflammatory action [162]. In cell culture models, IL10 consistently blunts the secretion of TNFα from LPS-stimulated macrophages, demonstrating potent anti-inflammatory activity.

1.8.3.3 IL10 in obesity and T2D

There is some evidence suggesting that IL10 may be involved in T2D. Although few studies have linked IL10 and T2D in humans, there are some cross-sectional studies that demonstrate this association. For example, in the Leiden 85-Plus Study there was an association of low IL10 production capacity with higher levels of serum glucose and triglycerides, higher hemoglobin A1c scores and a higher prevalence of T2D [165]. Additionally, Blüher et al. [166] observed lower IL10 in individuals with IGT or T2D and a negative correlation between IL10 and fasting plasma glucose (FPG) and insulin, and BMI. These cross-sectional studies indicate an association between T2D and IL10 but cannot directly demonstrate altered IL10 function during T2D. Some studies have tried to identify if IL10 functions differently during T2D. For example, mice who were engineered to ectopically express IL10 through a muscle cell-specific transgene [167] or gene transfer [168] were protected from a high-fat diet (HFD)-induced muscle insulin resistance and HFD-induced inflammation, including a reduction in pro-inflammatory cytokines and inhibition of macrophage infiltration. IL10 has been demonstrated to play some role in obesity and T2D; however, this role is unclear and requires further elucidation.

1.8.3.4 JAK/STAT signaling: A common signaling pathway of IL10 and IL6

As discussed above, IL10 and IL6 are both able to signal through the JAK/STAT pathway; however, they can mediate different downstream effects. The difference in cellular effects of IL10 and IL6 may depend upon activation of STAT3, which is the main regulator in the JAK/STAT pathway. For example, the activation of STAT3 via IL6 signaling is transient, whereas the activation during IL10 signaling is sustained over a greater duration [150, 169]. This difference in duration of STAT3-activation may be explained through the termination of IL6, but not IL10, signaling by SOCS3. SOCS3 terminates the IL6 signaling cascade by specifically binding to phosphotyrosine residues of the JAK2-gp130 complex of the IL6R complex [106] but does not associate with any phosphotyrosine motifs of the IL10R [150] and therefore does not affect IL10 signaling. Thus, although IL6 and IL10 may share some common signaling pathways and actions, there appear to be clear differences between the anti-inflammatory actions of these two cytokines.

1.8.4 Chemokine and chemokine receptors

Chemokines are chemoattractant cytokines that function in the movement of leukocytes up a chemokine gradient called leukocyte chemotaxis [73]. Chemokine nomenclature is based from the characteristic tetra-cysteine motif within the chemokine. The two major chemokine subfamilies are CC and CXC, whereas the two minor subfamilies are CXC3 and XC [170], which are based on the position at the N-terminus of two conserved cysteines [171, 172]. Therefore the designation of chemokines is based off their subfamily with the “L” designating it as a ligand (i.e., CCL, CXCL, CXC3L, and XCL). In addition to these four subfamilies of

chemokines based on their structure, the majority of chemokines also have a historical name related to their initially characterized function (e.g., MCP1 is the common name for CCL2).

Chemokine receptors are G protein-coupled receptors (GPCR) [173]. They are designated based on the subfamily of chemokines that they bind to and are further designated as a receptor with an "R" (i.e. CCR, CXCR, CXC3R, and XCR) [171]. Chemokine receptors are differentially expressed on all leukocytes but in general, the CC and CXC subfamilies are the most important in leukocyte migration/recruitment with the CC subfamily involved in monocyte and T-cell migration and the CXC subfamily involved in neutrophil migration [174, 175]. Although there are specific chemokine-chemokine receptor interactions that play important biological roles, chemokines are able to bind and activate several different chemokine receptors. This ability for different chemokine-chemokine receptors interactions is most often thought to represent redundancy but some argue that it may point to a strategy to overcome different inflammatory situations by specifically adjusting or adapting leukocyte responses [170]. Based on the chemokine subfamilies there are four main chemokine receptor subfamilies, which play important biological roles based on their chemokine-chemokine receptor interaction.

1.8.4.1 Chemokine receptor signaling

Chemokines mediate their effects via the chemokine receptors, which are GPCRs. GPCRs are a seven trans-membrane domain receptors that consists of the trimeric G protein made up of three subunits Gα, Gβ, and Gγ. When a chemokine binds its respective chemokine receptor, a conformational change is induced resulting in the exchange of a guanosine triphosphate (GTP) for the guanosine diphosphate (GDP) bound at the Gα subunit (GDP is normally bound to Gα when the GPCR is unoccupied). There is a resultant dissociation of the G

protein into a monomer of Gα-GTP and a dimer of Gβγ, both of which mediate chemokine-induced signaling. Signal termination is mediated by GTPase activating proteins and occurs once the GTP associated with Gα becomes hydrolyzed to GDP and subsequent reformation of the G protein occurs [73].

Several signaling pathways are initiated by the G protein subunits. For example, the Gβγ dimer subunit can result in downstream effects via the Ras/MAPK pathway leading to increased transcription of activator protein 1 and an upregulation of integrins or can also help mediate signaling via the phospholipase C-β and results in increased activity of NF-κB. Furthermore, Gα-GTP can activate signaling via Rho, which mediates cell movement. In addition to the G protein subunits, JAK is able to associate directly with the chemokine receptor and initiate signaling through phospholipase C and AKT to increase cell survival and increased transcription of activator protein 1 [73].

1.8.4.2 Chemokine-mediated leukocyte migration and infiltration

Chemotaxis of leukocytes occurs down a chemokine gradient to the site of inflammation through the following cascade: capture/tethering, rolling, slow rolling, adhesion strengthening and spreading, intravascular crawling, and paracellular and transcellular migration [176]. Leukocytes express chemokine receptors on their surface in order to recognize chemokines. Once bound, the chemokine-chemokine receptor interaction initiates a conformational change and subsequent signaling to induce both the speed and direction of leukocyte movement [73]. Some of the main mediators involved in this cascade include selectins and P-selectin glycoprotein ligand 1 [177], the immunoglobulin superfamily (ICAM1 and VCAM1) and

integrins [176]. Migration and infiltration of leukocytes is a key event that propagates tissue inflammatory responses.

1.8.4.3 Leukocyte infiltration in obesity and T2D

Although obesity and T2D are associated with high levels of pro-inflammatory cytokines such as TNFα [72, 178], the inflammatory environment in obesity is ultimately driven by activation and recruitment of leukocytes, including neutrophils, T cells, and monocytes/macrophages [27, 179]. During obesity, there are higher levels of circulating monocytes [180], and increased infiltration of macrophage and T cells into adipose and other tissues [27, 181, 182]. The importance of leukocyte chemotaxis in driving obesity and T2D is highlighted by findings showing that various chemokine or chemokine receptor knockout models are protected from HFD-induced obesity and insulin resistance [69, 183].

Although leukocytes appear to express multiple chemokine receptors to respond to various different chemokines [173], some chemokine–chemokine receptor interactions appear more important for specific leukocyte subsets. Although there are numerous identified chemokines, only certain chemokines have been implicated in the immune cell infiltration into adipose and other tissues in obesity. Three main chemokines are C-C motif ligand 2 (CCL2; also known as MCP1), CCL3 (also known as macrophage inflammatory protein [MIP] 1α), and C-X-C motif ligand 8 (CXCL8; also known as IL8). Specific chemokine receptors, differentially expressed on the surface of leukocytes, facilitate migration of immune cells into tissues in response to a chemokine gradient [173]. The CCL2-C-C motif receptor (CCR) 2 interaction is considered the major interaction involved in obesity-associated macrophage recruitment/infiltration into adipose [181] with further supporting evidence showing increased

levels of CCL2 and CCR2 in obese adults [184-186]. As CCR5 and C-X-C motif receptor (CXCR) 2 are also expressed on monocytes, interactions with CCR5 (CCL3-CCR5) and CXCR2 (CXCL8-CXCR2) may also play a role in chemotaxis of T cells and neutrophils, respectively [173]. The important role played by chemokines and their cognate chemokine receptors indicate that both of these aspects of leukocyte chemotaxis should be measured when attempting to understand chronic inflammation in obesity or T2D.

1.9 Inhibiting pro-inflammatory signaling during obesity and T2D

The majority of research on inflammation in T2D has been focused on reducing inflammation through the inhibition of pro-inflammatory signaling and/or neutralization of pro-inflammatory cytokines [178, 187, 188]. Research has revealed mixed results with some studies indicating benefits in metabolic outcomes of T2D [187, 189, 190] while others have not [191]. The role that anti-inflammatory cytokines and their signaling play in T2D has received much less attention. Accordingly, a major focus of this dissertation research was to better understand anti-inflammatory cytokine function in obesity and T2D.

1.10 Physical activity in prevention and treatment of obesity and T2D

Physical activity is a cornerstone in the management, prevention, and treatment of obesity and its related comorbidities. Physical activity targets many of the pathophysiological features that contribute to T2D, including its well-known effects at improving insulin sensitivity [192-197], body composition [198-201], and metabolic control [202-204]. Collectively, physical activity also improves cardiovascular health to aid in the reduction of CVD in people with T2D

[205-207]. The anti-inflammatory effects of exercise are believed to be a unifying mechanism by which physical activity lowers the risk of many chronic diseases, including T2D [208-212].

1.11 Immunomodulatory effects of exercise

The health benefits of exercise, and in particular during chronic low-grade inflammation associated with obesity and T2D, may be due to anti-inflammatory effects of exercise [208]. Most of these anti-inflammatory effects have been seen with moderate-intensity exercise; however, during higher volumes or intensities of exercise there may be immunosuppressive or even pro-inflammatory effects [213, 214]. The "elite athlete" paradox demonstrates an immunosuppressive effect of high volumes of exercise as elite athletes are more susceptible to upper respiratory tract infections [214-216]. This greater susceptibility may be somewhat explained due to increased production of IL10 from immune cells and may result in a less effective immune response [217, 218]. Some studies have also indicated that high-volumes or high-intensity exercise could have pro-inflammatory effects, commonly suggested by increases in leukocyte numbers and/or circulating pro-inflammatory cytokines [213, 219]. Additional research is required to elucidate the immunomodulatory effects of different types of exercise.

1.11.1 Proposed mechanisms underlying the anti-inflammatory effects of exercise

1.11.1.1 Decrease in visceral adipose tissue

One possible mechanism underlying the anti-inflammatory effects of physical activity and exercise training may be a decrease in mass of VAT. As adipose tissue becomes dysfunctional there are increased levels of pro-inflammatory adipokines including TNFα, MCP1, and IL6 [220, 221] and increased infiltration of monocyte/macrophages [222]. The reduction of

visceral fat mass through exercise results in lower pro-inflammatory adipokine release and subsequently less infiltration of monocytes/macrophages and other leukocytes into adipose [208, 223]. Evidence from animal models suggest that infiltration of monocytes/macrophages into adipose can be prevented through regular exercise training in the context of diet-induced obesity [224, 225]. However, it is unclear whether these anti-inflammatory effects on monocyte/macrophage migration are a direct result of exercise or are secondary to weight/fat loss [226] and whether exercise training in humans will result in altered markers of leukocyte migration.

1.11.1.2 Reduction in leukocyte migration

Chemokines and chemokine receptors are the main mediators of leukocyte migration. It has been suggested that exercise may alter chemokine receptor expression and therefore reduce leukocyte migration. Bishop et al. showed that an acute session of aerobic exercise, performed at 60% VO2peak for 2 h, reduced both CD4+ and CD8+ T lymphocyte migration towards the supernatants of human-rhinovirus infected bronchial epithelial cells in trained males [227]. The observation that these T cells migrated in the presence of supernatants only, without the presence of endothelial cells, suggested that the reduction in T cell migration was independent of changes in adhesion molecule expression. Therefore, in the absence of changes in adhesion molecules, physical stress could downregulate or desensitize the chemokine receptors that bind to the chemokines within the supernatants from infected bronchial epithelial cells. However, the suggestion that reduced T cell migration is caused by altered chemokine receptor expression is speculative only because the authors did not specifically measure any markers of chemokine receptor expression. Additional suggestions have been made that possible repeated elevations in

circulating chemokines during exercise may result in downregulation of their complementary chemokine receptor and conversely that reduced chemokine secretion may limit leukocyte migration/filtration [208]. Very little research on chemokine receptors in the context of exercise exists and therefore the impact of exercise on expression of chemokine receptors requires further research in humans.

1.11.1.3 Release of anti-inflammatory cytokines

During exercise, contracting muscle releases myokines such as IL6, which may mediate anti-inflammatory effects. Exercise has been shown to increase IL6 up to 100-fold in prolonged ultra-endurance exercise [132]; however, this increase is more modest following shorter duration exercise [228]. Fischer et al. reviewed 74 exercise trials and their effect on IL6 and indicated that 51% of the variation could be explained by exercise duration [229]. In addition to exercise duration, exercise intensity may also explain variations seen in exercise-induced IL6 production. For example, IL6 has been shown to consistently be increased following high intensity intermittent exercise [230, 231] and to a greater degree than moderate intensity continuous exercise [232]. Although elevated levels of exercise-induced IL6 are transient, there is a subsequent increase in the circulating levels of anti-inflammatory cytokines, IL1RA and IL10 [110]. Despite this work showing that anti-inflammatory cytokines, such as IL6 and IL10 are altered by exercise, the impact of exercise or exercise training on the anti-inflammatory function of these cytokines has not been studied.

1.11.1.4 Reduced expression of toll-like receptors (TLRs)

TLRs are pathogen recognition receptors and are essential in the detection of pathogen-associated molecular patterns found on microbial pathogens and damage-associated molecular patterns such as endogenous danger signals [73, 233]. TLR signal transduction results in increased production of pro-inflammatory cytokines and therefore TLR signaling plays an important role in systemic inflammation [234]. TLR-induced inflammation also plays a role during inflammatory conditions such as obesity, metabolic syndrome and T2D [235-237]. Therapeutically, both acute exercise and exercise training have been shown to reduce TLR expression during obesity [238, 239] and T2D [240]. Although exercise is associated with reduced TLR expression, the mechanism remains unclear [208].

1.11.1.5 Additional anti-inflammatory mechanisms of exercise

The two main populations of monocytes are classical (CD14+/CD16-) and non-classical (CD14+/CD16+) monocytes. The non-classical monocytes comprise of ~10% of the total monocyte population but have a greater contribution to the inflammatory potential of monocytes [241]. These inflammatory monocytes are upregulated in inflammatory conditions such as obesity [180, 185] and T2D [180, 242]. Acute exercise results in a transient increase in monocytes with a rapid return to baseline, which typically occurs 30 – 60 minutes post-exercise. Regular exercise can reduce basal levels of circulating inflammatory monocytes. Timmerman et al. [243] show that inactive older adults have a two-fold higher percentage of circulating inflammatory monocytes and that 12 weeks of regular exercise reduced the percentage to that of an active age-matched control. Exercise may elicit anti-inflammatory effects by reducing levels of inflammatory monocytes, which are associated with obesity and T2D.

1.12 Current exercise guidelines

Based primarily on the results of large diabetes prevention trials [9, 244] and other epidemiological risk factor reduction data, current guidelines advocate the accumulation of 150 minutes per week of moderate-intensity physical activity for the prevention and treatment of T2D. The ADA guidelines [245] also recognize the potential of other types of exercise, including resistance training and vigorous exercise for prediabetes and T2D. As an alternative to 150 minutes/week of moderate-intensity exercise, they recommend at least 90 minutes of vigorous aerobic activity. In addition, three days/week of strength training is recommended alongside aerobic exercise. How different types of exercise compare to standard care moderate-intensity exercise for improving risk factors and preventing T2D are not fully known. Through researching the effects of different exercise strategies on the major pathophysiological risk factors that contribute to T2D, it may be possible to identify alternative, or even more optimal, exercise prescriptions.

1.13 High-intensity interval training (HIIT)

Recently, HIIT has gained attention for its ability to elicit physiological benefits in a time-efficient manner [246]. HIIT comprises short, repeated bursts of high-intensity exercise that are separated by periods of rest or light exercise. Many different HIIT protocols exist that differ in interval length and intensity, which makes comparison amongst studies difficult. Nevertheless, recent meta-analyses are beginning to provide evidence that HIIT may be superior to traditional moderate-intensity continuous training (MICT) for improving cardiometabolic health. In a meta-analysis by Weston et al. [247], HIIT (studies primarily focused on “aerobic” interval training models involving 4 X 4-min vigorous intensity efforts at ~90% maximal heart rate [HR]

interspersed with 3-min rest periods, with studies lasting between 4 – 16 weeks but with the majority of studies lasting 12 weeks in duration), demonstrated greater exercise-induced adaptations compared to MICT, including improved cardiac function and greater improvements in peak oxygen uptake (VO_2peak). The overall increase in VO_2peak was 5.4 mL/kg/min in HIIT compared to 2.6 mL/kg/min in MICT. An increase in 3.5 mL/kg/min or 1 metabolic equivalent is associated with a 10 – 25% improvement in survival [248] demonstrating that HIIT appears to be more effective with respect to improving cardiorespiratory fitness at a clinically meaningful level.

As VO_2max (sometimes referred to as VO_2peak if a true plateau in oxygen consumption is not explicitly demonstrated in the maximal exercise test) is a strong predictor of mortality, the greater increase in VO_2max following HIIT may provide greater benefits than lower intensity exercise. Likewise, a recent meta-analysis by Jelleyman et al. [249] reported that HIIT (various protocols grouped together) led to greater improvements in insulin sensitivity and glucose control compared to MICT in subjects ranging from healthy young adults to T2D patients. However, the number of studies included in the meta-analysis was low and authors noted that more research in people at risk for T2D is needed.

1.13.1 HIIT: Metabolic improvements

There are also metabolic adaptations that may be influenced to a greater extent with HIIT compared to MICT. In insulin resistant states, the skeletal muscle is one of the main tissues affected. HIIT is hypothesized to elicit greater improvements in muscle insulin sensitivity as a result of higher degree of muscle fibre recruitment [250], greater muscle glycogen depletion, higher activation of AMPK [251], and increased protein content of GLUT4 [252, 253].

In support that these effects on muscle insulin sensitivity translate to improved glucose control, studies have used continuous glucose monitoring (CGM) to show that a single bout of HIIT [254] and two weeks of time-efficient HIIT involving 10 X 1-min vigorous efforts at ~90% HRmax separated by 1-min rest, can reduce glucose levels in patients with T2D [253]. Other studies support that HIIT has greater effects on glucose control using CGM. Little et al. [255] demonstrated that HIIT (10 X 1-min) had greater and longer-lasting effects in reducing postprandial glucose in overweight/obese adults when compared to MICT. Specifically, an acute bout of HIIT was compared to MICT (both exercise sessions performed in a randomized order two hours following breakfast). Although both HIIT and MICT were both effective at reducing postprandial glucose at the same day dinner, only HIIT showed a reduction on the next day's breakfast. Francois et al., [256] compared HIIT "exercise snacks" (6 X 1-min) to moderate walking before meals and their effect on postprandial hyperglycemia in prediabetes and T2D. Uphill walking interval "snacks" before meals (breakfast, lunch and dinner) reduced postprandial hyperglycemia greater than a single bout of pre-dinner moderate walking. Karstoft et al. [257] studied the effects of a longer-term HIIT (60 minutes, 5 days/week for 16 weeks) comprised of walking exercise (3-min intervals interspersed with 3-min rest periods) compared to MICT (60 minutes of steady state exercise) in T2D. CGM measurements, including 48h mean and 48h maximum values, were reduced after HIIT but not MICT. Therefore, the evidence is accumulating that HIIT has beneficial effects on metabolic outcomes, particularly glucose control.

1.13.2 Impact of HIIT on inflammation

Although the improvements in insulin sensitivity and reductions in hyperglycemia seen following HIIT suggest potential anti-inflammatory effects, there is a lack of research available on the immunomodulatory effects of HIIT. Since HIIT demonstrates improvements in cardiometabolic health in healthy and clinical populations, similar or superior to MICT [246, 249, 258] the impacts of HIIT on inflammation and immune function clearly require further study in order to fully understand and appreciate the potential value of this type of exercise training. The question of whether HIIT during obesity and related conditions is anti-inflammatory [238, 240], immunosuppressive [214-216], or even pro-inflammatory [213] still remains largely unknown.

Recent studies have, however, explored the anti-inflammatory effects of both HIIT and MICT by assessing leukocyte phenotype and circulating cytokines. Robinson et al. [238] demonstrated that two weeks of HIIT and MICT in overweight/obese adults led to reductions on lymphocyte and monocyte TLR4 and lymphocyte TLR2, whereas only MICT demonstrated a reduction in neutrophil TLR4. Another study confirmed similar findings with a reduction in monocyte, but not neutrophil, TLR2 and 4 after both HIIT and MICT [259]. Conversely, exercise can lead to an increase in pro-inflammatory mediators. Oliveira-Child et al. [260] observed increased monocyte TLR4, but not TLR2, following two weeks of HIIT.

The effects on other inflammatory mediators have also been compared between HIIT and MICT in preliminary studies. Changes in IL6 or TNFα were not seen after six weeks of either HIIT or MICT in overweight/obese adults [261]; however, a 16 week program demonstrated a decrease in TNFα following MICT and a decrease in IL6 but an increase in TNFα following HIIT [262]. Additionally, an acute exercise session of HIIT increased pro-inflammatory cytokines and chemokines CCL2 and CXCL8 in young healthy men [213]. Although many of

these studies indicate a more “anti-inflammatory” response of both MICT and HIIT, other studies suggest that HIIT may have more “pro-inflammatory” effects.

In contrast to the above-mentioned studies examining distinct leukocyte markers and circulating cytokines, less is known on the effects of HIIT versus MICT on immune function in obesity. The migration of neutrophils is preserved in physically active older adults, compared to age-matched controls, indicating a possible immune-enhancing effect of neutrophil migration in active individuals [263]. Furthermore, these same authors explored leukocyte function and showed that 10 weeks of HIIT and MICT both improved the phagocytic ability and oxidative burst of neutrophils and monocytes [264]. The limited data available indicates that HIIT and MICT both impact aspects of leukocyte function but more work is needed.

Collectively, the available literature suggests that HIIT and MICT may differentially impact inflammation and despite the improvements seen in cardiometabolic health, it still remains unclear whether HIIT has the same anti-inflammatory potential as MICT or if the effects are more pro-inflammatory in nature.

1.14 Aims and hypotheses

The overarching aim of this thesis was to better understand the potential mechanisms contributing to chronic inflammation in obesity and T2D, and to determine how exercise impacts anti-inflammatory cytokine function and chemokine receptors. To accomplish this, a cross-sectional study was conducted in Chapter 2 to compare the anti-inflammatory action of IL10 in individuals with T2D and age- and BMI-matched control participants. For Chapters 3 and 4, the influence of short-term exercise training on immune cell phenotype and function was investigated. In order to do this, a subsample of individuals with obesity from a larger

randomized controlled trial (RCT) were studied, which allowed for a comparison of MICT and HIIT in the absence of weight loss or body composition changes.

Chapter 2: The majority of research on inflammation associated with obesity and T2D has been focused on mechanisms to reduce inflammation through inhibition of pro-inflammatory signaling and neutralization of pro-inflammatory cytokines. However, it is possible that altered function of anti-inflammatory cytokines may be involved.

Aim: To explore the ability of IL10 to inhibit inflammation in immune cells of individuals with T2D and to further examine underlying mechanisms using macrophage cell models.

Hypothesis: T2D may impact inflammation by interfering with IL10 signaling.

Chapter 3: Although IL6 and IL10 have been implicated in the anti-inflammatory effects of exercise, the effect of exercise on the anti-inflammatory action/function of these cytokines remains unexplored.

Aim: To determine the impact of short-term exercise on the anti-inflammatory action of IL6 and IL10.

Hypothesis: Improved responsiveness of IL10 and/or IL6 would be a novel anti-inflammatory effect of exercise training.

Chapter 4: The influence of exercise on chemokines and their receptors remain an unexplored area of research, which is surprising given the importance of leukocyte infiltration into adipose and other tissues to propagate inflammation during obesity.

Aim: To explore the impact of short-term exercise on chemokine and chemokine receptor expression.

Hypothesis: HIIT and MICT would differentially modulate the levels of chemokines and leukocyte chemokine receptor expression.

Chapter 2 Hyporesponsiveness to the anti-inflammatory action of interleukin-10 in type 2 diabetes

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2.1 Background

Chronic low-grade inflammation plays a critical role in the development and progression of insulin resistance and T2D. The pro-inflammatory environment in T2D is characterized by elevated circulating pro-inflammatory cytokines and acute phase reactants, increased markers of leukocyte activation, and increased macrophage infiltration into adipose and other tissues [265, 266]. Elevated glucose is the direct metabolic consequence of insulin resistance, and hyperglycemia has been shown to promote cellular inflammation in circulating monocytes [31, 267], macrophages [268], and adipocytes and to contribute to the pathogenesis of T2D and its complications [265, 266]. Thus, a vicious cycle is created whereby inflammation induces insulin resistance, leading to elevated glucose that can propagate further inflammation. Understanding the mechanisms underlying the pro-inflammatory state in T2D, and how to mitigate it, is therefore of great therapeutic value for the prevention and treatment of T2D and its costly complications.

Most previous research on inflammation in T2D has focused on inhibiting pro-inflammatory signaling and/or neutralizing pro-inflammatory cytokines as therapeutic targets [178, 187, 188]. These efforts have revealed mixed results with some studies showing benefits on T2D-related metabolic outcomes [187, 189, 190, 269], whereas others did not [191]. The role of anti-inflammatory cytokines and signaling in T2D has received much less attention.

IL10 is an anti-inflammatory cytokine normally released locally from immune cells to help resolve inflammation, and is best characterized for its ability to inhibit macrophage activation [136]. Deficiencies in IL10 expression, or IL10R signaling, in mice and humans results in inflammatory diseases such as colitis [270-273]. There is some evidence suggesting that IL10 may be involved in T2D-related inflammation as mice engineered to ectopically express IL10 through either gene transfer [167] or through a muscle cell-specific transgene [168] are partially protected from HFD-induced obesity and glucose intolerance. However, the impact of T2D on IL10 anti-inflammatory function has not been previously assessed.

IL10 signaling consists of a ligand-specific IL10R1 and a second subunit IL10R2, which is also found in other cytokine receptors [136]. Binding of IL10 to the IL10R activates the STAT3 transcription factor. Our previous research has also shown that activation of the SHIP1 inositol phosphatase is required for IL10 action [162, 163]. SHIP1 is expressed predominantly in hemopoietic cells and inhibits pro-inflammatory signaling (induced by agents such as bacterial LPS) by degrading PIP_3 [164, 274]. We have also shown that SHIP1-mediated IL10 signaling functions to inhibit translation of TNFα mRNA, contributing to its anti-inflammatory action [162]. Recent research has also shown that IL10 activates the cellular energy gauge AMPK [151, 275] and that AMPKα1 is required for the anti-inflammatory effects of IL10 via the PI3K/AKT/Mammalian target of rapamycin complex 1, and STAT3 pathways [151]. Given the emerging role for AMPK as a key integrator of cellular metabolism and inflammation [276] and studies indicating that high glucose reduces AMPK activation (reviewed in:[277, 278]), we hypothesized that T2D may impact inflammation by interfering with IL10 signaling. The purpose of this study was to determine how T2D and hyperglycemia influenced the anti-inflammatory abilities of IL10 in immune cells. We examined the ability of IL10 to inhibit

inflammatory activation of immune cells in humans with T2D and explored the underlying mechanisms using macrophage cell culture models.

2.2 Methods

2.2.1 Human participants and experimental methods

2.2.1.1 Participants

Individuals with T2D were recruited via poster advertisement at local medical laboratories, Internet message board advertisements, and word-of-mouth. Age and BMI-matched non-T2D controls were recruited through Internet message boards and word-of-mouth. Sample size was calculated based on IL10-mediated TNFα inhibition pilot studies conducted in whole blood cultures in our lab that demonstrated a large effect size between T2D and non-T2D controls (~1 SD difference) (n=7-8 per group). With 80% power and alpha of 0.05, sample size required was estimated to be n=16 per group (calculated using G*Power Version 3). To preserve power we aimed to recruit 20-25 per group. The study and experimental protocols were approved by the UBC Clinical Research Ethics Board. All procedures were conducted in accordance to approved protocols and all participants provided written informed consent. T2D patients were not on exogenous insulin and any interested participants were excluded if they reported regularly taking anti-inflammatory medications or had a current or recent self-reported infection. Thirteen T2D patients were not taking any anti-diabetic medications and eleven were taking anti-diabetic medications (six metformin, three combination of metformin and sulphonylureas, one combination of metformin and DPP-4 inhibitor, and one combination of metformin and GLP-1 receptor agonist). Ten T2D patients and three non-T2D controls were taking anti-hypertensive medications. Details of medications in the T2D patients are shown in Table 2.1.

Table 2.1. Medication usage in T2D patients.

Medication usage in T2D patients	(n=24)
Anti-Diabetic (No.)	
Biguanide	6
Biguanide + Sulphonylurea	3
Biguanide + GLP-1R antagonist	1
Biguanide/DPP-4 inhbitor	1
Anti-hypertensive (No.)	
Calcium channel blocker	2
ACE inhibitor	1
Angiotensin II receptor blocker	2
Beta blocker	1
Calcium channel blocker + ACE inhibitor	2
Calcium channel blocker + Angiotensin II receptor blocker	1
Angiotensin II receptor blocker + Beta blocker	1

The two main medication classes used in the T2D patients were anti-diabetic medications (11 out of 24) and anti-hypertensive medications (10 out of 24).

2.2.1.2 Anthropometrics

Body mass, height (Seca 700 Mechanical Colum Scale) and waist circumference (WC) (measured at the top of the iliac crest) were measured and BMI was calculated as body mass in kilograms divided by height in meters squared.

2.2.1.3 Blood sampling

Fasting venous blood samples were obtained by venipuncture from an antecubital vein and collected into a sodium heparin and an ethylenediaminetetraacetic acid-coated (EDTA) tube (BD Vacutainer). Blood collected in the sodium heparin tube was used for whole blood cultures. Plasma was collected by centrifugation of the EDTA tube at 1550 xg for 15 min at 4°C and stored at -80°C for further analysis.

Plasma glucose was measured in duplicate using the hexokinase method (G5717-120: Pointe Scientific INC, USA) on a Chemwell 2910 automated analyzer (Awareness Technologies, Palm City, USA). Plasma cytokines (IL10, IL6 and TNFα) were measured by custom multiplex immunoassay (Human High Sensitivity T Cell multiplex kit: HSTCMAG-28SK, Millipore, Billerica, MA, USA) and read using a MAGPIX™ Bio-Plex® reader (BioRad, Hercules, California, USA) according to the manufacturer's instructions. Plasma was spun at 1000 xg for 15 min at 4°C to remove any precipitates and the assay was performed in duplicate. Plasma cytokine concentrations were calculated using a 5-parameter logistic fit on Bio-Plex Manager ™ 6.1 Software.

2.2.1.4 Continuous glucose monitoring (CGM)

Real-time CGM was measured over 48 – 72 hours using the Guardian® REAL-Time CGMS and Enlite® sensor (Medtronic MiniMed, Northridge, CA). Sensors were inserted by a trained researcher and participants were instructed on how to obtain finger prick blood samples (OneTouch Ultra Mini) for calibration at least three times per day. Upon device removal, data was downloaded to CareLink Personal Therapy Management Software for Diabetes and 24-hour average glucose was calculated after exporting the data to Microsoft Excel.

2.2.1.5 Human whole blood culture

Whole blood was diluted 1:10 in serum-free RPMI media (Sigma) containing 5 mM glucose with penicillin (50 U/ml) and streptomycin (50 ug/ml). Diluted whole blood (540 ul) was seeded in 24-well plates (Costar). Whole blood cultures were stimulated with LPS (from Escherichia coli 055:B5; L6529, Sigma) at 1 or 10 ng/ml with or without 10 ng/ml IL10. Whole

blood cultures were incubated at 37°C in 5% CO_2 and supernatants were collected at six hours for analyses of secreted TNFα on duplicate samples by ELISA (Human TNFα DuoSet, R&D Systems) according to the manufacturer's instructions. Absorbance was read at 450 nm on an iMark Microplate Absorbance reader (Biorad, CA, USA) and data were analyzed using Microplate Manager 6.0 (Biorad) to calculate TNFα concentration using a cubic spline regression model. TNFα concentration was expressed in pg/ml or as percent of maximal LPS-stimulated secretion.

2.2.1.6 Flow cytometry

IL10R1 levels were measured on different monocyte subsets in human whole blood by flow cytometry. 8 ul of FcR Blocking Reagent (Miltenyi, Bergisch Gladbach, Germany) was added to 72 ul of whole blood and allowed to incubate for 10 minutes at 4°C in the dark. Following this, 2 ul of CD14-Vioblue (Miltenyi, Bergisch Gladbach, Germany), CD16-FITC (Miltenyi, Bergisch Gladbach, Germany), and CD210/IL10R-PE (BioLegend, San Diego, CA) were added and again allowed to incubate for 10 minutes at 4°C in the dark. After incubation, 1 ml of red blood cell lysis buffer (Miltenyi, Bergisch Gladbach, Germany) was added and the sample was incubated for 15 minutes at room temperature in the dark. In order exclude dead cells from analysis, 2 ul of propidium iodide (PI) (Miltenyi, Bergisch Gladbach, Germany) was added immediately before analyzing on a MACSQuant Analyzer flow cytometer (Miltenyi, Bergisch Gladbach, Germany). A total of 15000 CD14+ events were collected for analysis in each subject. Events positive for PI were excluded from analysis using a not-gate. The different monocyte subtypes were identified using light scatter characteristics as well as being positive for CD14-Vioblue but negative for CD16-FITC (CD14+/CD16- "classic" monocytes) or positive for

both CD14-Vioblue and CD16-FITC (CD16+ “pro-inflammatory” monocytes). Fluorescence minus one (FMO) controls were used to establish positive staining. IL10R1 expression, expressed as median fluorescence intensity (MFI), was then analyzed on these monocyte subtype populations.

2.2.2 Cell culture models and experimental methods

2.2.2.1 Macrophage cell culture

Culture of RAW264.7 cells and derivation of bone marrow derived macrophages were as described [162, 163]. DMEM of various glucose concentration were generated by mixing appropriate proportions of DMEM high glucose (Hyclone SH30022.01) and DMEM low glucose (Hyclone SH30021.01). Stimulations with LPS and measurement of TNFα production by ELISA were also as described [162, 163].

2.2.2.2 Immunoblotting

Preparation of cell lysates for sodium dodecyl sulphate-gel polyacrylamide gel separation and immunoblot analysis were as described [162, 163]. Antibodies to pSTAT3-Y705, STAT3 protein, pAMPKα-Thr172, and pAMPKα were from Cell Signaling Technologies (Whitby, Ontario, Canada). Antibodies to murine IL10R1 and SHIP1 (clone P1C1) were from Santa Cruz Biotechnology (Texas, USA).

2.2.3 Statistical analysis

Data were analyzed using SPSS Statistics. Normality of data was assessed using Q-Q plots and the Shapiro-Wilk test and variables that did not meet the assumptions of normality were log transformed prior to statistical analyses. Three outliers were removed from analyses of plasma cytokines based on values that were deemed non-physiological being >10 SD from the mean (one T2D for IL10 and one non-T2D for IL6) and >4 SD from the mean (one non-T2D for IL6). Removal of these outliers from the select plasma cytokine analyses did not change the statistical outcomes but due to their influence on the mean values, were omitted from the analyses and descriptive data. Differences between T2D patients and non-T2D controls were analyzed by independent t-tests. A two-factor (group X concentration) repeated measures ANOVA were used to compare changes in whole blood culture cytokine secretion and for cell culture experiments. Significant main effects or interactions were followed by Fisher LSD post-hoc testing. Statistical significance was set at $p \leq 0.05$.

2.3 Results

2.3.1 IL10 is less effective at inhibiting inflammation in humans with T2D

To determine how T2D *in vivo* impacted IL10 function, we first examined the ability of IL10 to inhibit pro-inflammatory cytokine secretion from whole blood cultures prepared from patients diagnosed with T2D (n=24, FPG >7.0 mmol/L and/or hemoglobin A1c >6.4%) compared to non-T2D (n=22) age and BMI-matched controls. Characteristics of the patients, including age, BMI, WC, FPG, and plasma cytokines are presented in Table 2.2. Patients with T2D had significantly higher FPG ($P<0.001$) but were similar age, BMI and WC (all $P>0.05$). CGM was also obtained on a subset of patients with (n=15) and without (n=11) T2D. Patients

with T2D had a significantly higher 24-hour CGM average blood glucose (7.7 ± 1.8 mmol/L) compared with non-T2D controls (5.8 ± 0.8 mmol/L), confirming that immune cells were exposed to chronically elevated glucose levels ($P<0.05$). As expected, the basal pro-inflammatory cytokines TNFα and IL6 were elevated in patients with T2D (both $P<0.05$). Fasting IL10 concentrations were not significantly different ($P>0.05$).

Table 2.2. Characteristics of the T2D patients and non-T2D control participants.

Variable	**T2D**	**Non-T2D**	
	n=24	n=22	**p-value**
Age (years)	57.8 (10.9)	53.4 (10.7)	0.173
Sex, n			
Male	8	4	
Female	16	18	
BMI (kg/m²)	32.1 (6.2)	30.1 (4.8)	0.233
WC (cm)	109.1 (19.4)	102.3 (13.5)	0.183
Fasting plasma glucose (mmol/L)	7.8 (0.8)*	5.8 (0.8)	**<0.001**
24 Hour CGM average glucose (mmol/L)	7.7 (1.8)*	5.8 (0.8)	**0.003**
Plasma TNFα (pg/ml)	9.57 (5.98)*	5.75 (2.72)	**0.009**
Plasma IL6 (pg/ml)	3.02 (2.27)*	1.76 (0.94)	**0.049**
Plasma IL10 (pg/ml)	7.07 (5.93)	4.52 (3.99)	0.163

Values are mean (SD). BMI, body mass index; WC, waist circumference.

Whole blood cultures were used to minimize any impact of sample processing and maintain physiological conditions of plasma constituents and cellular composition [279]. Cultures were stimulated with LPS (1 and 10 ng/ml) +/- IL10 (10 ng/ml). A two-way ANOVA revealed a significant main effect of LPS ($P=0.047$, as expected) with no significant difference between T2D and non-T2D (main effect of group, $P=0.623$; Figure 2.1a). As described previously [162, 163, 280], the ability of IL10 to inhibit LPS-induced TNF-α secretion was used as an index of the anti-inflammatory ability of IL10. IL10 inhibited LPS-induced TNF-α

secretion with results of the two-way ANOVA indicating that the anti-inflammatory action of IL10 was lower in T2D vs. non-T2D control participants (main effect of group, P≤0.05, Figure 2.1). The findings were similar when TNFα was expressed as an absolute concentration (P=0.05; Figure 2.1b) or as a percentage of maximal LPS-induced secretion (P=0.032; Figure 2.1c), with post-hoc testing revealing a significant difference (P<0.05) between T2D and non-T2D in the 10 ng/ml LPS + 10 ng/ml IL10 condition. Secretion of IL10 from whole blood cultures of T2D patients and non-T2D controls at both 1 ng/ml LPS (T2D: 4.5 ± 3.5 ng/ml; Non-T2D: 5.6 ± 4.9 ng/ml) and 10 ng/ml LPS (T2D: 4.7 ± 3.5 ng/ml; Non-T2D: 5.4 ± 3.9 ng/ml) were not significantly different (P>0.05).

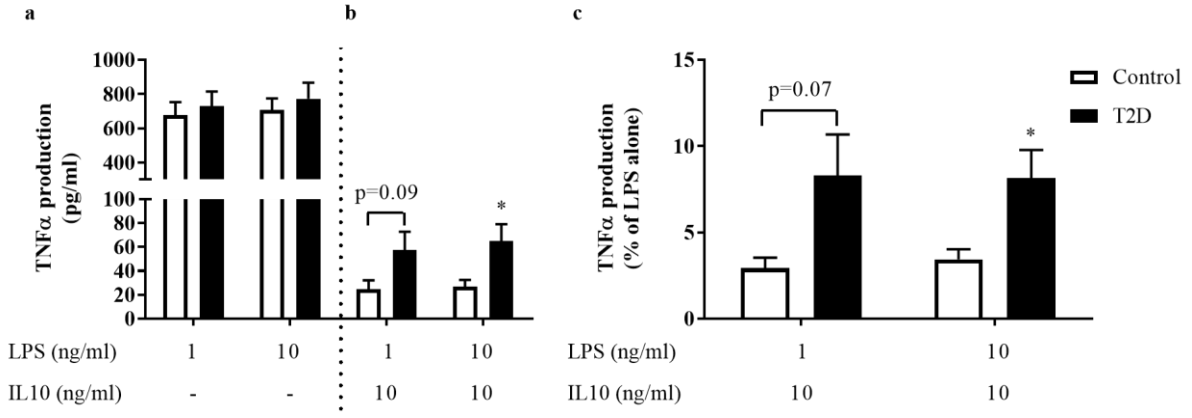

Figure 2.1. Humans with T2D display hyporesponsiveness to IL10. Whole blood cultures were stimulated with LPS only (1 and 10 ng/ml) or LPS + IL10 (10 ng/ml) and pro-inflammatory activation was assessed by TNFα secretion. A two-way ANOVA revealed no significant differences in (a) LPS-induced TNFα secretion between T2D patients (n=24) and age and BMI-matched non-T2D controls (n=22) at both 1 ng/ml and 10 ng/ml LPS. However, the ability of IL10 to suppress TNFα secretion was less effective in T2D (main effect of group, P<0.05) for both absolute (b) and % maximal (c) TNFα secretion with post-hoc tests revealing a significant difference at 10 ng/ml LPS combined with 10 ng/ml IL10 (P<0.05 for both). Biological replicates were cultured and TNFα secretion measured by standard ELISA. IL10 inhibition is expressed as % maximal TNFα secretion within each subject calculated as ([(LPS-induced secretion – LPS + IL10 secretion) / LPS secretion] X 100%). Values are mean ± SEM, *P<0.05 vs. Control.

Monocytes, the precursors to tissue macrophages, are the major cell type that produce pro-inflammatory cytokines (including TNFα), in whole blood cultures [241, 281]. In order to determine whether reduced expression of the IL10R might explain the lower anti-inflammatory function of IL10 in patients with T2D we performed flow cytometry experiments to analyze the surface protein expression of the IL10R1 on "classic" CD14+/CD16- monocytes and "pro-inflammatory" CD14+/CD16+ monocytes. We analyzed monocyte subsets because evidence indicate that CD16+ monocytes produce the majority of TNFα in human whole blood [241] and data suggesting that IL10 expression may be different in monocyte subsets [282]. There were no significant differences in IL10R1 detected (Figure 2.2), but there was a trend (P=0.057) for an increase in MFI of IL10R1 on classical CD14+/CD16- monocytes in T2D.

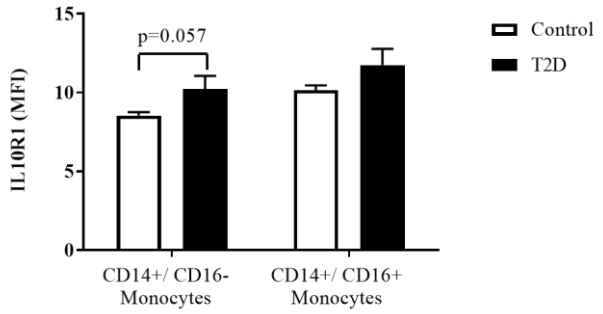

Figure 2.2. No significant difference in IL10R1 expression on monocytes in T2D. IL10R1 was measured on CD14+/CD16- and CD16+ monocytes by flow cytometry in the T2D and age and BMI-matched non-T2D control participants. Unpaired t-tests indicated that IL10R1 expression was not significantly different on either monocyte subset although IL10R1 tended to be lower on CD14+/CD16- classical monocytes in T2D (P=0.057). Values are mean ± SEM.

Thus, immune cells from individuals with T2D appear to be resistant to the anti-inflammatory effects of IL10, which are not explained by reductions in the surface protein expression of IL10R1.

2.3.2 High glucose promotes IL10 resistance in macrophages

In obesity and T2D, blood monocytes are recruited into tissues (e.g. adipose, liver) where they differentiate into macrophages and propagate a state of chronic low-grade inflammation [265]. In order to determine if hyperglycemia, the primary pathophysiological hallmark of T2D, might be responsible for reducing the anti-inflammatory function of IL10, we cultured RAW264.7 mouse macrophages in normal (5 mM) and high (15 mM) glucose and treated the cells with LPS +/- IL10. Similar to the findings in T2D patients, six hour exposure of macrophages to high glucose induced a state of IL10 resistance; IL10 was less effective at inhibiting LPS-induced TNFα secretion at 15 mM glucose compared to 5 mM glucose (Figure 2.3a) in RAW264.7 mouse macrophages. Findings were confirmed in primary mouse bone-marrow derived macrophages (BMDMs) (Figure 2.3b).

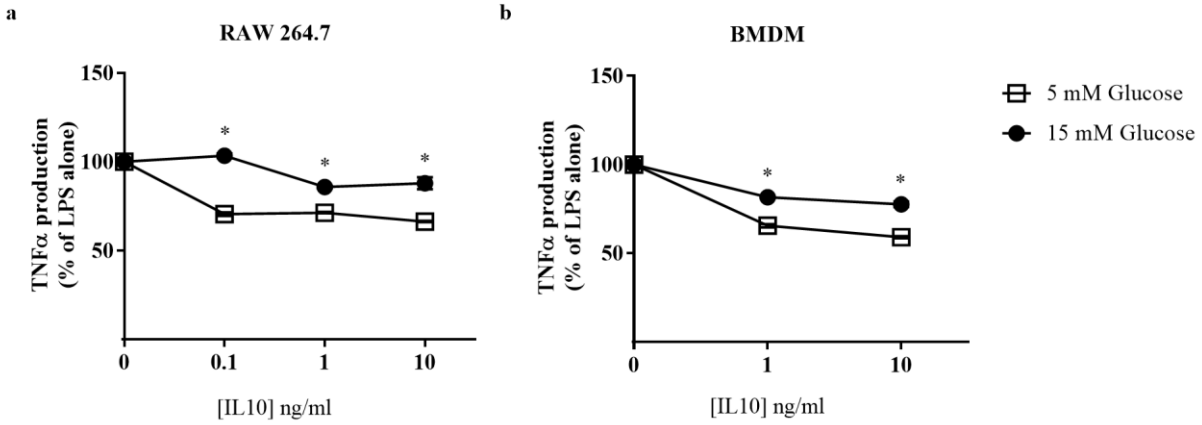

Figure 2.3. RAW264.7 cells and BMDMs (bone marrow-derived macrophages) grown in 15 mM glucose are hyporesponsive to IL10. RAW264.7 cells (a) and BMDMs (b) were cultured for six hours in 9% FCS in DMEM containing either 5 mM or 15 mM glucose. Cells were then stimulated for two hours with 1 ng/mL LPS in the presence of 0 – 10 ng/mL of IL10, and supernatants collected for TNFα production (expressed as % of that induced by LPS alone) by ELISA. Values are mean ± SEM (N=3). * $p<0.05$ vs. 5 mM glucose condition.

2.3.3 High glucose inhibits IL10-mediated STAT3 activation

To explore whether high glucose altered IL10 signaling, we first examined the total protein content of the key IL10 signaling component IL10R1 in RAW264.7 macrophages cultured for six hours in 15 mM compared to 5 mM glucose (Figure 2.4a). Quantification of immunoblots revealed no significant effects of high glucose on IL10R1, total STAT3, or total AMPKα protein levels (Figures 2.4b, 2.5b, and 2.6b). Thus, similar to humans with T2D, hyporesponsiveness to IL10 as a result of hyperglycemia in cultured macrophages did not appear to be explained by reduced protein levels of the IL10R or downstream signaling proteins. We next explored how exposure to high glucose influenced activation of IL10 signaling in macrophages. As expected, treatment of RAW264.7 macrophages treated with IL10 (10 ng/ml) in 5 mM glucose led to a robust activation of STAT3 as assessed by tyrosine-705 phosphorylation (Figure 2.5). When RAW264.7 cells were cultured in 15 mM glucose the ability of IL10 to induce STAT3 phosphorylation was significantly reduced ($P<0.001$). These findings indicated that high glucose impairs the ability of IL10 to activate intracellular STAT3.

We also examined IL10's ability to induce phosphorylation of AMPKα at threonine-172 as a marker of activation. Figure 2.6 shows that phospho-AMPKα levels appear constitutively higher in cells grown in 5 mM than 15 mM glucose, although this did not reach statistical significance. There appeared to be a marginal increase in threonine-172 phosphorylation of AMPKα in response to IL10 treatment in 5 mM glucose conditions, but this was not statically significant. We were not able to detect an increase phosphorylation of AMPKα1 in response to IL10 treatment in cells cultured in 15 mM glucose.

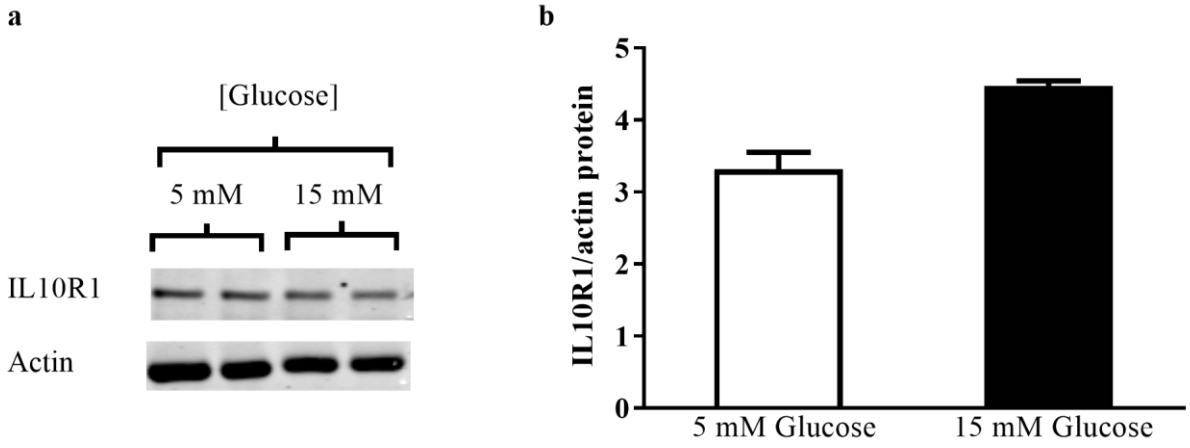

Figure 2.4. Protein levels of IL10R1 in macrophages are not affected by altered glucose levels. a) Cell lysates from RAW264.7 cells cultured in 5 mM or 15 mM glucose for six hours were immunoblotted with the indicated Abs. Representative immunoblots from duplicates are shown. b) Band intensities were quantified on a Li-Cor Odyssey scanner and normalized to the levels of actin and plotted in the bar graph. Paired t-tests revealed no significant differences between 5 mM and 15 mM glucose. Values are mean ± SEM (N=3). P>0.05.

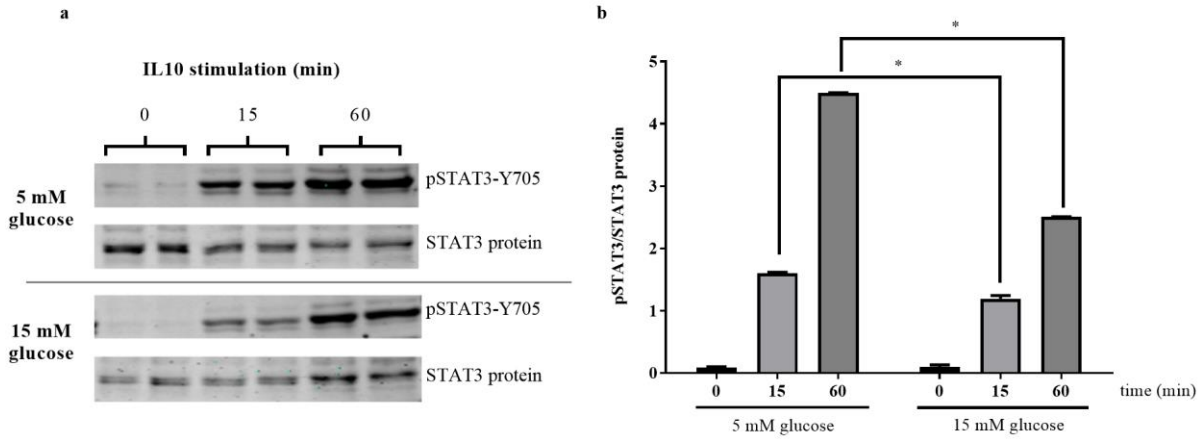

Figure 2.5. High glucose (15 mM) treatment reduces IL10's ability induce STAT3 Tyr 705 phosphorylation. RAW264.7 cells were cultured for six hours in either 5 mM or 15 mM glucose. Duplicate sets of cells were then stimulated with 1 ng/mL IL10. a) Cell lysates were separated by SDS-PAGE gel and immunoblotted with antibodies to P-STAT3 Y705 or total STAT3 protein. b) Band intensities were quantified on a Li-Cor Odyssey scanner and P-STAT3/STAT3 protein ratios are shown in the bar graph. Values are mean ± SEM (N=3). *P<0.05 vs. 5 mM glucose condition.

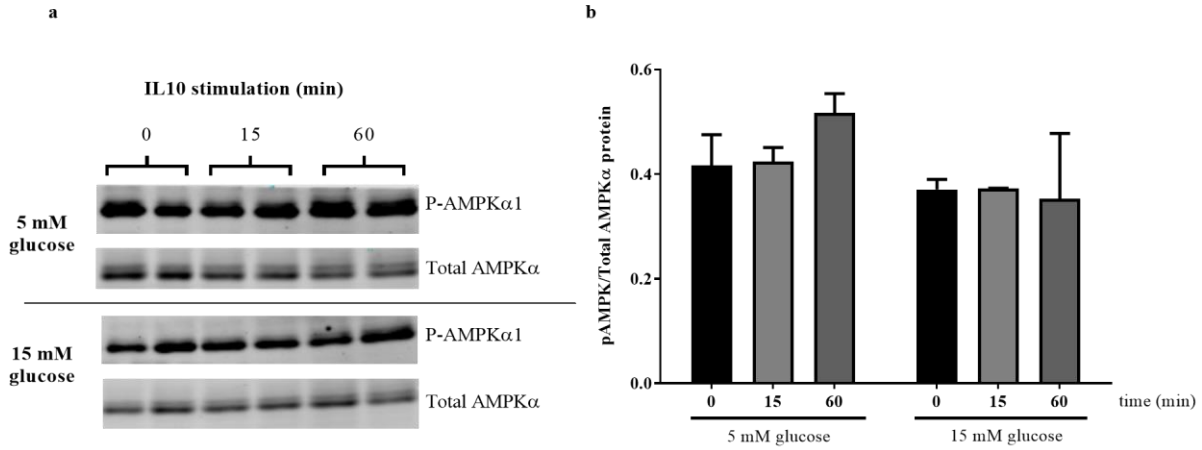

Figure 2.6. P-AMPK levels are not affected by IL10 treatment in RAW264.7 cells. RAW264.7 cells were cultured for six hours in either 5 mM or 15 mM glucose. Duplicate sets of cells were then stimulated with 1 ng/mL IL10. **a**) Cell lysates were separated by SDS-PAGE gel and immunoblotted with antibodies to P-AMPKα (Thr-172) and total AMPKα protein. **b**) Band intensities were quantified on a Li-Cor Odyssey scanner and P-AMPKα / total AMPKα protein ratios shown in the bar graph (no effects of time or condition, all P>0.05) Values are mean ± SEM (N=3).

2.3.4 The SHIP1 agonist AQX-MN100 can overcome high glucose-induced IL10 resistance

Given the importance of SHIP1 in mediating the anti-inflammatory actions of IL10 [162], we next determined whether the small molecule SHIP1 agonist AQX-MN100 [283] was able to overcome IL10 hyporesponsiveness in macrophages cultured under high glucose conditions. Despite the cells being resistant to the anti-inflammatory effects of IL10 in 15 mM glucose, AQX-MN100 was equally effective at inhibiting LPS-induced TNFα secretion in 15 mM and 5 mM glucose conditions (Figure 2.7) demonstrating the ability of a SHIP1 agonist to act as an anti-inflammatory agent under hyperglycemic conditions.

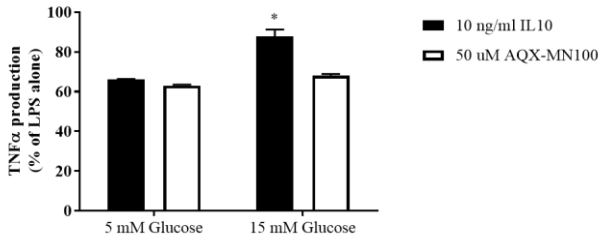

Figure 2.7. AQX-MN100 can bypass IL10 hyporesponsiveness to inhibit inflammation in cells grown in high glucose. Cells were cultured for six hours in 9% FCS in DMEM containing either 5 mM (Low) or 15 mM (High) glucose. Cells were then stimulated for two hours with 1 ng/mL LPS in the presence of 10 ng/mL IL10 or 50 uM AQX-MN100. TNFα production was assessed by ELISA. IL10 was less effective in high glucose whereas the anti-inflammatory actions of AQX-MN100 were not influenced. Values are mean ± SEM (N=3). * $p<0.05$ as compared to low glucose.

2.4 Discussion

In this study we provide evidence from both human and cell culture studies that T2D *in vivo* and hyperglycemia *in vitro* are related to a reduced anti-inflammatory function of IL10. The hyporesponsiveness to IL10 in the presence of high glucose appears linked to reduced intracellular signal transduction through STAT3, whereas the anti-inflammatory actions of AQX-MN100, a small molecule activator of SHIP1, is not affected by hyperglycemia. Collectively, the results support the novel idea that chronic low-grade inflammation in T2D might be explained, at least in part, by a reduction in the natural anti-inflammatory actions of IL10. Strategies aimed to overcome IL10 resistance, including SHIP1 agonists, might therefore hold therapeutic potential for reducing inflammation associated with metabolic disease.

2.4.1 IL10 function is impaired in immune cells from T2D

Previous studies examining a role for IL10 in T2D-related pathology have produced mixed results. Hong et al. demonstrated that muscle-specific overexpression of IL10 protected mice from HFD-induced insulin resistance and inflammation [168]. Similarly, Gao et al. demonstrated that treatment with IL10 though plasmid injections preserved insulin sensitivity and prevented glucose intolerance in mice fed a HFD [167]. Both of these studies provide evidence that artificially elevating IL10 to supraphysiological levels may have beneficial effects on T2D-related metabolic control, but they were not designed to address the function of IL10 in the context of established T2D. Kowalski et al. used bone marrow transfer techniques to create hematopoietic-cell restricted deletion of IL10 in mice and reported no impact of IL10 deletion on HFD-induced inflammation or insulin resistance, arguing against a role for IL10 in T2D-related pathology [284]. However, deletion of IL10 in hematopoietic cells may not be representative of subtle changes in IL10 action that may occur in T2D. We aimed to study how IL10 functioned to inhibit inflammation in immune cells in the context of T2D and hyperglycemia. Our results indicate that the normal anti-inflammatory actions of IL10 are impaired in T2D, effects that appear to be linked to hyperglycemia. These data suggest that the metabolic consequences of T2D may perpetuate a state of chronic low-grade inflammation through a mechanism involving failure of IL10 to adequately resolve innate immune activation.

We are aware of two previous studies linking reduced anti-inflammatory actions of IL10 to chronic inflammation. Avdiushko et al. demonstrated that cultured macrophages isolated from mice chronically infected with the LP-BM5 retrovirus had reduced capacity to response to IL10 as assessed by reduced ability of IL10 to inhibit LPS-induced cytokine secretion [285]. This hyporesponsiveness to IL10 upon viral infection was not related to reductions in mRNA expression of the IL10R and the authors were unable to decipher the potential mechanisms

responsible but concluded that the chronic inflammatory environment promoted by the LP-BM5 retroviral infection rendered macrophages less responsive to IL10 via a mechanism that was downstream of the IL10R. Yuan et al. also found that monocytes isolated from patients with systemic lupus erythematosus (SLE) were less responsive to IL10, assessed by reduced ability of IL10 to suppress human Ig-induced TNFα and IL6 secretion [286]. The IL10 hyporesponsiveness coincided with reduced IL10-induction of STAT3 phosphorylation in the presence of human Ig, while IL10R and STAT3 expression levels were the same in control and SLE monocytes.

2.4.2 IL10 function is impaired in macrophage cells exposed to high glucose

Our findings indicate that exposure to high glucose can also reduce the capacity of macrophages to respond to IL10, linking hyperglycemia in T2D to IL10 hyporesponsiveness. Our findings in cultured macrophages show that hyperglycemia impacts IL10 signaling at the level of STAT3, a key node in intracellular IL10 signal transduction [287]. IL10 has been reported to induce phosphorylation of AMPKα1 in primary bone marrow derived macrophages culture [151, 275]. IL10 was not able to inhibit TNFα mRNA induction in AMPKα1 knockout macrophages [151, 275]. Interestingly, AMPKα1 deficiency results in elevated LPS-induced TNFα levels regardless of the presence of IL10 [151, 275]. This suggests that AMPKα1 might be basally active even in the absence of IL10, and its presence may restrain LPS action. In fact, AMPKα1 activation state can be controlled by many signals including low glucose levels [288]. In our hands we saw no clear effects of IL10 on AMPK using threonine-172 phosphorylation as a marker of AMPKα1 activation. IL10 led to marginal but non-significant AMPK activation in low glucose conditions and no impact in high glucose (Figure 2.6b). It is possible that the higher

basal levels of phospho-AMPKα1 and elevations in total AMPKα1 protein when cells were cultured in low glucose (Figure 2.6a) may have masked any potential enhancing effects of IL10 treatment.

2.4.3 AQX-MN100, a SHIP1 agonist, can overcome impaired IL10 signaling

AQX-MN100 is a small molecular activator of SHIP1 that has previously been shown to mimic the anti-inflammatory actions of IL10 [163, 283]. Despite showing impaired ability to respond to IL10 under high glucose conditions, AQX-MN100 was equally effective at inhibiting LPS-induced TNFα secretion from macrophages grown in high or low glucose concentration. Thus, high glucose does not impair the intrinsic ability of SHIP1 to inhibit inflammation but the ability of IL10 to activate SHIP1 appears to be impaired.

2.4.4 Limitations

Our results show that IL10 hyporesponsiveness exists in humans with T2D compared to age and BMI-matched non-T2D controls; however, the exact mechanisms contributing to this effect *in vivo* cannot be determined. The parallel cell culture experiments support the notion that hyperglycemia is involved but it is important to acknowledge that differences in medications or other factors between the T2D patients and the non-T2D controls may have impacted the human experiments. Approximately half of the individuals with T2D included in this study (11 out of 24) were taking glucose-lowering medications (either metformin or a combination of metformin with one other drug; see Table 2.1). Exactly how these diabetes medications interact with hyperglycemia to impact IL10 function is not known. However, we feel that studying individuals

on glucose lowering (anti-diabetic) and other medications is more representative of a typical T2D patient. As well, patients did not take their metformin or other drugs on the morning of the fasted blood samples. Thus, the findings of altered IL10 function in individuals with T2D, who displayed evidence of chronic low-grade inflammation based on plasma TNFα and IL6 levels, suggests that IL10 resistance may have clinical relevance across a diverse group of T2D patients. We were not adequately powered to compare differences between T2D patients on different medications in this study but it may be interesting to explore potential interaction between T2D medications and IL10 function in future research. Another limitation of the current study is that we focused on the impact of elevated glucose for the macrophage culture experiments and results were based on N=3 independent experiments performed in duplicate. Future studies exploring how other factors that may be elevated in T2D (e.g., lipids, insulin) impact IL10 responsiveness may provide further insight.

2.5 Summary

The present findings indicate that IL10 hyporesponsiveness or "IL10 resistance" occurs in immune cells from humans with T2D and in macrophages cultured in physiologically-relevant hyperglycemia. Hyporesponsiveness to IL10 does not appear to be mediated by downregulation of the IL10R but there is impaired downstream IL10 signaling under hyperglycemic conditions that can be overcome with the small molecule SHIP1 agonist AQX-MN100. This is the first study to our knowledge implicating diminished anti-inflammatory IL10 functioning in T2D. A more thorough understanding of potential dysfunction in the IL10 signaling pathway in T2D is required to identify key mechanisms and uncover potential therapeutic options that target relieving IL10 resistance in T2D.

Chapter 3 Short-term exercise training reduces anti-inflammatory action of interleukin-10

Manuscript in preparation.

3.1 Background

During obesity, chronic low-grade inflammation plays a pivotal role in the development of insulin resistance, contributing to increased risk of T2D and CVD [289, 290]. Chronic inflammation is propagated by infiltration of immune cells into tissues [181, 182] and production of pro-inflammatory cytokines such as TNFα [291]. Our recent findings indicate that the function of anti-inflammatory cytokines may also be important [292]. In leukocytes isolated from individuals with T2D, the ability of the anti-inflammatory cytokine IL10 to inhibit TNFα production in endotoxin-stimulated whole blood cultures is impaired, suggesting that "resistance" to the normal anti-inflammatory action of IL10 may also contribute to inflammation in obesity-related metabolic disease [292].

Physical activity remains a cornerstone in the prevention and treatment of obesity and related metabolic diseases, with many of its benefits thought to occur through anti-inflammatory mechanisms [208]. Reductions in visceral fat mass, which results in lower pro-inflammatory cytokine/chemokine release and reduced infiltration of immune cells into adipose, is likely one overarching anti-inflammatory mechanisms of regular exercise [223]. Exercise can also directly effect the immune system through alterations in leukocyte phenotype [219, 293] and increased production of skeletal muscle-derived IL6 [209]. IL6 has been characterized as both pro- and anti-inflammatory; however, when released as a myokine during exercise [132, 294] it demonstrates anti-inflammatory properties [89, 108]. The release of IL6 during exercise is

followed by a subsequent increase in anti-inflammatory cytokines, including IL10 [89, 110]. Despite substantial research implicating IL6 and IL10 in immunomodulatory effects of exercise [108, 295], the ability of exercise to impact the anti-inflammatory or immunosuppressive *function* of these cytokines is unclear. Studying how IL6 and IL10 impact immune cell function is important because changes in circulating levels may not reflect how these cytokines impact cellular functions in their local environment where the concentrations are much higher [92, 296].

Although moderate-intensity exercise has demonstrated anti-inflammatory effects, higher volumes or intensities of exercise may be immunosuppressive. For example, elite athletes have been shown to be more susceptible to upper respiratory tract infections [214-216]. This greater susceptibility may be partly explained by greater immune cell production of IL10, which may result in a less effective pathogen-specific immune response [217, 218]. However, less research is available on the immunomodulatory effects of HIIT. HIIT is an alternative approach to standard care MICT and has gained popularity due to its time efficiency. HIIT results in improvements in cardiometabolic health that could reduce the risk for T2D and CVD in both healthy and clinical populations [246, 249, 258] but the impacts of HIIT on inflammation and immune function are less clear. Whether HIIT in obesity or related conditions is anti-inflammatory [238, 240], immunosuppressive [214-216], or even pro-inflammatory [213] still remains largely unknown.

The purpose of this study was to determine how exercise training impacts IL10 and IL6 anti-inflammatory function and further to compare immunomodulatory effects between MICT and HIIT. We tested the hypothesis that short-term HIIT and MICT, in the absence of changes in fat mass, would result in differential effects on IL10 and IL6 immunomodulatory function.

3.2 Methods

3.2.1 Participants

Participants met all eligibility criteria including: between 30 – 65 years of age, inactive (two or less bouts of >30 min moderate-to-vigorous aerobic physical exercise per week; assessed by a standard 7-day physical activity recall interview), completion of the Canadian Society for Exercise Physiology (CSEP) Physical Activity Readiness Questionnaire-Plus (PAR-Q+) and, if required, clearance for participation in vigorous exercise by a CSEP Certified Exercise Physiologist or their physician (PAR-MedX) [297]. Exclusion criteria included uncontrolled hypertension, history of CVD, previous myocardial infarction or stroke, diagnosed diabetes, use of glucose-lowering or immunomodulatory medications, or any contraindications to exercise. This study design was approved by the University of British Columbia Clinical Research Ethics Board and all subjects provided written informed consent. This study represents a subsample of participants from a larger RCT (N=99; https://clinicaltrials.gov; NCT02164474) that was conducted in waves of 10-12 participants each. Participants from the first five waves of the RCT were included in this sub-study if they were classified as obese (BMI > 30 kg/m^2 and/or a WC > 102 cm [men] or 88 cm [women][298] and/or a BF percent > 25% [men] or >30% [women] [299]). Recruitment of the first five waves occurred between June 2014 and September 2014 with the waves starting the program between July 2014 (Wave 1) and February 2015 (Wave 5). A total of 43 participants, who met the inclusion criteria, were randomized to HIIT (n=22) or MICT (n=21). Medication use by these participants was minimal. One participant in the HIIT group was taking an anti-hypertensive medication, whereas in the MICT group, one participant was on transdermal hormone replacement therapy with another participant taking a statin.

3.2.2 Pre-testing

Following an overnight fast (≥ 8 h), blood pressure was measured manually according to the Canadian Hypertension Education Program guidelines [300]. A fasting venous blood sample was obtained by venipuncture from an antecubital vein and was collected into a sodium heparin and an EDTA tube (BD Vacutainer). Blood collected in the sodium heparin tube was kept at room temperature and transported to the laboratory for whole blood culture and flow cytometry analyses. The blood collected in the EDTA tube was kept on ice for 10 – 20 min and then centrifuged at 1550 xg for 15 min at 4°C to isolate plasma, which was then stored at -80°C for further analysis. Height and body mass were measured to the nearest 0.1 cm and 0.1 kg, respectively (Seca 700 Mechanical Column Scale). Body composition was assessed using whole-body-dual energy X-ray absorptiometry (DXA; Discovery A, Hologic, Country) performed by the same technician. Prior to each scan, participants were instructed to remove all jewellery and metal. Results were analyzed using Hologic Discovery software Version 13.4.2 and measurements for total body (lean mass, fat mass, and fat percent) were recorded. Participants then completed a continuous incremental ramp maximal exercise test using an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands). Peak HR (HRpeak), defined as the highest HR achieved, was determined in order to prescribe and monitor the exercise training intensities. The test comprised of a four min warm-up at 30 W with a ramp increase of 15 W/min until either volitional exhaustion or revolutions per min fell below 50.

3.2.3 Training intervention

The training intervention consisted of 10 exercise sessions that were performed from Monday – Friday over two weeks, where Saturday and Sunday were set as rest days. We have previously published using this training protocol demonstrating significant improvements in

fitness and changes in immune cell parameters in the absence of weight loss or changes in body fat [238, 301]. The training intervention for HIIT and MICT was designed as progressive and matched for external work but the exercise intensity for both HIIT and MICT was individualized to each participant using their HRpeak, determined during the baseline maximal fitness test. HIIT consisted of 1 min at ~90% HRpeak interspersed with 1 min recovery periods at a low intensity. Each session of HIIT also included a three min warm-up and two min cool down. HIIT progressed from 4x1 min intervals (day 1) to 10x1 min intervals (day 10) with a total duration of 12 min and 25 min, respectively. Based on our past experience conducing this style of HIIT in many participants [238, 292, 302], the appropriate intensity is determined by a participant achieving 90% of HRpeak by the end of the third interval. Training intensity/effort is modified if the participant is above or below this benchmark. MICT consisted of exercise eliciting ~65% HRpeak progressing from 20 min (day 1) to 50 min (day 10). Days 1 and 10 were completed on a stationary bike with power output closely controlled but the remaining days modality was self-selected by the participant (treadmill walking, stationary cycling, elliptical training or walking outdoors). All supervised sessions were closely monitored for intensity using HR monitors (FT7, Polar, Kempele, Finland). Out of the 10 sessions, three sessions (days 4, 7, and 9) were completed independently by the participant, outside of the laboratory, with confirmation of compliance using HR monitors.

3.2.4 Post-testing

Participants returned for post-testing ~72 h (60 – 74 h) following their final training session. Post-testing was identical to pre-testing.

3.2.5 Blood analyses

3.2.5.1 Plasma cytokines

Concentrations of fasting IL10, IL6 and TNFα were measured using a custom Human High Sensitivity T Cell Panel Multiplex Kit (Cat# HSTCMAG-283K, Millipore, Billerica, MA, USA). Plasma samples were centrifuged at 1000 xg for 15 min at 4°C to pellet any debris. The assay was completed according to the manufacturer's protocol and all samples were measured in duplicate using the MAGPIX Bio-Plex reader (Bio-Rad, Hercules, CA). The intra-assay coefficient of variation (CV) based on duplicates was <5% and the inter-assay CV (based on controls run on each plate) was <3.5%. Cytokine concentrations were determined using the Bio-Plex Manager 6.1 software. The minimal detectable concentration for each analyte (in pg/ml), calculated by the software, was 0.56 (IL10), 0.11 (IL6), and 0.16 (TNFα).

3.2.5.2 Whole blood culture

Whole blood was diluted at 1:10 in serum-free RPMI media (Sigma) containing 5 mM glucose with 50 U/ml penicillin and 50 ug/ml streptomycin. Diluted whole blood (540 ul) was seeded in 24-well plates (Costar). Whole blood cultures were stimulated using LPS (from Escherichia coli 055:B5; L6529, Sigma) at 1 or 10 ng/ml with or without recombinant human IL10 (expressed and made in-house) or recombinant human IL6 (CAT#200-06, Peprotech) (1 ng/ml, 5 ng/ml or 10 ng/ml). The cultures were incubated at 37°C in 5% CO_2 and supernatants were collected at six hours for analyses of secreted TNFα on duplicate samples by ELISA (Human TNFα DuoSet, R&D Systems) according to the manufacturer's instructions. Absorbance was read at 450 nm on an iMark Microplate Absorbance reader (Biorad, CA, USA) and data were analyzed using Microplate Manager 6.0 (Biorad) to calculate TNFα concentrations using a

cubic spline regression model. TNFα concentrations were expressed in pg/ml or as percentage of maximal LPS-induced production. This assay assesses the ability of IL10 (or IL6) to inhibit TNFα production as an index of anti-inflammatory action [162, 296].

In order to measure IL6 and IL10 production from whole blood cultures, concentrations of IL10 and IL6 from supernatants were measured using the Human Th17 Magnetic Bead Panel Multiplex Kit (Cat# HTH17MAG-14K, Millipore). Samples were centrifuged at 1000 xg for 15 min at 4°C to pellet out debris. The assay was completed according to the manufacturer's protocol and all samples were measured in duplicate using the MAGPIX Bio-Plex reader (Bio-Rad, Hercules, CA). The intra-assay CV based on duplicates was <8.2% and the inter-assay CV (based on controls run on each plate) was <9.3%. Cytokine concentrations were determined using the Bio-Plex Manager 6.1 software. The minimal detectable concentration for each analyte (in pg/ml), calculated by the software, was 0.3 (IL10) and 1.7 (IL6). IL10 and IL6 were measured in LPS-stimulated cultures only as production of these cytokines in unstimulated whole blood is undetectable.

3.2.5.3 IL10 and IL6 receptor expression

The surface protein expression of IL10R1 and IL6Ra were measured on different leukocyte subsets using flow cytometry. Within 10 min of sample collection 10 ul of FcR Blocking Reagent (130-059-901; Miltenyi Biotech, Bergisch Gladbach, Germany) was added to 90 ul of whole blood and incubated in the dark for 10 minutes at 4°C. Next, 2 ul of CD14-Vioblue (130-094-364, Miltenyi Biotech), 2 ul CD16-FITC (130-091-244, Miltenyi Biotech), 10 ul CD210-PE (IL10R1; 308804, BioLegend, San Diego, CA) and 5 ul CD126 PE-Vio770 (IL6Ra; 130-104-102, Miltenyi Biotech) were added and further incubated in the dark for 10 minutes at 4°C. After which, 1 ml of red blood cell lysis buffer (120-001-339; Miltenyi Biotech)

was added and then incubated for 15 minutes at room temperature in the dark. Dead cells were excluded from analysis using the addition of 2 ul of PI (130-093-233, Miltenyi Biotech) with analysis performed on a MACSQuant Analyzer flow cytometer (Miltenyi Biotech). Leukocyte populations were determined using established gates based on characteristic forward and side scatter, and staining for common leukocyte markers to establish CD14+ monocytes and CD16+ granulocytes (neutrophils). Fluorescence minus one (FMO) controls were used to establish positive staining of IL10R1 and IL6Ra. A total of 10,000 CD14+ events for each subject were collected for analysis. In a separate panel, monocyte, neutrophil, and T lymphocyte count per ml were also measured by flow cytometry based on characteristic scatter profile and staining for CD14 (CD14 PE-Cy7, 561385; BD Pharmingen), CD16 (APC, 130-091-246; Miltenyi), and CD8 (APC-H7; 561423; BD Pharmingen), respectively. Optimization of the antibody panels were performed prior to the study and compensation was performed prior to analyses to control for fluorochrome spillover. Bank instrument settings were applied in order to standardize drift that may occur in laser strength over time. Leukocyte populations, quantified as count per microliter, and IL10R1 and IL6Ra median fluorescence intensity (MFI) were measured using MACSQuant software (Miltenyi Biotech).

3.2.6 Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 22. Statistical outliers were identified using an interquartile range (IQR) with a multiplier of 2.2 based off the method by Hoaglin, Iglewics and Tukey [303]. The 25^{th} and 75^{th} percentile weighted average values were determined on SPSS and further used to calculate “lower” and “upper” limits with a multiplier of 2.2 IQR. Based on these limits, values that fell outside were deemed to be statistical outliers and thus removed from all analyses. Normality was assessed using Q-Q plots and

Shapiro-Wilks test and when variables did not meet the assumptions of normality, transformation was completed prior to statistical analyses. All descriptive statistics are reported as mean (SD). Two-factor (group X time) mixed ANOVA with repeated measures on time was used to examine changes in anthropometric measures, circulating cytokines, IL10R1 and IL6Ra receptor expression. In order to confirm that IL6 and IL10 were indeed anti-inflammatory in our assays in baseline (pre-training) samples, the inhibition of TNFα production by both IL10 and IL6 was examined using separate two-factor (LPS concentration X IL10 or IL6 concentration) mixed ANOVAs with repeated measures on LPS and IL10 (or IL6) concentration. In order to determine the influence of HIIT or MICT on anti-inflammatory IL6 and IL10 action, a three-factor (group X time X concentration) mixed ANOVA with repeated measures on time and concentration was used to examine changes in LPS-stimulated TNFα production. LPS-induced IL6 and IL10 production before and after training were analyzed similarly with a three-factor (group X time X concentration) mixed ANOVA with repeated measures on time and concentration. Significant interactions were followed with pre-planned contrasts to compare pre vs. post intervention effects within HIIT and MICT separately with a Holm-Sidak correction for multiple comparisons. Level of significance was set at $p \leq 0.05$. Effect sizes were calculated for pairwise comparisons using Cohen's *d.*

Six participants (three HIIT and three MICT) were unable to complete the training intervention or post-testing, five of which dropped out immediately after pre-testing stating "lack of time" and one of which who completed only a portion of the training intervention and did not complete post-testing due to illness. Additionally, technical limitations precluded whole blood culture for four participants, three of which were from the HIIT group and one of which was from the MICT group. Results were analyzed per protocol so these ten participants were not included in the analysis, leaving 16 participants in the HIIT group and 17 in the MICT group. A

formal sample size calculation was not performed for this study as, to our knowledge, the impact of exercise training on the ability of IL10 or IL6 to inhibit inflammation has never been studied. However, a sample size of 16 per group would yield 80% power to detect a medium effect size (Cohen's f of 0.25) at the alpha level of 0.05 assuming a correlation amongst repeated measures of 0.8 (calculated using G*Power v3.0, ANOVA repeated measures, within-between interaction).

3.3 Results

Baseline characteristics of the 33 participants who completed the training intervention and post-testing are shown in Table 3.1.

Table 3.1. Baseline characteristics of participants prior to the training intervention.

Variable	**All** n=33	**HIIT** n=16	**MICT** n=17
Age (years)	47.0 (9.7)	50.4 (7.6)	43.8 (10.6)
Sex, n			
Male	5	2	3
Female	28	14	14
BMI (kg/m²)	32.2 (7.2)	33.3 (8.7)	31.1 (5.3)
WC (cm)	110.2 (17.5)	111.7 (20.7)	108.7 (14.4)
% BF	36.0 (7.0)	36.6 (6.3)	35.4 (7.8)

Values are mean (SD). BMI, body mass index; WC, waist circumference; BF, body fat.

3.3.1 Anthropometrics

There were no differences observed for measures of body composition (all $p>0.05$; Table 3.2) after HIIT or MICT.

Table 3.2. Measures of body composition before and after two weeks of HIIT and MICT.

Variable	HIIT			MICT			P-Value	
	Pre	Post	N	Pre	Post	N	Time	Interaction
BMI (kg/m²)	33.3 (8.9)	33.3 (8.6)	16	31.1 (5.3)	30.9 (5.1)	17	0.234	0.393
% BF	36.6 (6.3)	36.5 (6.4)	16	35.4 (7.8)	35.3 (7.8)	17	0.591	0.861
Total fat mass (kg)	34.4 (14.4)	34.3 (14.1)	16	31.4 (11.2)	31.3 (11.4)	17	0.637	0.854
Total lean mass (kg)	55.4 (14.4)	55.4 (14.0)	16	53.7 (10.1)	53.9 (10.0)	17	0.771	0.666

Values are mean (SD). BMI, body mass index; BF, body fat.

3.3.2 Plasma cytokines

No changes were observed in circulating plasma cytokines after HIIT or MICT (Table 3.3). Three plasma samples, two of which were from HIIT and one was from MICT, were not analyzed due to technical limitations during blood sampling. An additional two samples from HIIT were not included in the analysis for plasma IL10 as one was out of range (OOR)/undetectable and one was an outlier (determination of outlier as described in the Statistical Analysis).

Table 3.3. Circulating cytokine levels before and after two weeks of HIIT and MICT.

Variable	HIIT			MICT			P-Value	
	Pre	Post	N	Pre	Post	N	Time	Interaction
IL10 (pg/ml)	6.8 (5.3)	6.3 (5.2)	12	4.7 (2.5)	4.1 (2.6)	16	0.366	0.519
IL6 (pg/ml)	3.0 (2.0)	2.9 (2.5)	14	1.9 (0.9)	1.8 (0.6)	16	0.370	0.669
TNFα (pg/ml)	8.2 (2.7)	8.1 (3.0)	14	7.9 (3.4)	7.2 (2.0)	16	0.558	0.844

Values are mean (SD).

3.3.3 IL10 and IL6 anti-inflammatory action

We examined the ability of IL10 and IL6 to inhibit LPS-induced TNFα production before and after training as a measure of IL10 or IL6 anti-inflammatory action. Whole blood cultures were used as this model minimizes sample processing and retains physiological conditions (i.e. plasma constituents and cellular composition) relevant to in vivo conditions [279]. Using this

model, we have previously shown that IL10 inhibits LPS-induced TNFα production in individuals with T2D and age- and BMI-matched controls [296]. In the current study, we confirmed this anti-inflammatory action of IL10 and, given the ambiguity around the pro- vs. anti-inflammatory impact of IL-6 [92, 97, 108], tested the ability of IL6 to exert anti-inflammatory actions in this assay. In baseline samples, a two-way mixed ANOVA with repeated measures on LPS (1 ng/ml, 10 ng/ml) and IL10 (0 ng/ml, 1 ng/ml, 5 ng/ml, 10 ng/ml) concentration indicated a main effect of IL10 ($p<0.001$) when TNFα was expressed as either an absolute concentration in pg/ml or a percentage of maximal LPS-induced production (Figure 3.1). There was a dose-response inhibition of TNFα production with increasing concentrations of IL10 in cultures stimulated with 1 ng/ml and 10 ng/ml LPS, when expressed in absolute or as a percentage of maximal LPS-induced production.

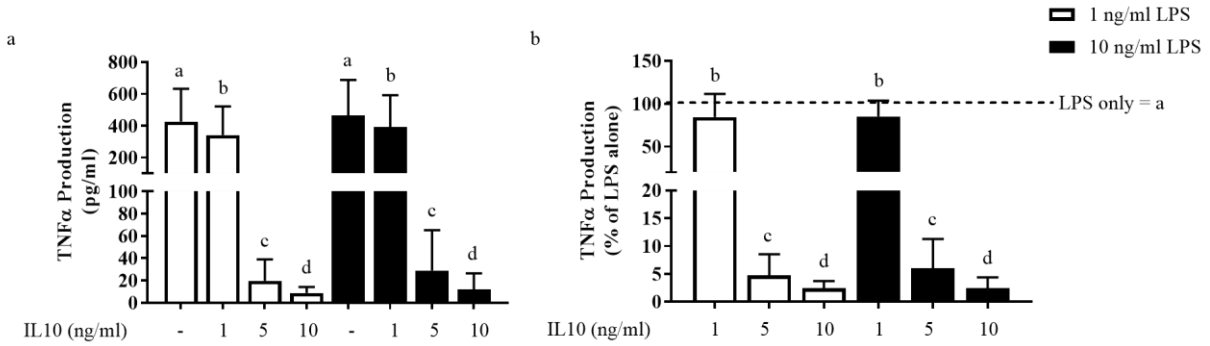

Figure 3.1. IL10 inhibits LPS-induced TNFα production. Baseline (pre-training) whole blood cultures were stimulated with LPS (1 ng/ml or 10 ng/ml) and IL10 (0, 1, 5, and 10 ng/ml). LPS-induced TNFα production was measured in the cultures and expressed as (a) absolute concentration and (b) percentage of maximal LPS-induced production. The overall ANOVA revealed a main effect of IL10 ($P<0.001$). Treatments without a common superscript are significantly different ($p<0.05$) within each respective concentration of LPS.

Using IL6 in this same assay setup, we next established whether IL6 inhibited LPS-induced TNFα production. As shown in Figure 3.2, the effect of IL6 was less potent than IL10 and differed dependent on LPS concentrations. A two-way mixed ANOVA with repeated measures demonstrated a main effect of LPS ($p=0.001$) and IL6 ($p<0.001$) and a LPS X IL6 interaction ($p=0.002$) on absolute TNFα production. Post-hoc tests indicated higher TNFα production at all IL6 concentrations when treated with 10 ng/ml LPS, compared to 1 ng/ml LPS, when expressed as either an absolute concentration or a percentage of maximal LPS-induced production. It can also be seen that overall, the degree of anti-inflammatory action exerted by equal concentrations of IL6 (Figure 3.2) is much less than IL10 (Figure 3.1).

There was a dose-response inhibition of TNFα production with increasing concentrations of IL10 in cultures stimulated with 1 ng/ml and 10 ng/ml LPS, when expressed in absolute or as a percentage of maximal LPS-induced production. However, there was no difference in reduction in absolute concentration between 0 ng/ml and 1 ng/ml IL6 (10 ng/ml LPS), and no difference in reduction in percentage of maximal LPS-induced production between 1 ng/ml and 5 ng/ml IL6 (1 ng/ml LPS), or between 0 ng/ml and 1 ng/ml IL6 (10 ng/ml LPS).

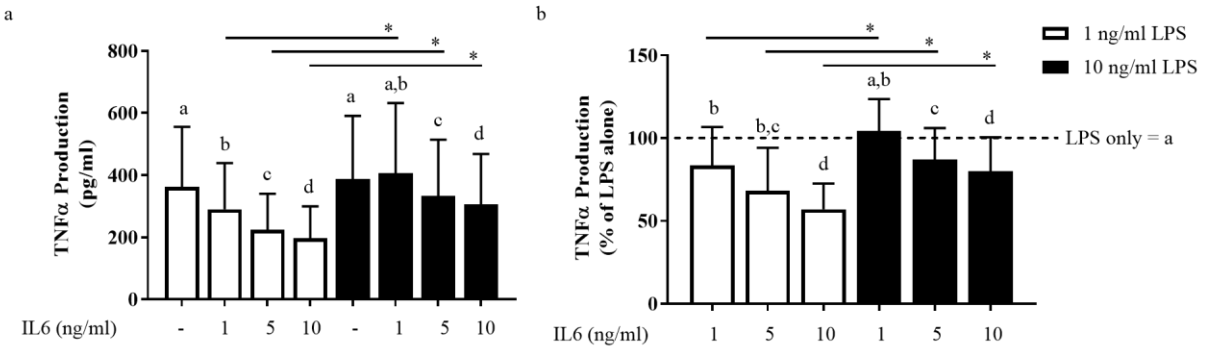

Figure 3.2. IL6 inhibits LPS-induced TNFα production. At baseline, whole blood cultures were stimulated with LPS (1 ng/ml or 10 ng/ml) and IL6 (0, 1, 5, and 10 ng/ml). LPS-induced TNFα production was measured in the cultures and expressed as (a) absolute concentration and (b) percentage of maximal LPS-induced production. The overall ANOVA revealed a main effect of IL6 ($P<0.001$). There was a main effect of LPS ($p<0.001$) and a LPS X IL6 interaction ($p=0.002$) on absolute TNFα production. Similarly, there was a main effect of LPS ($p=0.001$) and a LPS X IL6 interaction ($p=0.007$) on percentage of maximal LPS-induced production. Treatments without a common superscript are significantly different ($p<0.05$) within each respective concentration of LPS, *$p<0.05$ indicates significance between 1 ng/ml and 10 ng/ml LPS at each respective concentration of IL6.

3.3.4 Impact of exercise training on IL10 function

To determine how exercise training impacted IL10 function, we examined the ability of IL10 to inhibit LPS-induced TNFα production from whole blood cultures prepared from participants before (pre) and after (post) two weeks of HIIT or MICT (Figure 3.3). Whole blood cultures were stimulated with LPS (1 and 10 ng/ml) +/- IL10 (1, 5, and 10 ng/ml).

When expressed as an absolute concentration, treatment with 1 ng/ml LPS resulted in a main effect of time ($p=0.007$) and IL10 ($p<0.001$) with a time X IL10 interaction ($p=0.009$) (Figure 3.3a). Post-hoc tests indicated that absolute TNFα production, at both 1 and 5 ng/ml IL10 was significantly higher ($p=0.007$, $d=1.40$ and $p=0.028$, $d=1.05$, respectively) following HIIT and at 5 ng/ml IL10 ($p=0.050$, $d=0.94$) following MICT. When TNFα absolute concentration was expressed as a percentage of maximal LPS (1 ng/ml)-induced production, there was a main

effect of time (p=0.024) and IL10 (p<0.001) with a time x IL10 interaction (p<0.001) (Figure 3.3b). At 5 ng/ml IL10, TNFα was higher following MICT (p=0.028, d=1.57).

TNFα absolute production following treatment with 10 ng/ml LPS resulted in a main effect of time (p<0.001) and IL10 (p<0.001) with time X IL10 (p=0.031) and time X condition (p=0.030) interactions (Figure 3.3c). Post-hoc tests indicated that at both 1 and 5 ng/ml IL10 (p=0.032, d=1.12, and p=0.031, d=1.30, respectively) TNFα production was higher following HIIT but not MICT (1 ng/ml IL10: p=0.772, d=0.08; 5 ng/ml IL10: p=0.623, d=0.43). When TNFα absolute concentration was expressed as a percentage of maximal LPS (10 ng/ml)-induced production, there was a main effect of time (p=0.003) and IL10 (p<0.001) and a time X IL10 interaction (p=0.020) (Figure 3.3d). Post-hoc tests did not reveal any significant differences between pre and post at any IL10 concentration for either HIIT or MICT; however, the time X IL10 interaction indicates that there are differential effects of IL10 pre and post exercise training. Overall, these data indicate that short-term training reduced the ability of IL10 to inhibit LPS-induced TNFα production, with greater effects seen after HIIT.

The production of IL10 in whole blood cultures treated with LPS was not significantly different between pre and post exercise training (main effect of time, P=0.514; time X group interaction, P=0.653; main effect of LPS, P<0.001; LPS X group interaction, P=0.535; time X LPS interaction, P=0.086; time X LPS X group interaction, P=0.318). IL10 production [ng/ml] pre vs. post exercise training was as follows: 1 ng/ml LPS: HIIT, 2.36±1.36 vs. 2.29±1.04, N=14, d=0.03; MICT, 2.04±1.07 vs. 2.01±0.92, N=12, d=0.03; ALL, 2.21±1.22 vs. 2.16±0.98, N=26, d=0.03; and 10 ng/ml LPS: HIIT, 2.58±1.28 vs. 2.99±1.24, N=14, d=0.38; MICT, 2.49±1.22 vs. 2.51±0.94, N=12, d=0.17; ALL, 2.53±1.23 vs. 2.77±1.12, N=26, d=0.44).

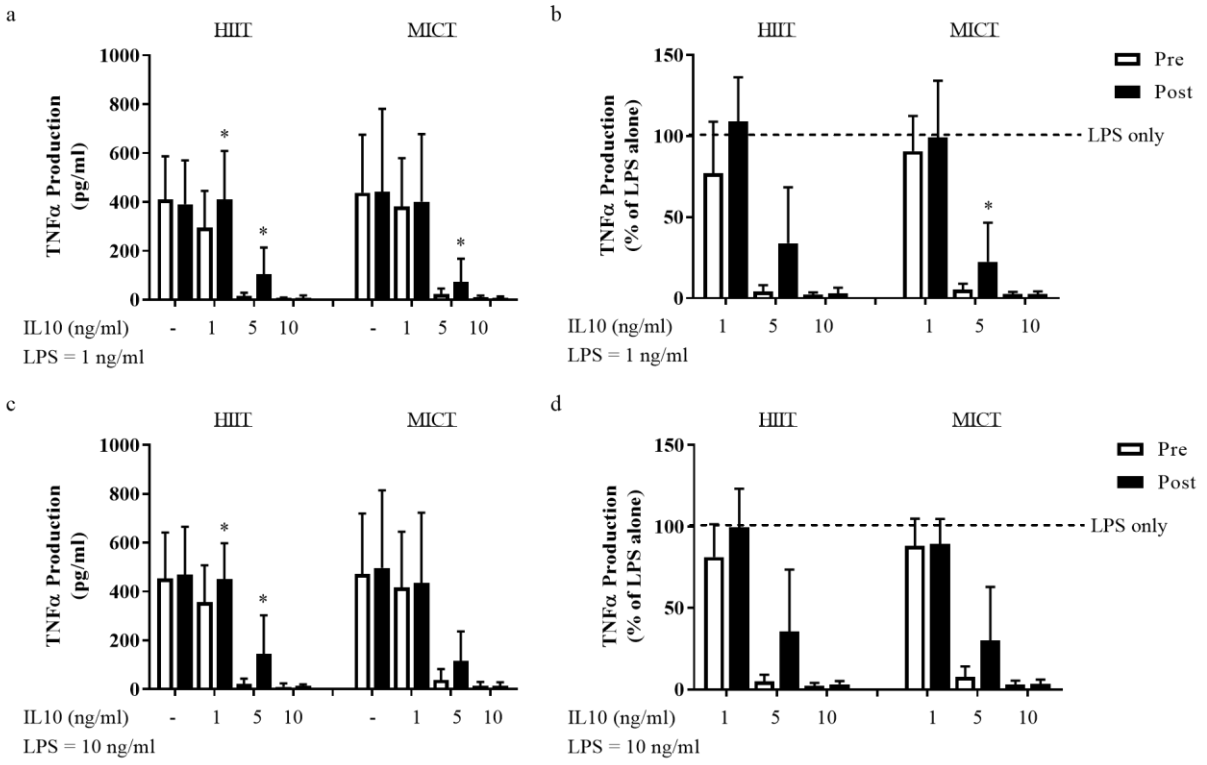

Figure 3.3. IL10 function is impaired following exercise training with greater effects seen with HIIT. Whole blood cultures were prepared from participants before (pre) and after (post) two weeks of HIIT or MICT and were treated with LPS (1 ng/ml or 10 ng/ml) and IL10 (0, 1, 5, and 10 ng/ml). LPS-induced TNFα production was measured in the cultures and expressed as (a, c) absolute concentration and (b, d) percentage of maximal LPS-induced production, following treatment with 1 ng/ml and 10 ng/ml LPS. *$p<0.05$ compared to pre within group at the same IL10 concentration.

3.3.5 Impact of exercise training on IL6 function

To determine how exercise effects IL6 function, we examined the ability of IL6 to inhibit LPS-induced TNFα production from whole blood cultures prepared from participants pre and post two weeks of HIIT or MICT (Figure 3.4). Whole blood cultures were stimulated with LPS (1 and 10 ng/ml) +/- IL6 (1, 5, and 10 ng/ml).

When expressed as an absolute concentration, treatment with 1 ng/ml LPS resulted in a main effect of IL6 ($p<0.001$) with a time X IL6 interaction ($p=0.048$) (Figure 3.4a). Post-hoc

tests did not indicate any significant pairwise comparisons within either HIIT or MICT. When TNFα absolute concentration was expressed as a percentage of maximal LPS (1 ng/ml)-induced production, there was a main effect of time ($p=0.010$) and IL6 ($p<0.001$) with time X IL6 ($p=0.049$) and IL6 X group ($p=0.037$) interactions (Figure 3.4b). There were no significant pairwise comparisons within either HIIT or MICT following post-hoc tests. No significant effects, other then a main effect of IL6 ($p<0.001$), occurred for treatment with 10 ng/ml LPS for either TNFα absolute concentration (Figure 3.4c) or TNFα expressed as a percentage of maximal LPS-induced production (Figure 3.4d).

The production of IL6 in whole blood cultures treated with LPS was not significantly different between pre and post exercise training (main effect of time, $p=0.219$; time X group interaction, $p=0.559$; main effect of LPS, $p<0.001$; LPS X group interaction, $p=0.341$; time X LPS interaction, $p=0.997$; time X LPS X group interaction, $P=0.024$). The three way interaction was followed up with a two-factor (group X time) mixed ANOVA with repeated measures on time completed separately for each LPS concentration with no significance between pre and post exercise training (1 ng/ml LPS: main effect of time, $p=0.225$, time X group interaction, $p=0.901$; and 10 ng/ml LPS: main effect of time, $p=0.250$, time X group interaction, $p=0.232$). IL6 production [ng/ml] pre vs. post exercise training was as follows: 1 ng/ml LPS: HIIT, 1248±544 vs. 1377±699, $d=0.21$; MICT, 1346±736 vs. 1488±867, $d=0.29$; ALL, 1299±640 vs. 1435±778, $d=0.25$; and 10 ng/ml LPS: HIIT, 1368±627 vs. 1633±771, $d=0.43$; MICT, 1529±778 vs. 1555±981, $d=0.01$; ALL, 1451±701 vs. 1592±870, $d=0.22$.

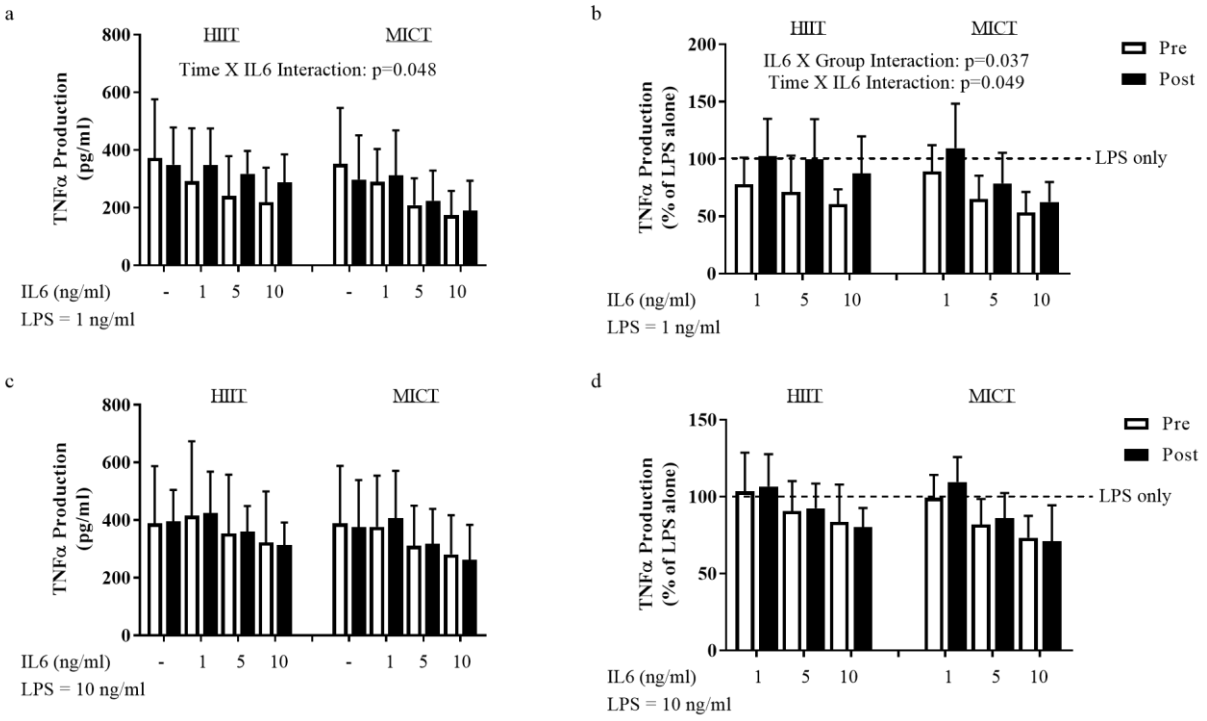

Figure 3.4. IL6 function is differentially affected by HIIT and MICT following exercise training. Whole blood cultures were prepared from participants before (pre) and after (post) two weeks of HIIT or MICT and were treated with LPS (1 ng/ml or 10 ng/ml) and IL6 (0, 1, 5, and 10 ng/ml). LPS-induced TNFα production was measured in the cultures and expressed as (a, c) absolute concentration and (b, d) percentage of maximal LPS-induced production, following treatment with 1 ng/ml and 10 ng/ml LPS.

3.3.6 Impact of exercise on IL10 and IL6 receptor expression

Surface protein expression of IL10R1 and IL6Ra on CD14+ monocytes, CD16+ granulocytes, and lymphocytes were measured using flow cytometry. There were no significant differences detected in either IL10R1 or IL6Ra MFI for either CD14+ monocytes, CD16+ granulocytes, or lymphocytes (Table 3.4).

Table 3.4. IL10R1 and IL6Ra expression on leukocytes.

Variable	HIIT			MICT			P-Value	
	Pre	Post	N	Pre	Post	N	Time	Interaction
CD14+ Monocytes								
IL10R1 MFI	4.28 (1.07)	4.00 (0.87)	14	4.05 (0.47)	4.02 (0.66)	17	0.247	0.359
IL6Ra MFI	6.78 (1.34)	6.97 (1.33)	15	7.00 (1.00)	6.85 (1.18)	17	0.940	0.455
CD16+ Granulocytes								
IL10R1 MFI	5.12 (0.54)	5.13 (0.83)	14	4.97 (0.63)	5.35 (0.62)	17	0.151	0.183
IL6Ra MFI	5.43 (0.83)	5.74 (0.97)	15	5.59 (0.72)	5.55 (0.69)	17	0.362	0.253
Lymphocytes								
IL10R1 MFI	3.16 (0.24)	3.04 (0.33)	14	3.32 (0.47)	3.29 (0.42)	17	0.460	0.677
IL6Ra MFI	3.49 (0.30)	3.40 (0.36)	15	3.50 (0.28)	3.47 (0.27)	16	0.311	0.593

Values are mean (SD).

HIIT or MICT did not result in significant differences in CD14+ monocytes, CD16+ granulocytes, or lymphocytes cell numbers measured by flow cytometry (Table 3.5).

Table 3.5. Leukocyte cell number ($x10^3 \cdot \mu l^{-1}$ blood).

Variable	HIIT			MICT			P-Value	
	Pre	Post	N	Pre	Post	N	Time	Interaction
White blood cells	3.05 (0.97)	3.16 (1.28)	14	2.56 (0.52)	2.47 (0.63)	15	0.497	0.573
CD14+ Monocytes	0.27 (0.08)	0.29 (0.11)	14	0.24 (0.11)	0.22 (0.08)	16	0.920	0.280
Neutrophils	2.45 (0.87)	2.52 (1.16)	14	2.02 (0.47)	1.91 (0.59)	15	0.866	0.342
T Cells	0.34 (0.18)	0.35 (0.23)	14	0.33 (0.11)	0.36 (0.13)	16	0.259	0.697

Values are mean (SD).

3.4 Discussion

This study suggests that short-term exercise, in the absence of weight or fat loss, influences anti-inflammatory IL10 and IL6 function in obese, previously inactive adults. Although both HIIT and MICT resulted in a reduced ability of IL10 to inhibit LPS-induced TNFα production, the effect of HIIT appeared more substantial. The effect of short-term training on IL6 function was less clear. Overall, these findings indicate that short-term training, and in particular HIIT, has a direct influence on IL10 anti-inflammatory function.

3.4.1 IL10 and IL6 inhibit LPS-stimulated TNFα production

Our results confirmed that both IL10 and IL6 were able to reduce LPS-stimulated TNFα production. This was important given the mixed results on whether IL6 is a pro- or anti-inflammatory cytokine [97, 108]. The anti-inflammatory action of IL6 to inhibit LPS-induced TNFα production has been shown in a human monocyte cell line (U937) and cultured human monocytes [107, 304, 305] but the majority of studies measure circulating IL6 as an index of a pro-inflammatory state [306]. Our results indicate that IL10 action, or its ability to reduce LPS-stimulated TNFα, was more consistent and effective than IL6. The response to IL10 was similar at both 1 and 10 ng/ml LPS and TNFα production was almost completely blocked at 10 ng/ml IL10. However, although IL6 showed an inhibition of LPS-stimulated TNFα at several concentrations of IL6, the overall inhibition was much lower than equal concentrations of IL10, and IL6 reduced LPS-stimulated TNFα production to a greater extent at 1 ng/ml LPS compared to 10 ng/ml LPS. Both IL10 and IL6 are known to activate STAT3 and induce STAT3-regulated genes, some of which are implicated in inhibiting TNFα production [149, 159]. However, IL10 also engages the SHIP1 pathway, as shown by Chan et al. [162] to potently inhibit TNFα production by a mechanism involving MAP kinase-interacting serine/threonine-protein kinase 1 signaling that causes a suppression of TNFα mRNA translation by rapidly shifting existing TNFα mRNA from polysomes to monosomes. These findings support that both IL10 and IL6 are anti-inflammatory cytokines that can directly inhibit LPS-stimulated TNFα production; however, IL10 shows greater anti-inflammatory effects.

Although evidence shows that exercise training can influence immune cell markers and inflammation, no studies to our knowledge have assessed IL10 or IL6 function following exercise training. Using a similar model of LPS-stimulated cytokine secretion from whole blood cultures, a study by Timmerman et al. has shown that longer periods (12 wk) of exercise training,

which included 20 min of endurance exercise at 70-80% heart-rate reserve and two sets of eight resistance exercises at 70-80% of one repetition maximum, can reduce TNFα production [243]. We did not see an influence on LPS-induced TNFα production following two weeks of HIIT or MICT, similar to our previously reported findings [238]. These findings support the notion that longer duration exercise training is needed to see reductions in LPS-induced TNFα production.

3.4.2 Short-term training reduces IL10 function with a greater effect seen with HIIT

Despite no effect on LPS-induced TNFα production, we demonstrate that short-term exercise training, and in particular HIIT, have immunomodulatory effects. Specifically, the ability of IL10 to inhibit TNFα production was reduced following two weeks of short-term exercise training of both MICT and HIIT. Although both training modalities impaired IL10 anti-inflammatory action, the effects of HIIT were more consistent. Our results demonstrated that both MICT and HIIT impaired IL10 function when whole blood cultures were treated with 1 ng/ml LPS; however, HIIT, but not MICT, reduced the ability of IL10 to inhibit LPS-induced TNFα production when whole blood cultures were treated with 10 ng/ml LPS (Figure 3.3c). This reduction in anti-inflammatory action, or "IL10 resistance" [296] did not appear to be explained by reduced surface protein expression of the IL10R1. There were also no changes in CD14+ monocytes, CD16+ granulocytes, or lymphocytes, all of which have been shown to produce IL10 [136]. It is possible that downstream intracellular signaling, either through the STAT3 or SHIP1 pathway, were influenced by HIIT but uncovering these mechanisms will require additional research.

3.4.3 The impact of short-term training on IL6 function was less clear

The effect of short-term training on the ability of IL6 to inhibit LPS-induced TNFα production was not as readily apparent as the IL10 findings. Although there were significant time X IL6 and IL6 X group interactions when treated with 1 ng/ml LPS (Figures 3.4a, and 3.4b), post-hoc tests did not reveal differences between pre- and post-training with either HIIT or MICT; however, interpretation of these interactions (Figures 3.4a & 3.4b) does seem to indicate that HIIT and MICT differentially affected the ability of IL6 to inhibit LPS-induced TNFα production. HIIT seemed to follow the same trend as IL10, with IL6 appearing less effective at inhibiting LPS-induced TNFα production after training (Figures 3.4a & 3.4b).

When whole blood cultures were treated with 10 ng/ml LPS there was no effect of short-term training on IL6 function. It may be possible that any effects of exercise training on IL6 function are lost at higher, more potent, concentrations of LPS. These results make sense as we did observe that IL6 was less effective at inhibiting TNFα at 10 ng/ml, as opposed to 1 ng/ml LPS; at a higher concentration of LPS, IL6 may become less effective and therefore less likely to be modulated by exercise training. There were no changes in the surface protein expression of IL6Ralpha, therefore the differential effects of HIIT and MICT on IL6 function were not explained by altered receptor expression.

3.4.4 Short-term training does not alter circulating cytokines

In the current study, we observed no changes in circulating plasma IL6, IL10 or TNFα following two weeks of either HIIT or MICT. Although acute exercise is known to increase plasma IL6 with a subsequent increase in IL10 [132], the effect of short-term exercise training on fasting levels of circulating IL6, IL10 and TNFα have shown mixed results. For example, no changes in plasma IL6 or TNFα were detected following three weeks of HIIT in

overweight/obese sedentary males [307] and our previous work indicates no impact of two weeks of HIIT or MICT on fasting plasma IL6, IL10, or TNF [238]. Additionally, no change in IL6 was detected following four weeks of aerobic/strength training in individuals with IGT or T2D [308]. Conversely, longer-term studies have reported that fasting IL10 may be increased following exercise training. Kadoglou et al. showed an increase in IL10 in overweight individuals with T2D following six months of aerobic exercise [309] and similarly after six months of HIIT, IL10 was increased in heart disease patients [310]. Other studies demonstrated increased IL10 following eight weeks of training in sedentary adults [311] and in patients following an acute myocardial infarction [312]. The mechanism(s) underlying the increase in fasting IL10 in these longer-term studies is not readily apparent. Given the relative "resistance" to IL10 seen following short-term HIIT in the current study it would be interesting to determine whether the increase in plasma IL10 seen in these longer-term studies occurs as a subsequent compensatory response to immune cell IL10 resistance.

3.4.5 Limitations

The short-term nature of our exercise training was purposefully selected in order to avoid any possible confounding effects of weight loss or body composition changes, with DXA results confirming no changes in these variables. Therefore the effects of exercise on IL10 and IL6 function are reflective of the initial adaptive response to exercise and future work is required to determine the long-term impacts of exercise training on IL6 and IL10 function.

Our previous study demonstrated an impaired function of IL10 or "IL10 resistance" in individuals with T2D [296]. In our current study, we explored this concept in obese individuals at high risk of developing T2D instead of individuals with T2D. The main reason for excluding patients with T2D from the current study was to reduce the confounding effects of glucose- and

lipid-lowering medications, many of which can have off-target effects on inflammatory signaling pathways [313]. It will be interesting in future work to determine if the influence of exercise training on anti-inflammatory cytokine function is different depending on disease status.

Due to the unequal representation of males and females, with 28 out of the 33 participants being female, we were unable to explore sex differences in the present study. Of the 28 female participants, 12 (43%) were premenopausal, three (11%) perimenopausal and 13 (46%) were postmenopausal. Within our study, we did not standardize the menstrual phase of each female participant for the pre-testing and post-testing because our study protocol required the testing measures to be taken ~23 days apart based on the study design. Specifically, pre-testing was completed on a Friday one week prior to the start of the training intervention and post-testing was completed on the Monday following completion of the training intervention. Within a randomized study it would be unlikely that systematic differences in the menstrual cycle for pre-testing and post-testing would occur; however, we acknowledge that menopausal status or menstrual cycle was not specifically controlled in this study.

3.4.6 Considerations

The findings of reduced ability of IL10 to inhibit LPS-induced TNFα production after training clearly demonstrate immunomodulatory effects of exercise, particularly HIIT, in previously inactive obese adults. However, it is unclear whether these effects represent an impairment in anti-inflammatory ability, a potential increase in pro-inflammatory potential, and/or an immune-enhancing response. Effective inhibition of TNFα production by anti-inflammatory cytokines (i.e., IL10 and IL6) is hypothesized to help limit chronic inflammation in obesity [296] and reduced ability after HIIT might be viewed in this light as a potentially negative pro-inflammatory response. Alternatively, increased TNFα production in the presence

of higher IL10 or IL6 could contribute to greater innate immune responses to pathogens to enhance the ability to fight off infection. Further studies will be needed to understand the clinical significance of reduced IL10 and IL6 action following interval and continuous exercise.

3.5 Summary

To our knowledge, this is the first study to explore how exercise impacts IL10 or IL6 *function* and our findings suggest that HIIT and MICT may differentially impact anti-inflammatory cytokine action. Short-term training, and in particular HIIT, reduces the ability of IL10 to LPS-induced TNFα production. Although HIIT and MICT differentially affected IL6 function, their specific impact was less clear. As neither HIIT nor MICT impacted fasting plasma concentrations of IL10, IL6 or TNFα, the results indicate that future studies exploring exercise immunology should consider how anti-inflammatory cytokines function instead of simply measuring their circulating concentrations.

Chapter 4 Short-term exercise training alters leukocyte chemokine receptors in obese adults

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4.1 Background

Obesity continues to be a worldwide health concern. In the United States, the prevalence of obesity in adults has reached 36.5% [314]. Obesity is a primary risk factor that drives the development of insulin resistance, which contributes to T2D and CVD. Chronic low-grade inflammation plays a key role in the development of insulin resistance and its complications [289]. The inflammatory environment in obesity is ultimately driven by activation of immune cells, including monocytes/macrophages, T cells, and neutrophils [27, 179]. Obesity is associated with higher levels of circulating monocytes [180] and greater infiltration of macrophages and T cells into adipose and other organs/tissues [27, 181, 182].

Leukocytes are recruited to sites of inflammation by chemotactic cytokines known as chemokines [173]. Chemokines implicated in immune cell infiltration into adipose and other tissues in obesity include CCL2 (also known as MCP1), CCL3 (also known as MIP1α), and CXCL8 (also known as IL8) [181, 315]. Specific chemokine receptors, differentially expressed on the surface of leukocytes, facilitate migration of immune cells into tissues along a chemokine gradient [173]. The importance of leukocyte chemotaxis in driving obesity and T2D is highlighted by findings showing that various chemokine or chemokine receptor knock-out models are protected from HFD-induced obesity and insulin resistance [69, 183]. CCL2, via interaction with its receptor CCR2, is believed to be the major chemokine involved in obesity-associated macrophage infiltration into adipose [181], which is supported by studies in humans

that show elevated CCL2 and CCR2 in obesity [184, 185]. Although leukocytes appear to express multiple chemokine receptors in order to respond to various different chemokines [173], some chemokine-chemokine receptor interactions appear more important for specific leukocyte subsets. For example, higher CCR2 expression has been shown on CD14+/CD16- classical monocytes when compared to CD14+/CD16+ monocytes [186], suggesting a greater role for CCL2 in migration of CD14+/CD16- monocytes. Furthermore, even though monocytes express CCR5 and CXCR2, CCL3-CCR5 interactions may also be influential in mediating chemotaxis of T cells, whereas CXCL8 primarily mediates neutrophil chemotaxis through interaction with CXCR2 [173].

Physical activity is a cornerstone in the management, prevention, and treatment of obesity and its related comorbidities. The anti-inflammatory effects of MICT are hypothesized to play a major role in the health benefits of exercise, particularly in the context of chronic low-grade inflammation associated with obesity [208]. Decreased visceral fat mass likely contributes to some of the anti-inflammatory effects of exercise training due to lower pro-inflammatory adipokine and chemokine secretion, which may be linked to reduced number of pro-inflammatory macrophages and other immune cells within adipose [208, 223]. Animal studies also suggest that regular exercise training can prevent infiltration of monocytes/macrophages into adipose tissue during the development diet-induced obesity [224]. However, whether the anti-inflammatory effects are due to a direct effect of exercise impacting monocyte migration or are secondary to weight/fat loss are not clear [208, 226].

Recently, HIIT has gained attention for its ability to elicit cardiometabolic benefits, often in a time-efficient manner [316]. HIIT involves brief, repeated bursts of vigorous exercise separated by periods of rest. HIIT represents an alternative approach to traditional MICT. HIIT has been shown to promote adaptations similar, or superior, to MICT in healthy and clinical

populations [316]. However, the potential for HIIT, as opposed to MICT, to have anti-inflammatory effects are much less studied. In an initial study, Robinson et al. [317] found that both HIIT and MICT led to reductions in monocyte TLR4 expression but only MICT led to reductions in neutrophil TLR4 in overweight and obese adults, suggesting that HIIT and MICT might have different impacts on receptor expression on immune cells. Other research has argued that HIIT may have pro-inflammatory effects because an acute session of HIIT led to an increase in pro-inflammatory cytokines, including the chemokines CCL2 and CXCL8, in young healthy men [213]. Therefore, despite potential improvements in cardiometabolic health markers [316], it is unclear whether HIIT has the same anti-inflammatory potential as MICT, particularly in the context of obesity.

The purpose of this study was to determine the impact of HIIT versus MICT on circulating chemokine levels and expression of chemokine receptors on leukocytes. We employed short-term training modeled after previous studies (30) in attempts to isolate the direct effects of exercise training in the absence of weight loss or changes in body composition. Based on our recently published work showing evidence of potentially greater anti-inflammatory effect of MICT on leukocytes [317], we hypothesized that HIIT and MICT would differentially modulate levels of chemokine and chemokine receptors.

4.2 Methods

4.2.1 Experimental design

This study represents a sub-study from a larger RCT examining cardiometabolic adaptions and adherence to HIIT versus MICT in individuals at elevated risk of developing T2D (NCT02164474). After participants were screened and deemed eligible, they were randomized

into two groups: HIIT or MICT. A research assistant accessed a password-protected website to retrieve the randomization, which was based on variable permuted blocks. After randomization, both groups underwent the same experimental protocol, which consisted of: i) baseline (pre) testing, ii) a 10-session training intervention over a two-week duration, and iii) post-testing ~72 h following the final training session in order to avoid confounding effects of acute exercise.

4.2.2 Participants

Eligibility criteria included: inactive (two or less aerobic bouts of >30 min moderate-to-vigorous physical exercise per week; assessed by a standard 7-day physical activity recall interview), between the ages of 30–65 years, completion of the CSEP Physical Activity Readiness Questionnaire-Plus (PAR-Q+) and, if required, cleared for participation in vigorous exercise by a CSEP Certified Exercise Physiologist or their physician (PAR-MedX) [297]. Exclusion criteria included history of CVD, uncontrolled hypertension, previous myocardial infarction or stroke, diagnosed diabetes, taking glucose-lowering or immunomodulatory medications, or any contraindications to exercise. This study design was approved by the University of British Columbia Clinical Research Ethics Board and all subjects provided written informed consent.

The larger RCT was conducted in waves of 10-12 participants each. For the present sub-study on the impact of HIIT versus MICT on chemokines and chemokine receptors, sample size was calculated a priori using data from Krinninger et al. [185] to determine the smallest meaningful difference in CCR2 expression on CD14+/CD16+ monocytes. Using data from Krinninger et al., we calculated an effect size of 0.93 as the difference between lean and obese females and defined this as the smallest meaningful difference. Using means and SD for CCR2

expression on CD14+/CD16+ monocytes from an optimized antibody panel from data collected on our flow cytometer (n=24) a sample size of 17 per group was needed with 80% power and alpha of 0.05, assuming a correlation between repeated measures of r=0.5 (Calculated using G-Power version 3.1.9.2). To account for ~20% dropout or missing blood samples we aimed to recruit at least 42 (n=21 per group) obese participants from the larger RCT.

Participants who were classified as obese (BMI > 30 kg/m2 and/or a WC > 102 cm [men] or 88 cm [women] [298] and/or a body fat (BF) percent > 25% [men] or >30% [women]) from the first five waves of the RCT were included in this sub-study. Recruitment occurred between June 2014 and September 2014 with the five waves starting the program between July 2014 (Wave 1) and February 2015 (Wave 5). A total of 43 participants met the inclusion criteria and were randomized to HIIT (n=22) or MICT (n=21). Medication use in the sample was minimal. One participant from the HIIT group was taking an anti-hypertensive medication. In the MICT group, one participant was taking a statin and another was on transdermal hormone replacement therapy.

4.2.3 Pre-testing

After an overnight fast (≥ 8 h), manual blood pressure was measured according to the Canadian Hypertension Education Program guidelines [300] and a fasting blood sample, collected into two separate EDTA-coated Vacutainer (BD) tubes, was obtained from an antecubital vein. Whole blood from one EDTA tube was kept at room temperature and transported to the laboratory flow cytometry analyses. The remaining blood was kept on ice for 10-20 minutes before being centrifuged at 1550 xg for 15 min at 4°C to isolate plasma, which was stored at -80°C for further batch analysis. Body mass and height were measured to the

nearest 0.1 kg and 0.1 cm, respectively (Seca 700 Mechanical Column Scale). Body composition was measured by whole-body DXA (Discovery A, Hologic, Country) and analyzed using Hologic Discovery software Version 13.4.2. Participants were instructed to remove all jewellery and metal prior to DXA scans, which were all performed by the same technician. Measurements for total body (lean mass, fat mass and fat percent) and estimated VAT mass were recorded. Participants completed a continuous incremental ramp maximal exercise test on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) to determine HRpeak in order to prescribe and monitor training intensities. The maximal test consisted of a 4 min warm-up at 30 W and a ramp increase of 15 W/min until volitional exhaustion or the revolutions per min fell below 50. HRpeak was defined as the highest HR achieved.

4.2.4 Training intervention

The training intervention involved ten exercise sessions performed Monday-Friday over two weeks, with Saturday and Sunday set as rest days. Training sessions for HIIT or MICT were designed to be progressive and matched for external work. Exercise intensity was determined using HRpeak measured from the baseline maximal exercise test. HIIT consisted of 1 min eliciting ~90% HRpeak interspersed with 1 min of low intensity recovery periods in between each interval, as we have described previously [301, 317]. Each HIIT session included a standard 3 min warm up and 2 min cool down. Participants progressed from 4x1 min intervals (day 1) to 10x1 min intervals (day 10) with the total duration of each training session lasting from 12 min to 25 min, respectively. MICT consisted of continuous moderate-intensity exercise eliciting ~65% HRpeak, progressing from 20 min (day 1) to 50 min (day 10). Participants completed days 1 and 10 on a stationary bike and were given the option of several exercise modalities for the

other sessions including treadmill walking, stationary cycling, elliptical training or walking outdoors with intensity closely monitored by a research assistant using HR monitors (FT7, Polar, Kempele, Finland). Of the 10 sessions, 3 sessions (days 4, 7 and 9) were completed independently by the participant at home, with compliance confirmed using downloadable HR monitors. This training protocol was modeled after our previously published work [301, 317].

4.2.5 Post-testing

Participants returned to the lab ~72 h (range 60-74 h) following their last training session for post-testing, which was identical to pre-testing.

4.2.6 Blood analyses

4.2.6.1 White blood cell count

Total and differential (lymphocyte, monocyte, and granulocyte) white blood cell counts were measured in fasting blood samples using the Coulter AcT diff 2 Analyzer (Beckman Coulter, USA).

4.2.6.2 Plasma chemokines and adipokines

Fasting plasma concentrations of chemokines/adipokines were measured using multiplex immunoassay. CXCL8 (IL-8) and CCL3 (MIP-1α) were measured using the Human High Sensitivity T Cell Multiplex Kit (Cat# HSTCMAG-283K, Millipore, Billerica, MA, USA). Leptin and CCL2 (MCP-1) were measured using the Human Adipokine Multiplex Kit (Cat#

HADK2MAG-61K-01, Millipore, Billerica, MA, USA). Briefly, plasma samples were spun at 1000xg for 15 min at 4°C to pellet out any debris. The assay was completed according to manufacturer's protocol and samples measured in duplicate using the MAGPIX Bio-Plex reader (Bio-Rad, Hercules, CA). The intra-assay CV based on duplicates was <4% (for both Multiplex Kits) and the inter-assay CV (based on controls run on each plate) was <2% (CXCL8 and CCL3) and <5% (leptin and CCL2). Plasma concentrations were determined using the Bio-Plex Manager 6.1 software. The minimal detectable concentration for each analyte (in pg/ml), calculated by the software, was 0.25 (CXCL8), 1.28 (CCL3), 37 (leptin), and 2.2 (CCL2).

4.2.6.3 Chemokine receptors

Chemokine receptor (CCR2, CCR5 and CXCR2) levels were measured on different leukocyte subsets in whole blood by flow cytometry. Blood was transported to the laboratory and within 10 minutes of sample collection 10 µl of FcR Blocking Reagent (Miltenyi, 130-059-901) was added to 90 µl of whole blood and incubated in the dark for 10 min at 4°C. Following which, 2 µl of CD195 (CCR5) BV421 (BD Horizon, 562576), 5 µl CD182 (CXCR2) FITC (BD Pharmingen, 551126), 2 µl CD192 (CCR2) PE (Miltenyi, 130-103-829), 2 µl CD14 PE-Cy7 (BD Pharmingen, 561385), 2 µl CD16 APC (Miltenyi, 130-091-246), and 2 µl CD8 APC-H7 (BD Pharmingen, 561423) were added and incubated in the dark for 10 min at 4°C. Following incubation, 1 ml of red blood cell lysis buffer (Miltenyi, 120-001-339) was added and incubated in the dark for 15 min at room temperature. To exclude dead cells from the analysis, 2 µl of PI (Miltenyi, 130-093-233) was immediately added before sample analysis on a MACSQuant Analyzer flow cytometer (Miltenyi Biotech, Bergisch Gladbach, Germany). The leukocyte populations had established gates based on the characteristic forward and side scatter, as well as

staining for common leukocyte markers to establish CD14+/CD16- monocytes and CD14+/CD16+ monocytes, CD16+ granulocytes (neutrophils) and CD8+ lymphocytes (T cells). Fluorescence minus one (FMO) controls were used to establish positive staining of CCR2, CCR5 and CXCR2. A total of 10000 CD14+ events were collected for analysis in each subject. Gating strategy is shown in Figure 4.1. Antibody panel optimization was performed prior to the study. Compensation was performed prior to analyses to control for fluorochrome spillover and bank instrument settings were applied to standardize any drift in laser strength over time. CCR2, CCR5, and CXCR2 expression on the leukocyte populations were quantified as median fluorescence intensity (MFI), count per μl and percent positive cells using MACSQuant software (Miltenyi Biotech).

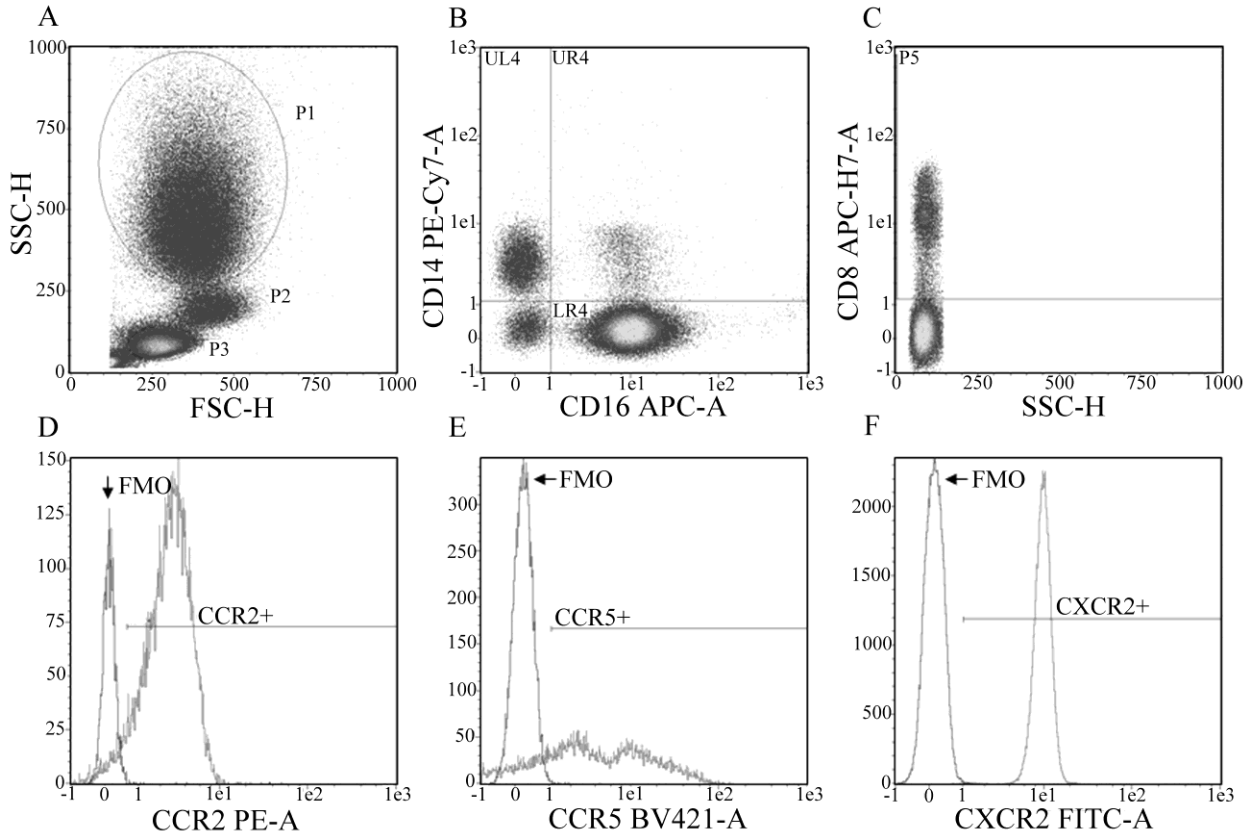

Figure 4.1. Flow cytometry gating strategy. Live cells (negative for PI staining) were first categorized by characteristic scatter (A) as either granulocytes (P1), monocytes (P2), or lymphocytes (P3). Each cell population was then confirmed by positive expression of cell surface markers within their respective populations: (B) monocytes were confirmed by positive expression of CD14 (UL4) or CD14 and CD16 (UR4), granulocytes were confirmed by positive expression of CD16 (LR4), and (C) lymphocytes were confirmed by positive expression of CD8 (P5). Cell surface expression of CCR2 (D), CCR5 (E), and CXCR2 (F) were then measured on the cell types relative to FMO controls for each chemokine receptor (indicated on each graph).

4.2.7 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 22. Statistical outliers were determined using an IQR with a multiplier of 2.2 based off the method by Hoaglin, Iglewics and Tukey [303]. Briefly, the 25th and 75th percentile weighted average value were determined using SPSS and then used to calculate the lower and upper values with a multiplier of 2.2 IQR. Based off of these limits, values that fell outside were deemed to be outliers and

removed from the analyses. Normality was assessed using Q-Q plots and by Shapiro-Wilks test. Data was log-transformed or square root-transformed if a normal distribution did not exist. All descriptive statistics are reported as mean (SD). Two-factor (group x time) mixed ANOVA with repeated measures on time was used to examine changes in anthropometric measures, blood cell counts, chemokine and chemokine receptor expression. Significant interactions were followed up with post-hoc tests comparing pre to post within group. Pre-planned contrasts were also conducted to compare pre vs. post intervention effects within HIIT and MICT separately. Level of significance was set at $p \leq 0.05$. Effect sizes were calculated using Cohen's *d.*

Six participants (three HIIT and three MICT) did not complete the intervention or post-testing, five of which dropped out immediately after pre-testing due to lack of time and one of which who completed part of the intervention but could not complete the training or post-testing due to illness. Results were analyzed per protocol so these six participants were not included in the analysis, leaving 19 participants in the HIIT group and 18 in the MICT group.

4.3 Results

Baseline characteristics of the 37 participants who completed the intervention are shown in Table 4.1.

Table 4.1. Baseline characteristics of participants before the training intervention.

Variable	All	HIIT	MICT
	n=37	n=19	n=18
Age (years)	46.7 (9.7)	48.6 (8.2)	44.7 (11.0)
Sex, n			
Male	5	2	3
Female	32	17	15
BMI (kg/m²)	31.6 (7.1)	32.6 (8.4)	30.6 (5.4)
WC (cm)	108.7 (17.2)	109.6 (19.8)	107.7 (14.6)
% BF	35.9 (6.6)	36.4 (5.8)	35.4 (7.5)

Values are mean (SD). BMI, body mass index; WC, waist circumference; BF, body fat.

4.3.1 Anthropometrics

No differences were observed for body composition measures including BMI, estimated VAT mass, percent BF, total fat mass, and total lean mass following HIIT or MICT (Table 4.2).

Table 4.2. Body composition measures before and after 2 wk of HIIT and MICT.

Variable	HIIT			MICT			P-Value	
	Pre	Post	N	Pre	Post	N	Time	Interaction
BMI (kg/m²)	32.6 (8.4)	32.6 (8.2)	19	30.6 (5.4)	30.4 (5.3)	18	0.971	0.911
Estimated VAT mass (g)	661.9 (298.5)	667.1 (296.3)	19	542.4 (229.5)	641.0 (296.0)	18	0.101	0.200
% BF	36.4 (5.8)	36.3 (5.9)	19	35.4 (7.5)	35.1 (7.6)	18	0.393	0.717
Total fat mass (kg)	33.0 (13.7)	32.9 (13.5)	19	30.7 (11.2)	30.6 (11.4)	18	0.536	0.697
Total lean mass (kg)	53.7 (14.2)	53.8 (13.7)	19	52.8 (10.7)	53.0 (12.1)	18	0.238	0.705

Values are mean (SD). BMI, body mass index; VAT, visceral adipose tissue; BF, body fat.

4.3.2 Blood cell count

No significant effects of training were seen on white blood cell, lymphocyte, monocyte or granulocyte counts (Table 4.3)

Table 4.3. Hematology counts before and after two weeks of HIIT and MICT.

Variable	HIIT			MICT			P-Value	
	Pre	Post	N	Pre	Post	N	Time	Interaction
White blood cells (x10³/µl)	5.72 (0.77)	5.88 (1.13)	15	5.05 (1.11)	4.79 (0.91)	13	0.711	0.132
Lymphocytes (x10³/µl)	2.43 (0.95)	2.57 (0.54)	15	2.22 (1.03)	1.98 (0.31)	13	0.731	0.162
Monocytes (x10³/µl)	0.41 (0.10)	0.51 (0.21)	15	0.44 (0.19)	0.40 (0.25)	13	0.721	0.127
Granulocytes (x10³/µl)	2.87 (0.85)	2.79 (1.05)	15	2.38 (1.2)	2.42 (0.78)	13	0.917	0.814

Values are mean (SD).

4.3.3 Chemokine and chemokine receptors

No significant changes were observed in plasma chemokine or leptin concentrations (Table 4.4).

Table 4.4. Circulating chemokine and adipokine levels before and after 2 wk of HIIT and MICT.

Variable	HIIT			MICT			P-Value	
	Pre	Post	N	Pre	Post	N	Time	Interaction
CCL2 (pg/ml)	326.4 (98.2)	343.4 (122.2)	17	350.9 (133.6)	349.1 (127.6)	15	0.620	0.543
CCL3 (pg/ml)	15.7 (5.1)	14.8 (5.1)	13	16.0 (6.8)	14.6 (7.4)	15	0.085	0.684
CXCL8 (pg/ml)	4.9 (1.5)	4.8 (1.4)	16	4.5 (1.2)	4.7 (1.6)	15	0.774	0.302
Leptin (ng/ml)	65.0 (46.4)	60.3 (42.3)	17	68.2 (58.9)	61.6 (46.5)	16	0.379	0.865

Values are mean (SD). Four plasma samples (two MICT and two HIIT) did not have sufficient volume to measure circulating chemokines or leptin. For CCL3, four HIIT samples and one MICT sample were measured as OOR. OOR, out of range.

4.3.3.1 CCR2

There were no statistically significant differences found in surface expression of CCR2 (Figures 4.2a-d). There was a group x time interaction (P = 0.019) for CD14+/CD16+ monocytes positive for CCR2 (Table 4.5) with post-hoc tests indicating a reduction (~3.5 %) following MICT only (P=0.027, Cohen's *d*=0.6), with no change after HIIT (P=0.368, Cohen's *d*=0.2). No differences were observed for percent CCR2 positive cells in any of the other leukocyte subsets (Table 4.5). There was a group x time interaction (P=0.048) for the neutrophil cell count per µl

blood (Table 4.5); however, post-hoc tests indicated no statistically significant differences within groups over time: MICT (P=0.127, Cohen's *d*=0.4) and HIIT (P=0.195, Cohen's *d*=0.3). No changes in CCR2 positive cell counts per µl blood for the other leukocyte subsets were observed (Table 4.5).

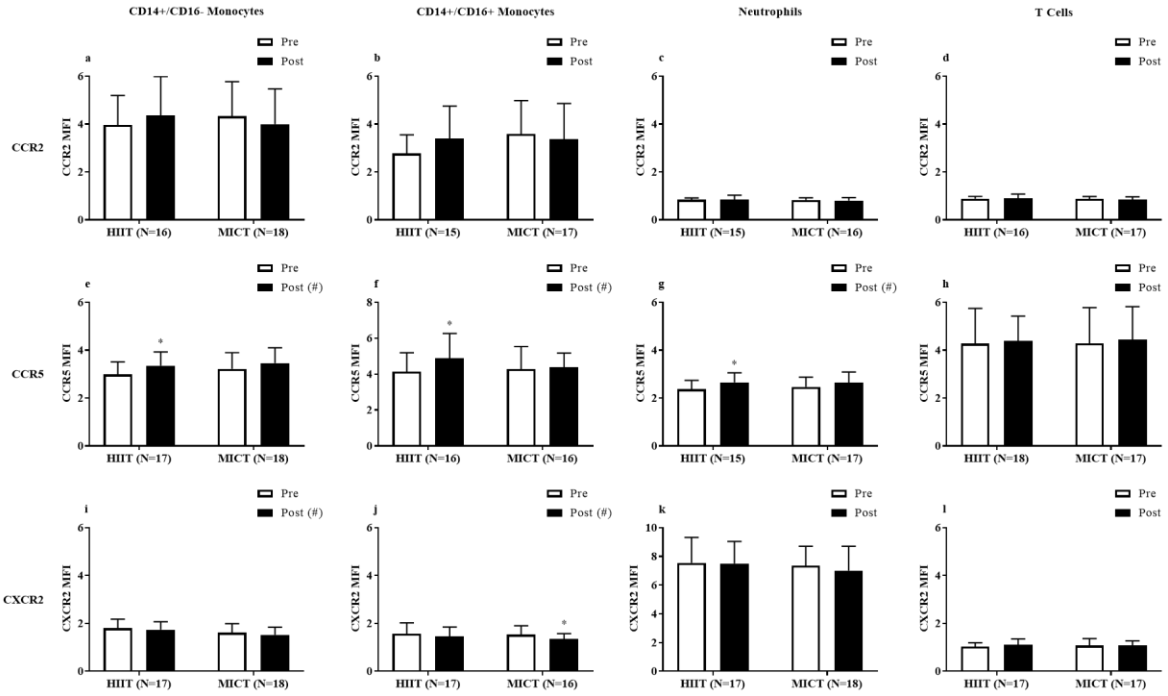

Figure 4.2. Short-term HIIT and MICT result in different responses on leukocyte chemokine receptor surface expression. Surface expression of CCR2 (A – D), CCR5 (E – H) and CXCR2 (I – L) was measured on CD14+/CD16- monocytes, CD14+/CD16+ monocytes, neutrophils, and T cells by flow cytometry before and after training. MFI, median fluorescence intensity. *p<0.05 pre vs. post within-group, #p<0.05 main effect of time.

Table 4.5. Chemokine receptor percent positive cells and cell counts per µl before and after two weeks of HIIT and MICT.

	HIIT			MICT			P-Value	
Variable	Pre	Post	N	Pre	Post	N	Time	Interaction
CCR2								
CD14+/CD16- Monocytes								
Positive Cells (%)	98.07 (1.25)	98.18 (1.14)	15	98.57 (1.00)	98.60 (0.88)	15	0.650	0.770
Cell number (x10³/µl blood)	0.26 (0.07)	0.30 (0.09)	15	0.23 (0.10)	0.22 (0.08)	17	0.310	0.087
CD14+/CD16+ Monocytes								
Positive Cells (%)	92.58 (4.36)	93.39 (5.17)	15	95.88 (4.51)	92.55 (5.99)*	17	0.140	**0.019**
Cell number (cell/µl blood)	7.4 (5.4)	5.9 (.3)	15	3.9 (4.2)	3.6 (3.0)	16	0.577	0.371
Neutrophils								
Positive Cells (%)	50.43 (7.34)	51.34 (6.42)	17	49.43 (5.26)	49.31 (5.67)	17	0.782	0.722
Cell number (x10³/µl blood)	1.08 (0.58)	1.23 (0.63)	16	1.07 (0.33)	0.94 (0.28)	17	0.923	**0.048**
T cells								
Positive Cells (%)	51.50 (5.79)	51.00 (6.20)	15	51.82 (5.98)	50.53 (5.77)	16	0.547	0.788
Cell number (x10³/µl blood)	0.15 (0.08)	0.16 (0.07)	16	0.16 (0.06)	0.18 (0.07)	17	0.330	0.680
CCR5								
CD14+/CD16- Monocytes								
Positive Cells (%)	78.23 (8.60)	81.52 (4.59)	17	80.44 (5.81)	81.18 (4.59)	17	0.166	0.378
Cell number (x10³/µl blood)	0.20 (0.06)	0.23 (0.09)	17	0.19 (0.09)	0.18 (0.06)	17	0.487	0.401
CD14+/CD16+ Monocytes								
Positive Cells (%)	87.61 (6.81)	89.29 (6.25)	17	87.82 (8.31)	90.21 (3.97)	17	0.130	0.789
Cell number (cell/µl blood)	6.9 (4.8)	5.5 (3.9)	16	3.6 (4.4)	3.7 (3.9)	16	0.683	0.221
Neutrophils								
Positive Cells (%)	65.18 (9.04)	70.53 (6.43)*	17	66.22 (5.67)	70.78 (6.58)*	17	**0.003**	0.794
Cell number (x10³/µl blood)	1.52 (0.51)	1.72 (0.90)	17	1.42 (0.40)	1.34 (0.41)	17	0.536	0.144
T cells								
Positive Cells (%)	77.99 (5.12)	80.36 (3.60)*	17	79.17 (5.80)	80.76 (3.70)	17	**0.033**	0.664
Cell number (x10³/µl blood)	0.27 (0.15)	0.29 (0.18)	17	0.25 (0.09)	0.28 (0.10)	17	0.382	0.975
CXCR2								
CD14+/CD16- Monocytes								
Positive Cells (%)	92.64 (5.40)	91.39 (5.00)	16	88.91 (6.53)	87.44 (9.56)	17	0.090	0.890
Cell number (x10³/µl blood)	0.23 (0.07)	0.26 (0.09)	17	0.21 (0.09)	0.19 (0.08)	17	0.877	0.504
CD14+/CD16+ Monocytes								
Positive Cells (%)	83.14 (13.09)	81.24 (13.16)	17	85.17 (10.58)	80.67 (11.65)	17	0.097	0.494
Cell number (cell/µl blood)	6.7 (5.1)	4.9 (3.6)	16	3.3 (3.2)	3.0 (2.2)	16	0.342	0.268
Neutrophils								
Positive Cells (%)	99.87 (0.09)	99.84 (0.12)	16	99.87 (0.07)	99.83 (0.14)	17	0.069	0.604
Cell number (x10³/µl blood)	2.37 (0.86)	2.44 (1.26)	17	2.14 (0.58)	1.90 (0.56)	17	0.450	0.166
T cells								
Positive Cells (%)	26.31 (6.88)	29.67 (9.16)	17	29.23 (10.36)	29.88 (12.74)	17	0.339	0.350
Cell number (x10³/µl blood)	0.09 (0.06)	0.11 (0.07)	17	0.09 (0.05)	0.10 (0.06)	17	0.196	0.436

Values are mean (SD).*P<0.05 pre vs post within-group.

4.3.3.2 CCR5

There was a main effect of time (P=0.012) on the surface expression of CCR5 on CD14+/CD16- monocytes (P=0.012), CD14+/CD16+ monocytes (P=0.033) and neutrophils (P=0.002). Significant increases occurred after HIIT but not MICT for all of these cell types (CD14+/CD16+ monocytes: HIIT: P=0.025, Cohen's *d*=0.6; MICT: P=0.178, Cohen's *d*=0.3; CD14+/CD16+ monocytes: HIIT: P=0.024, Cohen's *d*=0.6; MICT: P=0.545, Cohen's *d*=0.1; and, and neutrophils: HIIT: P=0.006, Cohen's *d*=0.8; MICT: P=0.094, Cohen's *d*=0.4) (Figures 4.2e-g). Surface expression of CCR5 was increased following HIIT on these cell subsets by ~12%, ~18%, and ~12%, respectively. The surface expression of CCR5 on T cells was not affected (Figure 4.2h) but there was a main effect of time (P=0.033) on T cells positive for CCR5 (Table 4.4). Pre vs. post-intervention contrasts revealed a ~3% increase after HIIT (P=0.019, Cohen's *d*=0.7) but not MICT (P=0.214, Cohen's *d*=0.3). A main effect of time (P=0.033) on neutrophils positive for CCR5 (Table 4.5) was also seen, with pre-planned contrasts indicating increases in both HIIT (~8%, P=0.024, Cohen's *d*=0.6) and MICT (~7%, P=0.049, Cohen's *d*=0.4). No differences in CCR5 positive cell counts per µl blood were observed for the leukocyte subsets (Table 4.5).

4.3.3.3 CXCR2

A main effect of time (P=0.043) was observed for the surface expression of CXCR2 on CD14+/CD16- monocytes. Pre-planned contrasts within groups did not reveal statistically significant changes in HIIT (P=0.281, Cohen's *d*= 0.3) or MICT (P=0.080, Cohen's *d*=0.5). On CD14+/CD16+ monocytes, there was a main effect of time (P=0.003) on the surface expression of CXCR2, with a ~13% reduction after MICT (P=0.032, Cohen's *d*=0.8) but no difference

following HIIT (P=0.388, Cohen's *d*=0.4). There were no changes following training in surface expression of CXCR2 on T cells or neutrophils (Figures 4.2k, l) or any changes on percent positive cells for CXCR2 (Table 4.5) for any of the leukocyte subsets measured. No changes in CXCR2 positive cell counts per μl blood for the leukocyte subsets were seen (Table 4.5).

4.4 Discussion

The results of this study demonstrate that, in obese adults, short-term exercise training in the absence of weight loss has direct effects on leukocyte chemokine receptors. HIIT and MICT appear to differentially modulate chemokine receptors on specific immune cells, indicating that the intensity and/or pattern of exercise impact adaptive responses across immune cell subsets. Overall, two weeks of MICT led to a reduction in CCR2 and CXCR2 on CD14+/CD16+ monocytes. In contrast, HIIT resulted in an overall increase of CCR5 on CD14+/CD16- monocytes, CD14+/CD16+ monocytes, neutrophils and T cells. These changes occurred in the absence of weight loss or alterations in circulating chemokines, suggesting that exercise training has a direct influence on chemokine receptor expression in circulating leukocytes.

In a previous study by Krinninger et al. [185], obese women demonstrated higher surface expression of CCR2 and CCR5 on both CD14+/CD16- and CD14+/CD16+ monocytes compared to lean women. These monocytes also had greater chemotactic activity, which was suggested to be due to the increased CCR2 expression. Although studies indicate a greater expression of these chemokine receptors during obesity [185, 318], studies on the effects of exercise training on chemokine receptors are lacking. Given the importance of chemokine receptors in mediating chronic low-grade inflammation in obesity [69, 183] such information may improve our understanding of the anti-inflammatory effects of exercise training in obesity.

4.4.1 Impact of MICT on chemokine receptors

4.4.1.1 Changes in CCR2 expression

Our study shows that MICT, but not HIIT, decreased the percent of CD14+/CD16+ monocytes that were positive for CCR2. We are only aware of previous studies that have examined CCR2 after acute exercise, which have shown mixed results. CCR2 expression on CD14+/CD16- and CD14+/CD16+ monocytes was unchanged in the hours after moderate-intensity leg or arm cycling exercise in healthy young males [319]. Okutsu et al. also reported no change in CCR2 expression on CD14+ monocytes in young men after an acute bout of aerobic exercise, although, when PBMCs were incubated with post-exercise serum there was an increase in CD14+ monocyte CCR2 expression [320]. However, Wells et al. reported decreased CCR2 expression on CD14+ monocytes, collapsed across groups, after an acute bout of high-volume, moderate intensity or low-volume, high-intensity lower-body resistance exercise in resistance-trained young males [321]. These mixed results may be due to the difference in exercise modality (aerobic vs. resistance) and/or study sample (e.g., healthy vs. resistance trained). Our study is the first, to our knowledge, in obese adults and we are the first to report that aerobic training (two weeks of MICT) can lead to reductions in CD14+/CD16+ monocyte CCR2.

Although CD14+/CD16+ monocytes only constitute ~10% of total circulating monocytes, they show a greater inflammatory potential [208] and are involved in inflammatory-related pathologies such as obesity and CVD [180]. The main function of CCR2 and its ligand CCL2 is monocyte trafficking to sites of inflammation [173] and during obesity, there is a greater degree of accumulation of these pro-inflammatory monocytes into tissues such as adipose [181]. Although CCR2 is more highly expressed on CD14+/CD16- compared to CD14+/CD16+ monocytes [186], surface expression and percent positive CCR2 CD14+/CD16+ monocytes have

been reported to be higher in obese compared to lean women [185]. A decrease in the percent of CD14+/CD16+ monocytes that are positive for CCR2 after MICT may therefore be considered more substantial due to the higher inflammatory potential of this monocyte subset. A reduction of percent CD14+/CD16+ monocytes that are positive for CCR2 as seen in our study may indicate that MICT is better able to reduce potential for pro-inflammatory monocyte migration, which would potentially decrease the accumulation of pro-inflammatory monocytes into tissues such as adipose.

We also found an interaction for the percent neutrophils that were positive for CCR2. The mean differences and effect sizes indicated that MICT tended to be lower, whereas HIIT was higher, following training but specific post-hoc tests did not indicate statistical significance on the pairwise comparisons. This trend and significant interaction effect for granulocytes is in line with the potential for MICT to reduce CCR2 on monocytes.

4.4.1.2 Changes in CXCR2 expression

Two weeks of MICT also led to a decrease in CXCR2 MFI expression on CD14+/CD16+ monocytes. An overall decrease in CXCR2 MFI expression on CD14+/CD16- monocytes after exercise training was also observed. CXCR2 expression has been shown to increase following an acute bout of exercise in vascular endothelial cells of skeletal muscle in healthy young men, with this increase suggested to be beneficial in the stimulation of angiogenesis [322]. Although this increase in CXCR2 expression was described as beneficial, the study was focused on young healthy males who most likely do not have an accumulation of monocytes and other leukocytes into their tissues as seen during obesity. The overall decrease in CXCR2 expression on both monocyte subsets observed in our study may be seen as potentially anti-inflammatory based on

reduced potential for monocyte trafficking. Although CXCR2 is expressed on monocytes and T cells, its expression is highest on neutrophils and it is believed to play a predominant role in neutrophil chemotaxis [173]. We did not see any impact of exercise training on CXCR2 on neutrophils or T cells, indicating that the effects were specific to monocytes. These findings of different effects on immune cell subsets highlight the potential importance and added insight of studying chemokine receptors on different leukocytes.

4.4.2 Impact of HIIT on chemokine receptors

4.4.2.1 Changes in CCR5

Two weeks of HIIT showed an overall increase of CCR5 on all leukocyte subsets studied. There was an increase of CCR5 surface expression on CD14+/CD16- monocytes, CD14+/CD16+ monocytes and neutrophils. Further analysis demonstrated an increase in the percent of CD14+/CD16+ monocytes and T cells that were positive for CCR5. Although these consistent increases in CCR5 in all leukocytes were seen after HIIT, MICT did also lead to an increase in the percent CCR5 positive CD14+/CD16+ monocytes.

There are very few studies exploring the effects of exercise on CCR5. In obese adults who showed elevated CCR5 in adipose tissue, three months of exercise reduced adipose tissue expression of CCR5 alongside a decrease in percent body fat [318]. Dorneles et al. [323] also reported decreased expression of whole blood CCR5 24 hours following an acute session of strength training in young males (6). On the other hand, CCR5 on neutrophils was increased after an acute bout of high intensity aerobic exercise in young males [324]. These conflicting results may be due to the lack of leukocyte distinction in the study by Dorneles et al. [323] compared to the specificity of neutrophil CCR5 expression in the latter study [324]. Our results appear to

indicate that an increase in CCR5 following short-term HIIT is a consistent response across multiple immune cells including monocytes, neutrophils and T cells.

CCR5 is recognized for mediating T cell trafficking and type 1 adaptive immunity but is also involved in monocyte/macrophage migration and CCR5 is expressed on neutrophils [173]. Therefore, an increase in CCR5 expression, such as we observed on all leukocyte subsets after HIIT, could be indicative of increased potential for T cell, monocyte and/or neutrophil migration to tissues. However, the increase in CCR5 expression following HIIT may also be indicative of an alternative immunomodulatory function of exercise. Along with its function in T cell trafficking, CCR5 has also been implicated in muscular endurance. Lei el al. have shown that CCR5 activation by orosomucoid 1 increases muscle glycogen storage and enhances muscle endurance [325], an effect that appears related to CCR5-mediated activation of skeletal muscle AMPK [326]. These mechanistic studies have been completed in mice but studies in humans have shown upregulated levels of ORM-1 in PBMCs after an acute bout of resistance exercise [327], providing some preliminary evidence that ORM-1 and CCR5 interactions may be influenced by exercise.

4.4.3 Changes in chemokines and adipokines

There were no changes in plasma chemokines or the adipokine, leptin (Table 4.4). The short-term nature of our exercise intervention and the lack of weight loss or reduction in VAT likely explain why no changes in chemokines or leptin were seen. The lack of change in circulating inflammatory mediators is in line with a previous investigation that found no change in plasma IL6 and TNFα following six weeks of HIIT or MICT in overweight young men [261]. As levels of chemokines are known to impact receptor expression [320], our results indicate that

the changes seen in CCR2 and CXCR2 on monocytes after MICT and CCR5 on all leukocytes after HIIT do not appear related to altered circulating levels of their respective ligands.

4.4.4 Limitations

We purposefully chose to study short-term exercise in this initial study of leukocyte chemokine receptors in order to avoid the possible confounding effects of weight loss or body composition changes on these inflammatory parameters [226]. Thus, the effects seen on leukocyte chemokine receptors may only reflect aspects of the initial adaptive response to exercise in previously inactive obese adults. Longer-term studies are warranted to determine the impacts of prolonged exercise training.

A previous study has shown that acute infusion of lipids and/or glucose may impact levels of circulating CCL2 [328] and Wells et al. [329] have shown that protein ingestion following resistance exercise impacts monocyte CCR2 expression. It is therefore possible that nutrition may influence the impact of exercise on immune cell chemokine receptor expression. In our study, participants were instructed not to change their dietary habits over the course of the two-week intervention but we did not specifically control diet throughout. The objective measures of body mass and body composition by DXA provide evidence that energy balance was maintained but it is possible that subtle changes in diet, in addition to the HIIT or MICT exercise interventions, influenced the results.

Of our 32 female participants, 15 (40.5%) were premenopausal, three (8.1%) perimenopausal and 14 (37.8%) were postmenopausal. It was not possible to standardize the menstrual phase of each female participant for pre- and post-testing as the study protocol dictated that measures were taken 23 days apart (pre-testing always occurred on a Friday one week prior

to beginning the training intervention and post-testing was always completed on the Monday following completion of training). We are not aware of any data indicating that chemokines or their receptors are altered with menstruation, and it seems unlikely that systematic differences in the timing of menstrual cycle phase for pre- and post-testing would arise within a randomized study, but it should be noted that that our study sample and design were not able to control for menopause or menstrual cycle.

We were not able to determine the impact of chemokine receptor changes on infiltration of different leukocytes into tissues. Because visceral adipose and the arterial wall are considered most important inflammatory sites for metabolic and cardiovascular health [330, 331] such studies are not feasible in humans with existing methods. It would be interesting to determine whether the changes in chemokine receptors translate into altered tissue immune cell infiltration to impact inflammation in future work. Complementing circulating leukocyte measures with subcutaneous adipose tissue or skeletal muscle biopsies would be one potential approach. Similarly, the chemotactic potential or migratory capability of each leukocyte subset was not measured in the present study. Based on our findings, it would be of interest in future studies to measure chemotactic potential of different cell types (i.e. monocytes, neutrophils and T cells) following exercise training to determine if altered chemokine receptors translate into increased or decreased ex vivo migration.

4.5 Summary

Short-term exercise training resulted in changes in chemokine receptor expression in the absence of weight or visceral fat loss and without changes in circulating chemokines. Specifically, MICT led to decreased CCR2 and CXCR2 on monocytes and HIIT resulted in

increased CCR5 on monocytes, neutrophils and T cells in obese adults. These findings indicate that exercise has direct effects on immune cells in adults with obesity, in the absence of changes in systemic chemokines or fat loss, with the type of exercise (HIIT or MICT) resulting in different responses. The implication of reduced CCR2 and CXCR2 following MICT and of increased CCR5 after HIIT will require further research but overall these findings indicate, based on leukocyte chemokine receptor expression, that MICT may reduce monocyte infiltration whereas HIIT may increase monocyte, T cell and neutrophil infiltration. These findings indicate distinct effects of exercise on chemokine receptors and future studies are required to explore the effect of different exercise intensities and modalities on leukocyte trafficking during obesity [77].

Chapter 5 General discussion

5.1 Main findings

The collection of studies within this thesis sought to enhance the understanding of how T2D, obesity, and exercise impacted immune cell phenotype and function.

In Chapter 2, the effect of T2D on IL10 function was assessed in immune cells from individuals with T2D and the mechanisms explored using macrophage cell models (RAW264.7 and BMDMs) treated with high glucose. The primary finding of Chapter 2 was that the ability of IL10 to inhibit LPS-induced TNFα production was reduced in immune cells from individuals with T2D, suggesting an IL10 hyporesponsiveness or "IL10 resistance" during T2D. Furthermore, IL10 resistance was also demonstrated in two macrophage cell models exposed to high glucose conditions suggesting that hyperglycemia may be involved in the mechanism. This study introduced a novel role of reduced IL10 function during T2D.

It is now understood that the underlying pathological defects in T2D, insulin resistance and beta cell dysfunction, occur prior to the onset and diagnosis of T2D [12-14]. Estimates have indicated that these early metabolic defects can occur up to 10 years prior to diagnosis [20, 21]. In this regard, it may be more beneficial to test therapeutic treatments in individuals who are at high risk of developing T2D versus those who have a diagnosis of T2D, in order to intervene prior to more severe metabolic dysfunction. With this concept in mind, Chapters 3 and 4 sought to explore the effect of short-term exercise training, in the form of HIIT or MICT, on immune cell phenotype and function. Early literature indicating the anti-inflammatory effects of exercise in the context of obesity and T2D is founded on MICT. Recent research has compared HIIT to MICT; however, the results are mixed and whether HIIT is anti-inflammatory, pro-inflammatory, or even immunosuppressive remain to be elucidated.

The main findings of Chapter 3 showed that two weeks of HIIT and MICT resulted in reduced anti-inflammatory IL10 function, with a greater effect seen with HIIT. The effects of short-term exercise training on IL6 function was less clear but differential effects of HIIT and MICT were present. Chapter 4 demonstrated that short-term exercise training altered chemokine receptor expression with differential effects seen between HIIT and MICT. More specifically, MICT reduced chemokine receptors (CCR2 and CXCR2) on monocytes whereas HIIT increased expression of the chemokine receptor CCR5 on monocytes, neutrophils and T cells. The studies in Chapters 3 and 4 suggest that the impact of exercise on immune cell phenotype and function may be dependent upon the intensity or pattern of exercise. Overall, HIIT and MICT appear to differentially modulate immune cell phenotype and function.

5.2 Impact of T2D on immune cell phenotype and function

5.2.1 IL10 resistance occurs in T2D

It is commonly accepted that there is a link between T2D and elevated levels of pro-inflammatory cytokines such as TNFα [64, 332-335]. Recently, there has been increased recognition that anti-inflammatory cytokines are also involved in the pathogenesis of T2D [336]. IL10 is a potent anti-inflammatory mediator involved in resolution of inflammation [136] with some evidence indicating an association of low levels of IL10 with obesity and T2D [165, 166] and that increased expression of IL10 in mice models protects from HFD-induced obesity and inflammation [167, 168]. In Chapter 2, we showed that the ability of IL10 to inhibit LPS-induced TNFα production was reduced in immune cells from individuals with T2D, suggesting a reduced anti-inflammatory action of IL10. This hyporesponsiveness or “IL10 resistance” was also confirmed in two separate macrophage cell models when cultured in high but not low glucose

conditions, suggesting that elevated glucose as seen in T2D may mediate this effect. There were no changes in the expression of IL10R1 on immune cells of individuals with T2D or on macrophage cell models exposed to high glucose, providing evidence that altered receptor expression was not responsible.

There are only two previous studies that have linked reduced IL10 function with chronic inflammation [285, 286]. Yuan et al. [286] demonstrated IL10 resistance in individuals with systemic lupus erythematosus (SLE). Specifically, SLE monocytes demonstrated increased levels of heat-aggregated human IgG-induced TNFα and IL6 production, which were not as effectively inhibited by IL10 treatment in SLE monocytes. This IL10 resistance could also not be explained by any changes in the IL10R. It is of interest to note the similarities between this study in monocytes from individuals with SLE and Chapter 2, which assessed whole blood cultures from individuals with T2D. For example, the results from Chapter 2 indicated higher levels of plasma TNFα and IL6 in individuals with T2D. In addition, IL10 was less effective at reducing LPS-induced TNFα in individuals with T2D as well as in macrophages exposed to high glucose, neither of which were explained by changes in the IL10R. These studies indicate that IL10 resistance plays a role in inflammatory conditions such as SLE and T2D, and therefore should be considered in other inflammatory conditions.

5.2.1.1 IL10 resistance may be mediated by SHIP1 signaling

IL10 has been shown to inhibit ~20% of all LPS-induced cytokine secretion [165]. However, this inhibition cannot be fully attributed to IL10-induced genes via the traditional JAK/STAT pathway [146]. Chan et al. [166] have described SHIP1 as an alternate pathway through which IL10 can mediate its anti-inflammatory effects. The IL10 resistance demonstrated

in Chapter 2 was not explained by downregulation of the IL10R1 in immune cells of T2D or of IL10R1, STAT3, or AMPKα in macrophages exposed to high glucose. IL10-induced activation of STAT3, but not AMPKα, was reduced in macrophage cell models exposed to high glucose. Interestingly, treatment with a SHIP1 agonist, AQX-MN100, reversed the IL10 resistance observed in the macrophage cell models cultured in high glucose indicating a role for IL10-induced SHIP1 signaling. The findings from Chapter 2 suggested that IL10 resistance occurs in conditions such as T2D and that therapeutic approaches, such as utilization of SHIP1 agonists, should be considered for treating or reversing IL10 resistance. SHIP1 agonists are currently being tested in clinical trials for unstable chronic obstructive pulmonary disease (ClinicalTrials.gov Identifier: NCT01954628), atopic dermatitis (ClinicalTrials.gov Identifier: NCT02324972), and interstitial cystitis/bladder pain syndrome (ClinicalTrials.gov Identifier: NCT01882543) and have been previously tested in allergic and pulmonary inflammation [337, 338]. It may be of interest to explore how "mimicking" IL10 action using these compounds effects other inflammatory conditions, including T2D or obesity.

5.3 Impact of obesity and exercise on immune cell phenotype and function

5.3.1 IL10 and IL6 are anti-inflammatory

IL10 is a potent anti-inflammatory mediator and has been shown to strongly inhibit LPS-induced TNFα production [296]. Although evidence indicates that exercise-induced IL6 is anti-inflammatory [108, 339] there is a lack of evidence indicating the direct inhibitory effect of IL6 on pro-inflammatory cytokines like TNFα [107, 129, 340]. In Chapter 3, we confirmed that IL6 was in fact anti-inflammatory, at least in the context of reducing LPS-induced TNFα production, in a human whole blood culture model. The inhibitory function of IL10 was also confirmed;

however, IL10 showed more consistent and potent anti-inflammatory effects compared to IL6 in LPS-stimulated human whole blood cultures, similar to work in other cells [341, 342].

Findings from Chapter 2, along with two previous studies [285, 286], have indicated that the anti-inflammatory action of IL10, and more specifically the ability of IL10 to reduce TNFα production may be reduced during inflammatory conditions such as T2D and SLE. We did not study the effect of IL6 responsiveness in individuals with T2D. However, a study by Jiang et al. [343] has implicated IL6 resistance in glucose, but not lipid, metabolism in skeletal muscle from individuals with T2D. Specifically, an acute exposure of skeletal muscle cells to IL6 resulted in increased glucose uptake and glycogen synthesis in normal glucose tolerance (NGT) controls but not in individuals with T2D, and a similar increase in fatty acid oxidation in both groups. Although, IL6 activated STAT3 in skeletal muscle cells and cultured myotubes in individuals with NGT and T2D, there was a blunted response in those individuals with T2D. Furthermore, IL6-induced JAK2 phosphorylation was increased in NGT individuals but not individuals with T2D. The "IL6 resistance" was not explained by changes in IL6R or gp130 protein or IL6, IL6R or gp130 mRNA levels; however SOCS3 protein was elevated in T2D myotubes, which may partly explain the blunted response of IL6 signaling. Although the authors suggest that SOCS3 may play a role in this blunted effect, it is known that SOCS3 does not associate with the IL10R to affect IL10 signaling [150] and therefore the existence of IL10 resistance in inflammatory conditions points to other possible mechanisms for IL6 resistance in combination with or alternative to increased SOCS3 expression. Evidence for IL10 and IL6 resistance exists in inflammatory conditions such as T2D and therefore therapeutic approaches to combat these resistive states is of interest.

5.3.1.1 IL10 function is reduced following HIIT and MICT, but to a greater extent with HIIT

As demonstrated in Chapter 2, IL10 resistance may play a role in inflammatory conditions like T2D. Therefore, to better understand the impact of exercise on IL10 function we explored short-term exercise training in individuals who were at high risk of developing T2D. It was decided not to study the impact of exercise in T2D patients due to the potential confounding effects of medications in this population, which would be difficult to stratify between HIIT and MICT groups and given the mechanism behind IL10 resistance is not known we could not rule out drug interactions. Results from Chapter 3 demonstrated that two weeks of either HIIT or MICT led to a less effective IL10 response as the ability of IL10 to reduce LPS-induced TNFα production was reduced following exercise training. This reduced IL10 action occurred at both low and high LPS concentrations and at multiple IL10 concentrations following HIIT and at only1 ng/ml LPS + 5 ng/ml IL10 for MICT, indicating greater modulation of IL10 function following HIIT as compared to MICT.

Downregulation of the IL10R1 expression on leukocytes did not appear to explain these results. This is a novel finding as to my knowledge no other study has examined the impact of exercise on IL10 function in leukocytes or any other cell type. Since IL10 is known to mediate its inhibitory effect on TNFα through intracellular pathways including JAK/STAT and SHIP1, it may be possible that downstream signaling mediators are influenced by MICT and HIIT, with a greater or different effect with HIIT. Future research will be required to elucidate these mechanisms.

5.3.1.2 IL6 function is differentially influenced by HIIT and MICT

IL10 and IL6 have been implicated as anti-inflammatory mediators of exercise, therefore in addition to IL10, IL6 function may also be important when considering inflammatory conditions such as obesity and T2D. Evidence of IL10 resistance is demonstrated in Chapter 2 and a single previous study has provided evidence of IL6 resistance during T2D [343]; however, IL6 resistance was demonstrated in the context of glucose metabolism and not inflammation. Results from Chapter 3 demonstrated that HIIT and MICT differentially affected IL6 function in the context of inhibiting LPS-induced TNFα production; however, the interpretation of the findings were not straightforward. Although the statistical results indicated Time X IL6 and IL6 X Group interactions, the post-hoc tests did not indicate any significant differences within either HIIT or MICT. Without significant post-hoc tests it is more difficult to pinpoint where specific differences lie. Although speculative, it seems that the effect of HIIT on IL6 function paralleled the trend of HIIT on IL10 function as IL6 appeared to be less effective at inhibiting LPS-induced TNFα production following the two-week training intervention. No significant differences were seen at the higher (i.e., more potent) concentration of LPS. These results are perhaps not surprising as IL6 was less effective at inhibiting TNFα production at 10 ng/ml LPS compared to 1 ng/ml LPS. At the higher concentration of LPS, IL6 may become less effective and therefore less likely to be affected by exercise. Downregulation of the IL6Ra expression on leukocytes did not appear to explain these results. This finding is novel and to our knowledge, anti-inflammatory IL6 function in response to exercise has never been assessed.

5.3.2 Short-term exercise training does not alter cytokine expression

The majority of studies exploring the effects of exercise on inflammation use change in cytokine(s) as their main indicator of change in inflammation. Both acute exercise and exercise training models have been observed to alter circulating cytokine levels; however, these results are often mixed. For example, TNFα has been shown to be increased [344], decreased [345], or not changed [238, 307] following exercise training of various durations and intensities. Of course, these results should be interpreted dependent upon type and duration of exercise as well as population studied. In Chapter 3 and Chapter 4, circulating levels of plasma cytokines and chemokines were measured following short-term exercise training in the absence of weight/fat loss. Neither HIIT nor MICT altered circulating cytokines measured in Chapter 3 (IL10, IL6, and TNFα) nor chemokines measured in Chapter 4 (CCL2, CCL3, and CXCL8). Although there are no changes in systemic circulating cytokines this may not necessarily reflect local cytokine levels in different tissues such as adipose and skeletal muscle because there are many different sources of cytokines that end up getting measured in plasma cytokine assays. For example, monocytes/macrophages are considered the primary source of TNFα but other cells such as neutrophils and activated T cells also produce TNFα [73, 81]. In addition, IL6 is produced by many leukocytes [87] but is also produced by endothelial cells [73], adipocytes [88], and skeletal muscle [89]. Therefore, the implication(s) of changes in systemic circulating cytokines following exercise remains unclear because the exact source(s) of each cytokine cannot easily be elucidated. Given the myriad of physiological responses and adaptations across multiple body systems experienced as a result of exercise training, and the many different cellular sources of cytokines, it seems interpretation of plasma cytokines after exercise training may not be overly informative.

5.3.3 HIIT and MICT differentially modulate chemokine receptor expression

A pathological feature of obesity is the infiltration of monocytes/macrophages, and the subsequent increase in pro-inflammatory cytokines, in tissues such as adipose tissue [152]. The main mediators of monocyte/macrophage migration and infiltration are chemokines and chemokine receptors [69, 183]. CCL2/MCP1 – CCR2 has been the most studied and implicated as the main interaction involved in obesity-induced macrophage infiltration in adipose tissue [181, 184, 185]; however, other interactions may also play a role in leukocyte infiltration during obesity including CCL3/MIP1α – CCR5 [346, 347], and CXCL8/IL8 – CXCR2 [348]. For example, Krinninger et al. [185] demonstrated that obese women have higher expression of CCR2 and CCR5 on CD14+/CD16- and CD14+/CD16+ monocytes and a greater monocyte chemotactic activity. Certain chemokine-chemokine receptor interactions are considered more important in the pathology of obesity.

For the purpose of Chapter 4, these three chemokine-chemokine receptor pairs were explored. Overall, two weeks of MICT led to decreased expression of CCR2 and CXCR2 on CD14+/CD16+ monocytes and two weeks of HIIT led to increased expression of CCR5 on CD14+/CD16- monocytes, CD14+/CD16+ monocytes, neutrophils and T cells. These results suggest that that HIIT and MICT differentially modulated chemokine receptor expression on several different leukocyte subsets.

5.3.3.1 HIIT and MICT: Effects on CCR2

Results demonstrated a decrease of CCR2 percent positive CD14+/CD16+ monocytes after two weeks of MICT but not HIIT. Although there are several studies that have demonstrated the effects of exercise on chemokine receptors, these studies are in acute exercise

models only. For example, although no changes in CCR2 occurred after an acute bout of either high-intensity or high volume resistance training, when these groups were collapsed there was increased plasma CCL2/MCP1 and decreased CD14+ monocyte CCR2 expression [321]. The same authors studied the effects of an amino acid supplement following lower body resistance exercise, where they found that CCL2/MCP1 was maintained and "very likely greater" at 2 hour post-exercise and CCR2 was increased and "likely greater" immediately, 1 hour and 5 hours post-exercise and "very likely greater" 2 hours post-exercise (using magnitude based inferences) [329]. Unlike these studies, an acute bout of moderate exercise in healthy young men increased CCL2/MCP1 immediately after exercise but did not directly alter CCR2 expression on either CD14+/CD16- monocytes or CD14+/CD16+ monocytes [319] or in PBMCs [320]. However, in the latter study, incubation of PBMCs in post-exercise serum increased CCR2 expression and increased PBMC migration towards CCL2/MCP1 [319]. In addition, cortisol demonstrated similar results in respect to CCR2 expression and migratory potential. The glucocorticoid receptor antagonist, RU-486, blocked both the cortisol and post-exercise serum-induced increase in CCR2, thus implicating cortisol as the mediator within post-exercise serum for the effects on CCR2 expression and PBMC migration. Interestingly, the authors demonstrated similar findings in CXCR4 expression and migration to CXCL12 on natural killer cells following moderate exercise; however, RU-486 was only able to block the cortisol-induced, but not the post-exercise serum-induced, upregulation of CXCR4 and increased migratory ability to CXCL12 [349]. Therefore, it appears that factors other than cortisol present within post-exercise serum may be responsible for mediating changes in chemokine receptor expression.

5.3.3.2 HIIT and MICT: Effects on CXCR2

Results from Chapter 4 also demonstrated a decrease in CXCR2 CD14+/CD16+ monocytes following MICT. There are two previous studies that have shown an effect of exercise on CXCR2; however, the results are opposing. Hong et al. demonstrated that an acute bout of moderate exercise decreased CXCR2 in monocytes, with the greatest difference seen in CXCR2 CD14++CD16- monocytes [350], whereas Frydelund-Larsen et al. [322] showed increased CXCR2 in vascular endothelial cells of skeletal muscle following an acute bout of exercise. These differences may be explained by a few factors including exercise duration and sample population. Firstly, the duration of exercise in the study by Hong et al. was 20 minutes compared to the three hour exercise duration of Frydelund-Larsen's study. In addition, the sample population in Hong et al.'s paper was a mix of individuals with normal and elevated blood pressure, with an average age of 36 and 45 years respectively (subsequent analysis still indicated decreased monocytes CXCR2 in both of these populations), whereas the study population in Frydelund-Larsen's study had a mean age of 25 years and included only healthy males. The different responses of exercise on chemokine receptors may be partly explained by exercise type and population of individuals studied.

5.3.3.3 HIIT and MICT: Effects on CCR5

In Chapter 4, CCR5 was shown to increase following HIIT on CD14+/CD16- monocytes, CD14+/CD16+ monocytes, neutrophils and T cells, and following MICT on neutrophils. Evidence from acute exercise studies in healthy young men have shown that the expression of CCR5 in whole blood is decreased 24 hours following an acute bout of strength training [323], and increased on neutrophils following an acute session of high intensity aerobic exercise [324].

Unlike CCR2 and CXCR2, there is one article that has looked at the effects of exercise training on CCR5 expression. Three months of combined moderate intensity and resistance exercise training in obese adults reduced the expression of CCR5 in adipose tissue [318]; however, this change was accompanied by a decrease in body fat percentage and therefore cannot be attributed directly to exercise.

5.3.3.4 Regulation of chemokine receptors

Chemokine-chemokine receptor interactions are the main mediator of leukocyte migration/recruitment; however, they are also important in other responses such as cell proliferation, activation, and differentiation, tumor metastasis, remodelling of the extracellular matrix, and angiogenesis [170]. Due to the importance that these chemokine-chemokine receptor interactions play, there are various mechanisms involved in the regulation of chemokines and their cognate chemokine receptors. Regulatory mechanisms function to modulate changes in concentration, and molecular structure of chemokines, chemokine receptors, or of the chemokine-chemokine receptor pair.

During the normal process of inflammation the majority of chemokines and chemokine receptors become upregulated [173]. Furthermore the basal levels of chemokines and chemokine receptors are upregulated in inflammatory conditions such as obesity and T2D [351]. A main regulatory pathway of chemokine receptors is clathrin-mediated endocytosis. Once chemokines bind and activate their chemokine receptor, the receptor undergoes internalization via the clathrin-mediated endocytosis and then are either degraded or recycled. Receptor degradation occurs via lysosomal degradation and results in attenuated signaling, whereas receptor internalization results in a resensitization of signaling [351]. Another regulatory mechanism of

chemokine receptor expression is post-translational modification. Two modifications include glycosylation, which has been shown to protect from proteolysis [352], and sulfation, which has been implicated in altering chemokine binding affinity and activation [353]. Whether the changes in chemokine receptors seen in Chapter 4 were related to increased clathrin-mediated endocytosis in MICT and/or increased glycosylation or sulfation after HIIT remains to be determined.

5.3.3.5 Implications for changes in chemokine receptors

The clinical significance of altered chemokine receptor expression following exercise still remains unclear. Some studies suggest that an increase could be beneficial. For example Wells et al. [329] suggest that the increase in CCR2 expression following ingestion of an amino acid supplement in conjunction with an acute bout of high-volume, moderate intensity resistance exercise is beneficial. They suggest that this upregulation of CCR2 is part of a transient pro-inflammatory response and may act to remove CCL2/MCP1 in order to trigger an increase in CCL2/MCP1 secretion to maintain monocyte responsiveness. It should be noted that the authors mentioned that caution should be used in interpretation because subsequent inflammatory events may add further clarification. In addition, the increase in CXCR2 expression in vascular endothelial cells of skeletal muscle of healthy young men was suggested as an advantageous response in simulating angiogenesis [322]. Conversely, a decrease in adipose tissue CCR5 in obese individuals following three months of exercise training was seen as beneficial in respect to reducing obesity-induced inflammation [318]. Both increased and decreased chemokine receptor expression induced by exercise has been suggested to be beneficial, indicating that the

implications of altered expression are dependent upon the different study design and variables measured.

Findings from Chapter 4 indicated that based solely on leukocyte chemokine receptor expression, MICT may reduce monocyte infiltration, whereas HIIT may increase monocyte, T cell, and neutrophil migration/infiltration. However, leukocyte migration in obesity was not directly measured in the study but when taken in the context of previously published work on leukocyte migration with exercise [208, 227] it seems that altered chemotaxis could be one aspect of immunomodulatory impact of exercise. More research is required to explore the clinical significance of altered chemokine receptor expression and the subsequent effects on leukocyte migration not only in different populations but following different types of exercise as well.

5.3.4 Impact of short-term training on leukocytes

Acute exercise demonstrates a transient leukocytosis [354], which may be dependent upon individual fitness levels [355, 356], exercise intensity [357] and exercise duration [358]. Although leukocytosis is observed following acute exercise, the function or "destiny" of these immune cells remains relatively unclear. The results from Chapter 3 and Chapter 4 demonstrated that short-term exercise training did not result in any changes in leukocyte populations including total white blood cells, CD14+ monocytes, neutrophils, or T cells (as measured by flow cytometry) or total white blood cells, lymphocytes, monocytes, or granulocytes (as measured by hematology analyzer).

Prestes et al. [359] demonstrated higher levels of CD4+ T cell death (measured via annexin V+ apoptotic marker) immediately after resistance exercise designed to induce

hypertrophy, whereas there was higher CD4+ T cell migration (measured via CX3CR1 migration marker) following resistance exercise designed for local muscle endurance. Levels of CD4+ T cell death were higher, whereas the levels of CD4+ T cell migration were lower, 24 hours post-exercise in the hypertrophy group compared to the local muscle endurance group. Another study by Pereira et al. [360] demonstrated similar results in resistance exercise, designed to induce hypertrophy, resulting in increased levels of CD4+ and CD8+ T cell death and migration up to 24 hours post-exercise. These studies explored the effects of acute resistance exercise; however, aerobic exercise and longer-term training may not result in the same effects. Similarly, Navalta et al. [361] studied markers of T cell death and migration after several aerobic exercises at various intensities. CD4+ T cell death was increased with moderate intensity, whereas CD4+ T cell death and migration were elevated with high intensity. Similarly, CD8+ T cell death increased with moderate intensity but CD8+ T cell migration, and not T cell death, was elevated with high intensity. These studies suggest that a gradual or progressive adaptation to a hypertrophy resistance exercise program may minimize effects on CD4+ and CD8+ T cells and suggested by Prestes et al. may reduce any possible susceptibility to antigens that these changes may result in. In addition, Navalta et al. suggest that the elevated levels of CD4+ and CD8+ T cell death may create an immunological susceptibility, which high intensity exercise may play a protective role with increased T cell migration.

Although these studies are in acute exercise, taken together it indicates that inflammatory effects including changes in T cell apoptosis and migration, are different depending on exercise dose and intensity and it could be applied to the changes in immune cell phenotype and function observed within this thesis. Whether other leukocytes are modulated similarly by acute and chronic exercise is not clear but overall the results of Chapters 3 and 4 indicate that simple changes in leukocyte numbers do not explain altered immune function after HIIT or MICT.

5.4 Strengths and limitations

In chapter 2, IL10 function was assessed between individuals with T2D and an age- and BMI- matched control group. A limitation of this comparison was that around half of the individuals with T2D were taking glucose-lowering (11 of 24) and anti-hypertensive (10 of 24) medications. Therefore, although the control group was matched for both age and BMI, there is a possibility that the difference in medication usage and their possible interaction(s) on hyperglycemia could impact IL10 function. Unfortunately, due to the sample study of the size there was inadequate power to compare the effects of different medications on IL10 function within individuals with T2D. Although medication usage was not controlled for within the study population, the T2D group were able to abstain from taking their glucose-lowering medication prior to their fasting blood sample. Although the multitude of medication usage within the T2D group may be thought of as a limitation, it may in fact be a better representation of typical individuals with T2D.

In Chapter 2 IL10 resistance was observed in individuals with T2D. This concept was further explored in Chapters 3 and 4, sub-studies of a larger RCT, which assessed inflammatory parameters following a two-week training intervention of either HIIT or MICT in obese adults. Obese individuals who were at high risk of developing T2D were set as the study population instead of individuals with T2D in order to reduce the confounding effects of medications, as both glucose- and lipid-lowering medications can affect signaling pathways involved in inflammation [313].

A short-term training period (i.e. two weeks) was purposefully chosen in order to avoid any confounding effects of weight loss or body composition on inflammatory parameters. It is possible that the effects of a short-term exercise intervention may be reflective of the initial

adaptive response to exercise and that longer-term exercise may be required to determine the response following sustained or longer-term exercise training.

Although participants were instructed not to alter their diet during the two week training intervention, diet was not specifically controlled for. There is evidence that nutrition may influence inflammatory parameters. For example, CCL2/MCP1 is altered following infusion of either lipid or glucose [328] and monocyte CCR2 is altered following ingestion of protein following resistance exercise [329]. In addition, higher levels of IL6 have been associated with "unhealthy" diets in humans [362-364], and other dietary interventions such as high carbohydrate diets in a mouse model [365]. There is less evidence linking diet/nutritional status to IL10 with one study demonstrating lower levels of IL10 in mouse adipose tissue following a diet of lard, high in both saturated and monounsaturated fatty acids [366], and another study demonstrating a possible trend of decreased IL10 in mouse lymphocytes following a HFD of unsaturated fatty acids [367]. Although diet was not controlled for directly, measures of body mass (i.e. weight, BMI, WC) and body composition were objectively measured by DXA. All body measures were unchanged following two weeks of exercise training providing evidence that the dietary energy balance was maintained over the intervention. However, it cannot be ruled out that even subtle changes in dietary/nutritional composition or other factors including sleep, in conjunction with the exercise training, may have influenced the effects on the inflammatory parameters.

A limitation in our study sample was the unequal representation of males and females with 35 (81%) out of the 43 participants being female. Due to this unequal balance, sex differences could not be explored. Of the female participants, 16 (45.7%) were premenopausal, three (8.6%) perimenopausal, and 16 (45.7%) were postmenopausal. Menstrual cycle was not standardized for our female participants because it was not possible within the study design.

Specifically, pre- and post-testing was separated by 23 days with pre-testing occurring on a Friday one week prior to the start of the training intervention and post-testing was completed on the Monday following completion of the training intervention. Due to the mixed menstrual status of the female participants as well as the study design, menopause or menstrual cycle was not controlled for.

We are unaware of any studies demonstrating altered chemokines or their receptors with menstruation. Studies indicate that IL10 is not altered with menstruation [368]. Although menstrual cycle has been shown to affect plasma IL6 levels, the results are mixed indicating increased IL6 [369], decreased IL6 [370], or no change [371]. According to Angstwurm et al, there is no effect of menstrual cycle on LPS-induced cytokines including IL6, IL10 and TNFα within whole blood cultures [370], which may be more pertinent to the results in Chapter 4. Although menstrual cycle may have affect inflammatory parameters, it is unlikely that systematic differences would occur within a randomized study due to the different phases of the menstrual cycle of our female participants during pre- and post-testing.

In Chapter 4, short-term exercise training demonstrated altered chemokine receptor, with differential effects seen between HIIT and MICT. We suggest that the altered expression of chemokine receptors would affect the ability of leukocytes to infiltrate into tissues; however, we did not directly measure chemotactic ability of these leukocytes. In the context of inflammation during metabolic and cardiovascular health, VAT and the arterial wall are thought to be the most important sites [330, 331]; however studies exploring these major sites are not feasible in humans.

5.5 Implications and future directions

The finding that IL10 resistance occurs in individuals with T2D and in macrophages exposed to high glucose is novel. There are only two other studies that mention IL10 resistance, one of which is in individuals with SLE [286] and the other is in mouse macrophages infected with LP-BM5 retrovirus [285]. The majority of studies that research inflammation in T2D are focused on inhibiting pro-inflammatory cytokines/signaling, therefore, this study indicates that altered anti-inflammatory cytokine signaling may also play a role in the inflammatory pathology of T2D. For future studies, the impact of other factors involved in the pathology of T2D on IL10 resistance should be explored. For example, it is well known that both hyperinsulinemia and hyperlipidemia are involved in the pathology so it would be of interest to explore how insulin and/or lipids impact IL10 responsiveness during T2D. In addition, since the majority of individuals with T2D use glucose-lowering medications, which are often used in conjunction with other medications like lipid-lowering or anti-hypertensive medications, it would also be of interest to compare any interactions between these medications and IL10 responsiveness in T2D.

This thesis indicates that short-term exercise training results in direct effects on several inflammatory parameters, specifically IL10 function and chemokine receptor expression. The short-term nature of the training intervention avoided confounding effects such as reduced body mass and reduced body composition (i.e. estimated VAT, total lean and fat mass, and body fat percentage) allowing for exploration into the direct effects of exercise. In order to avoid the confounding effects of medications, the study was performed in individuals with obesity and not T2D. As the initial study implicated IL10 resistance in individuals with T2D, exploration into the influence of exercise training on IL10 and IL6 function, as well as chemokine receptor expression, in individuals with T2D will be required to determine whether there are differential

effects on anti-inflammatory cytokine function and chemokine receptor expression in T2D or other disease models.

Short-term exercise training, particularly HIIT, reduced the ability of IL10 to inhibit LPS-induced TNFα production. Although these immunomodulatory effects were seen following exercise training, the implication of whether these findings represent an impairment in anti-inflammatory, a potential increase in pro-inflammatory potential, and/or an immune-enhancing effect is not known. The results of this study could be seen as a pro-inflammatory effect as inhibition of TNFα by anti-inflammatory cytokines is considered to help limit inflammation during obesity [120]. Conversely, the results could be seen as immune-enhancing with greater TNFα production after exposure to a pathogen (i.e., LPS) seen as a more robust innate immune response. Future work should explore the clinical significance of reduced function of cytokines such as IL10 and IL6 following exercise training, and further explore whether the type (i.e. interval vs. continuous) of exercise training matters.

Short-term exercise training was also shown to result in direct effects on chemokine receptors, with differential effects of HIIT and MICT. Future research should extend this research to explore how these changes in chemokine receptors will translate into leukocyte infiltration into tissues during inflammatory conditions such as obesity and T2D. To assess leukocyte infiltration better, studies should use complementary assays on specific leukocyte chemotactic potential/migratory capability or assess leukocytes within biopsies of skeletal muscle and subcutaneous adipose tissue. Using these suggested complementary methods, any affects of exercise training on chemokine receptor expression can be further translated in respect to changes in leukocyte migration/infiltration.

5.6 Questions raised from this research?

The data within this thesis indicates that immune cell phenotype and function play an important role in the inflammatory pathology of T2D and obesity with an important therapeutic role of exercise. The main findings of my thesis provide a few thought provoking questions. **How much do changes in cytokine(s) mean?** The majority of exercise studies focused on inflammation assess changes in cytokines as their main indicator of whether the exercise intervention was pro-inflammatory or anti-inflammatory, which is then used to suggest whether that exercise intervention was "good or bad". Within this thesis, short-term exercise did not induce changes in circulating cytokines/chemokines but did promote changes in the immunomodulatory function of cytokines, namely IL10 and IL6. Solely measuring circulating cytokines could be misleading or misinformed as various sources of cytokines exist, and therefore it becomes impossible to clarify the specific contribution of the difference source cells/tissues to the systemic circulating levels of each specific cytokine. Although circulating cytokines may provide some insight into the effects of a particular exercise intervention, cytokine *function* should be measured in conjunction with circulating cytokines to better elucidate the effects of an exercise intervention on inflammation. **Does measuring immune cell numbers tell the whole story?** Exercise has been shown to result in a transient leukocytosis, or increased number of immune cells, but what happens to these cells? Studies have demonstrated that the "destiny" of immune cells, i.e. whether they undergo apoptosis or migration, may depend upon exercise protocol or intensity. The results in my thesis attempt to shed more light on the potential "destiny" of immune cells in response to exercise by measuring chemokine receptor expression. Within the current literature, few studies explore the effects of exercise on chemokine receptor expression. Furthermore, the concept that exercise directly alters chemokine receptor expression was, prior to embarking on this research, based on speculation only.

Although my thesis did not measure leukocyte migration/infiltration, the insights provided implicate that there are different responses of chemokine receptor expression, and therefore likely leukocyte migration, dependent upon leukocyte type and exercise intensity. **How do we interpret changes in immune cell phenotype and function?** The results of my thesis demonstrate a reduced function of IL10, an altered function of IL6, and an altered expression of chemokine receptors following short-term training of HIIT and MICT. The results of these studies could indicate that both HIIT and MICT are pro-inflammatory because they reduce the ability of IL10 to reduce LPS-induced TNFα production. These results could also implicate MICT as anti-inflammatory and HIIT as pro-inflammatory because overall MICT reduced chemokine receptor expression on monocytes, whereas HIIT increased chemokine receptor expression on monocytes, neutrophils, and T cells. On the other hand, these results could be interpreted completely opposite and instead, "pro-inflammatory" effects could be touted as beneficial in the context of enhancing or stimulating a greater immune response. Therefore, both as a researcher and as a reader, it is important to address the interpretation of whether an exercise intervention is anti-inflammatory/immunosuppressive or pro-inflammatory/immune-enhancing. The research question will often be framed by the authors of the study and should be considered when interpreted results. Overall the research presented within this thesis highlights the importance of measuring immune cell phenotype and function in order to better understand the chronic inflammation of obesity and T2D and explore the impact of exercise. Furthermore, it is interesting to think that if circulating plasma cytokines or immune cell numbers were the main outcomes measured within this thesis, the impact of T2D and exercise on inflammation would have been lost, or at least interpreted differently.

5.7 Conclusions

The overarching aim of this thesis was to explore the impact of T2D, obesity, and short-term exercise on immune cell phenotype and function. Our findings are novel because they explore not only the changes in circulating chemokines/cytokines but examine immune cell phenotype and, importantly, immune cell function as well. Specifically, it was demonstrated that IL10 function is reduced in T2D, which we refer to as "IL10 resistance". The impact of exercise in individuals at high risk for developing T2D indicated that HIIT and MICT differentially modulated immune cell phenotype and function. There was reduced anti-inflammatory function of IL10 after HIIT and MICT, but a greater effect seen with HIIT. Similarly, IL6 function was reduced only with HIIT. Further exploration into immune cell phenotype indicated that MICT downregulated, whereas HIIT upregulated, chemokine receptor expression. Further research is required to elucidate the immunomodulatory impact of different types of exercise; however, one major contribution of these studies is that it highlights how immune cell function, and not just circulating cytokine/chemokine levels, should be considered when attempting to decipher the impacts of exercise on the immune system.

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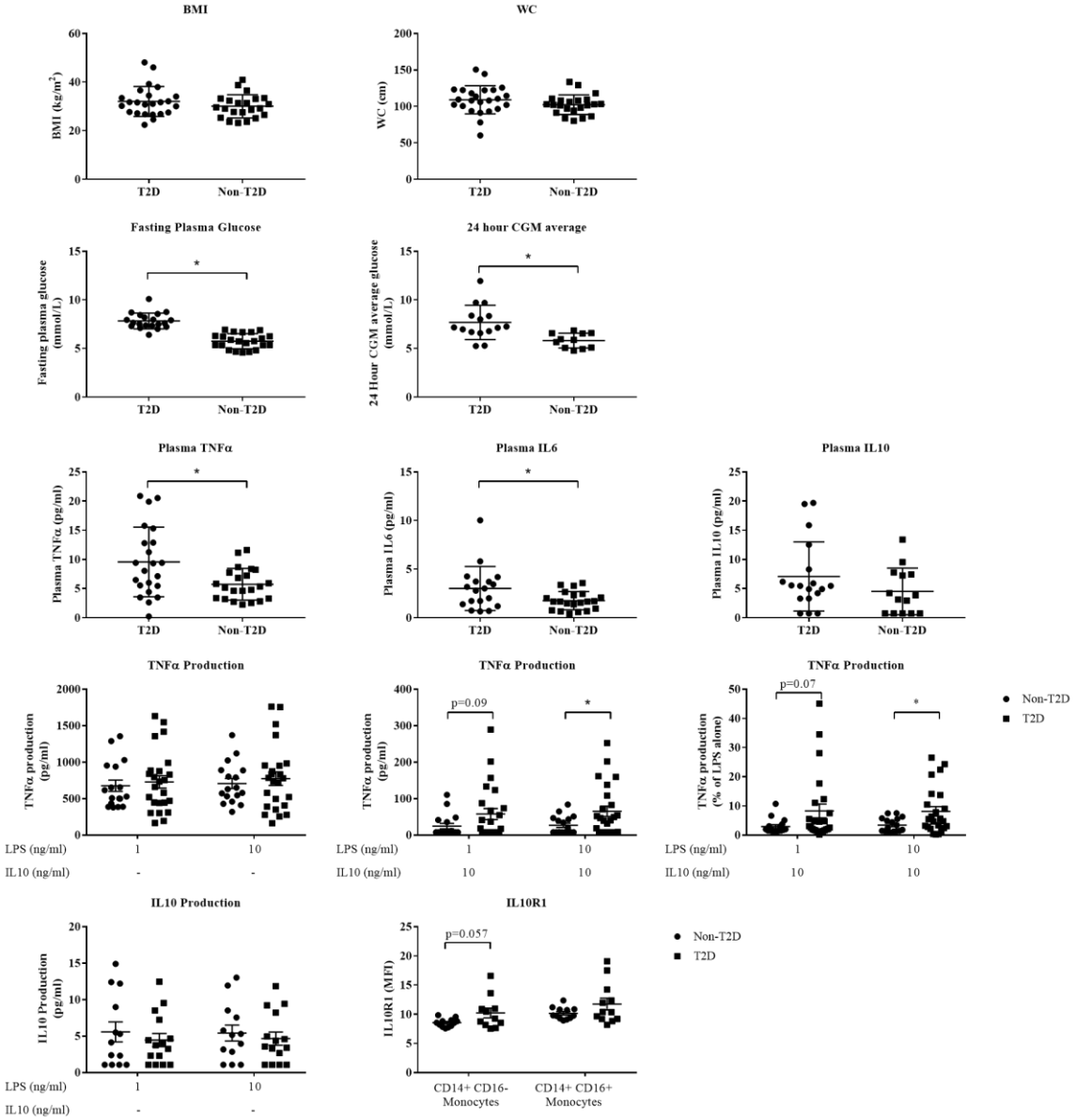
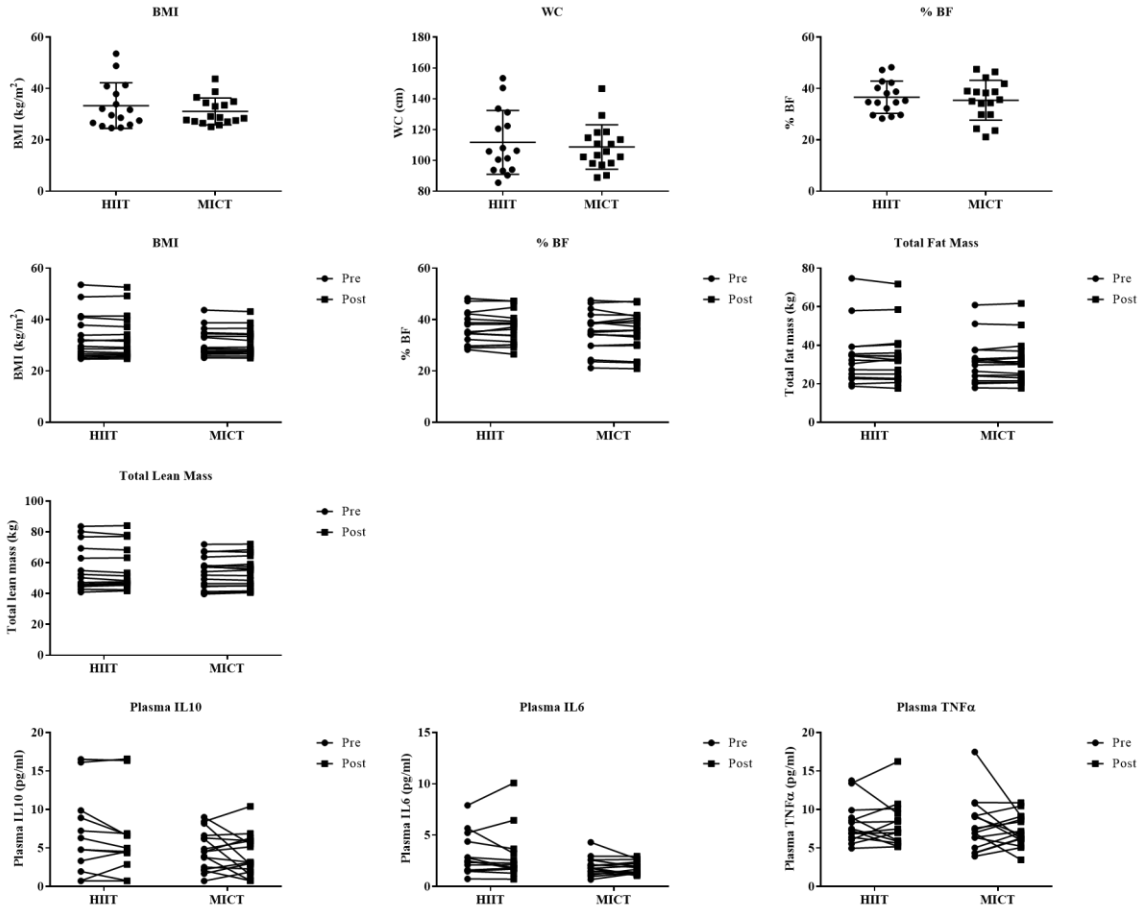
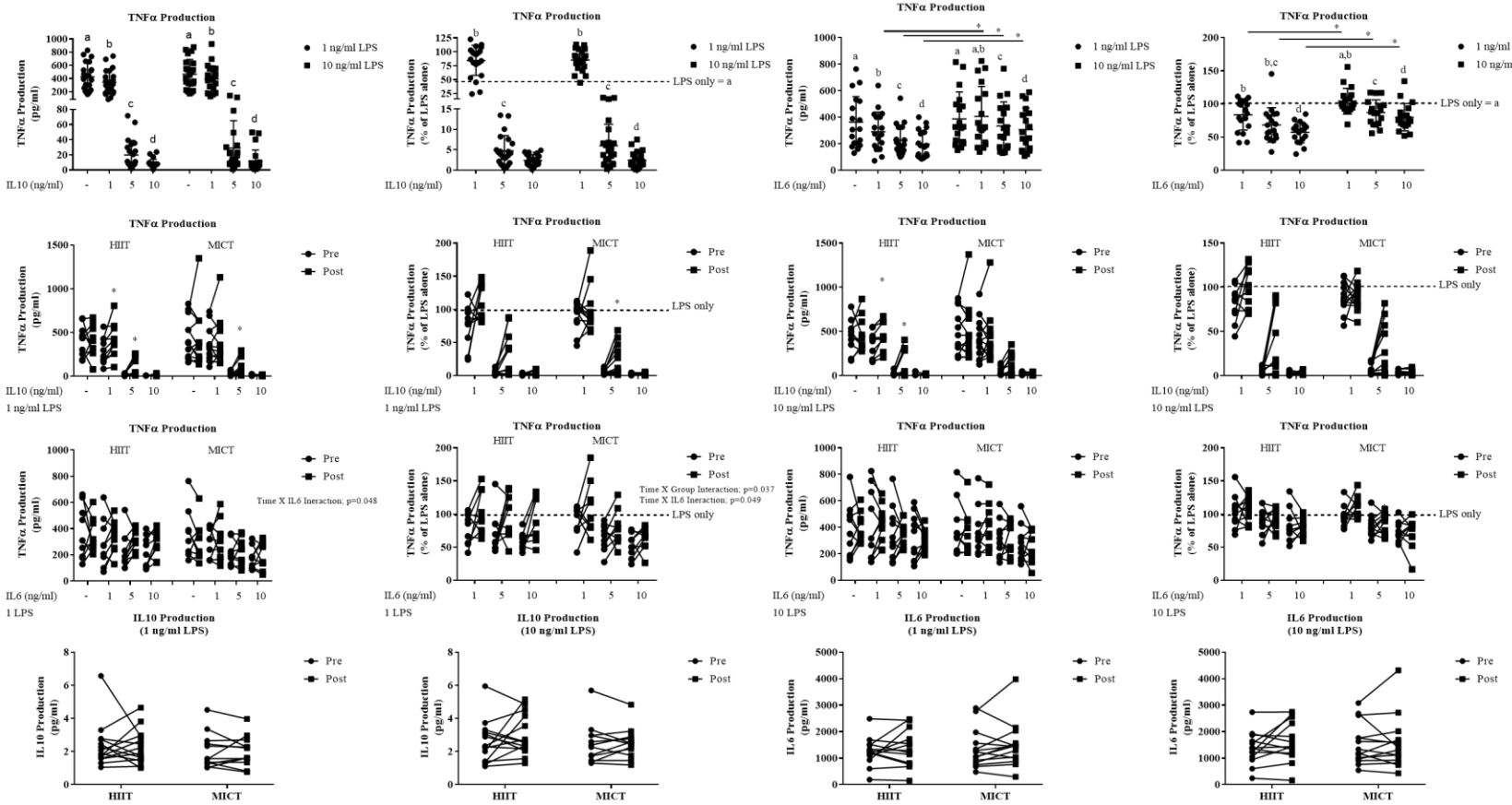
Appendices

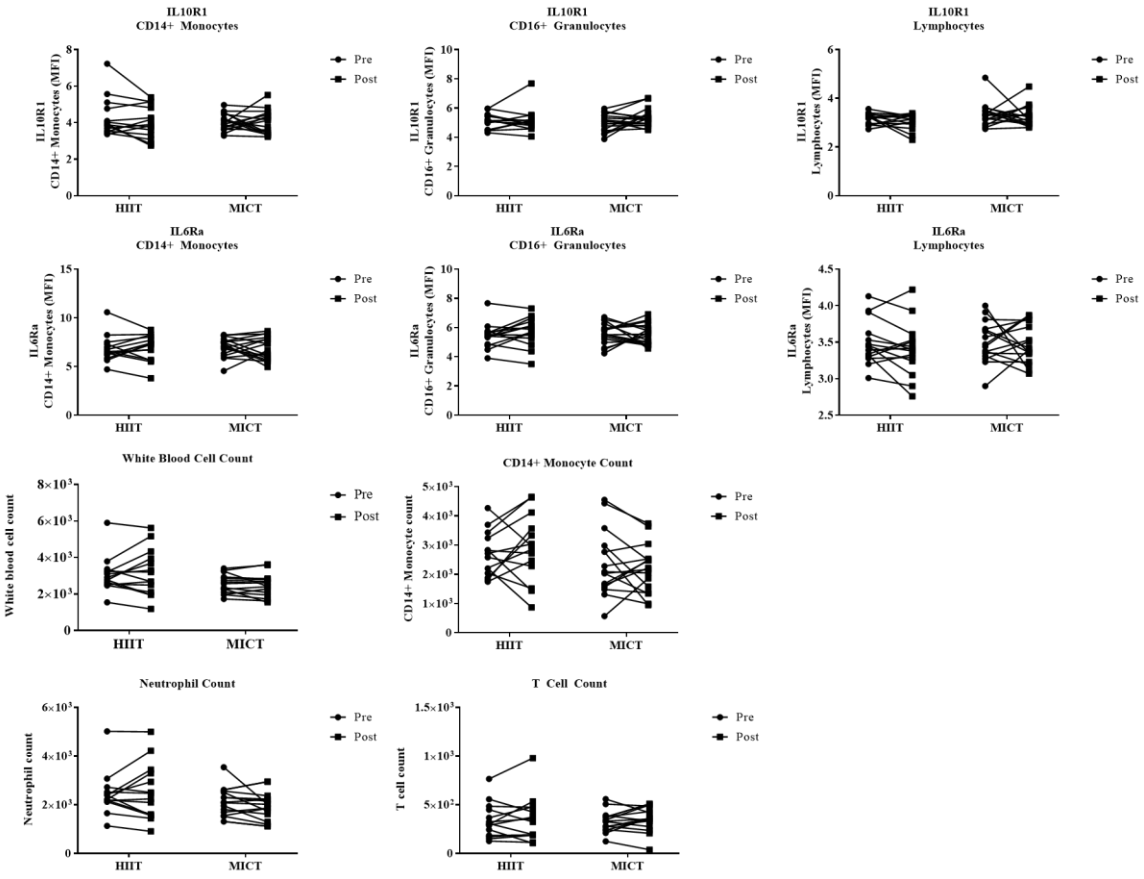
Appendix A: Individual data from Chapter 2



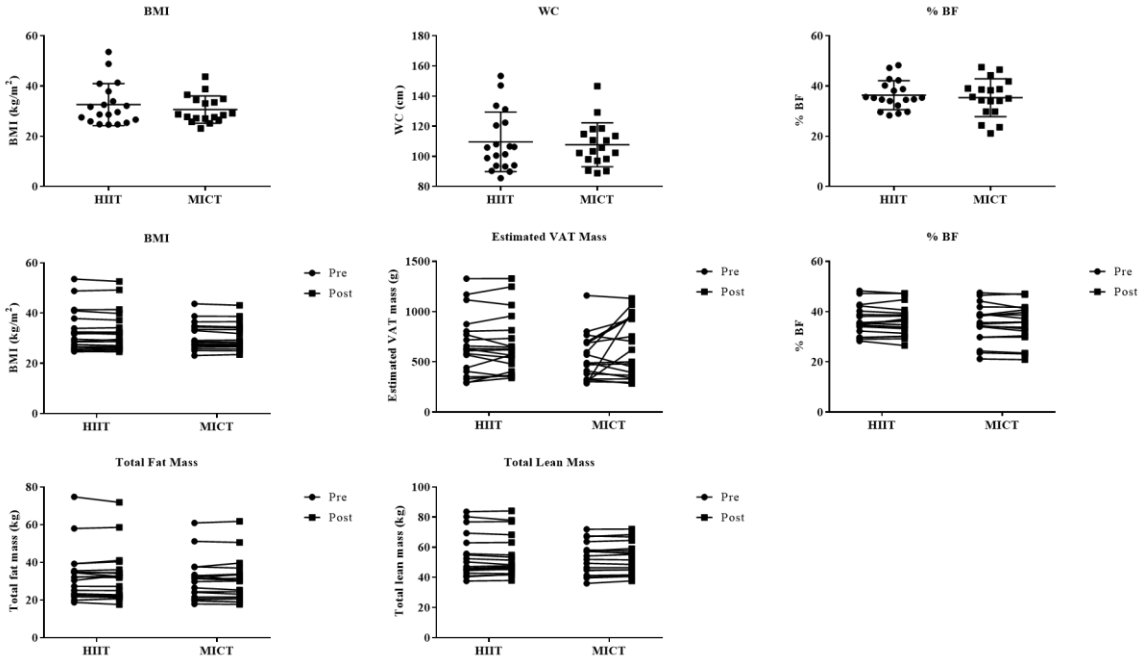
Appendix B: Individual data from Chapter 3

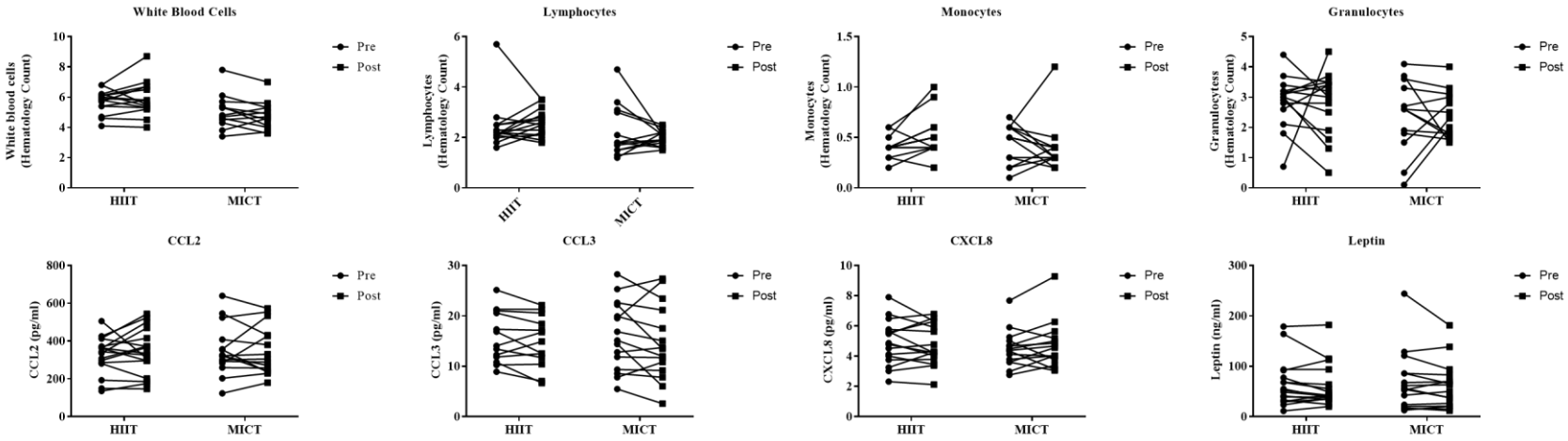


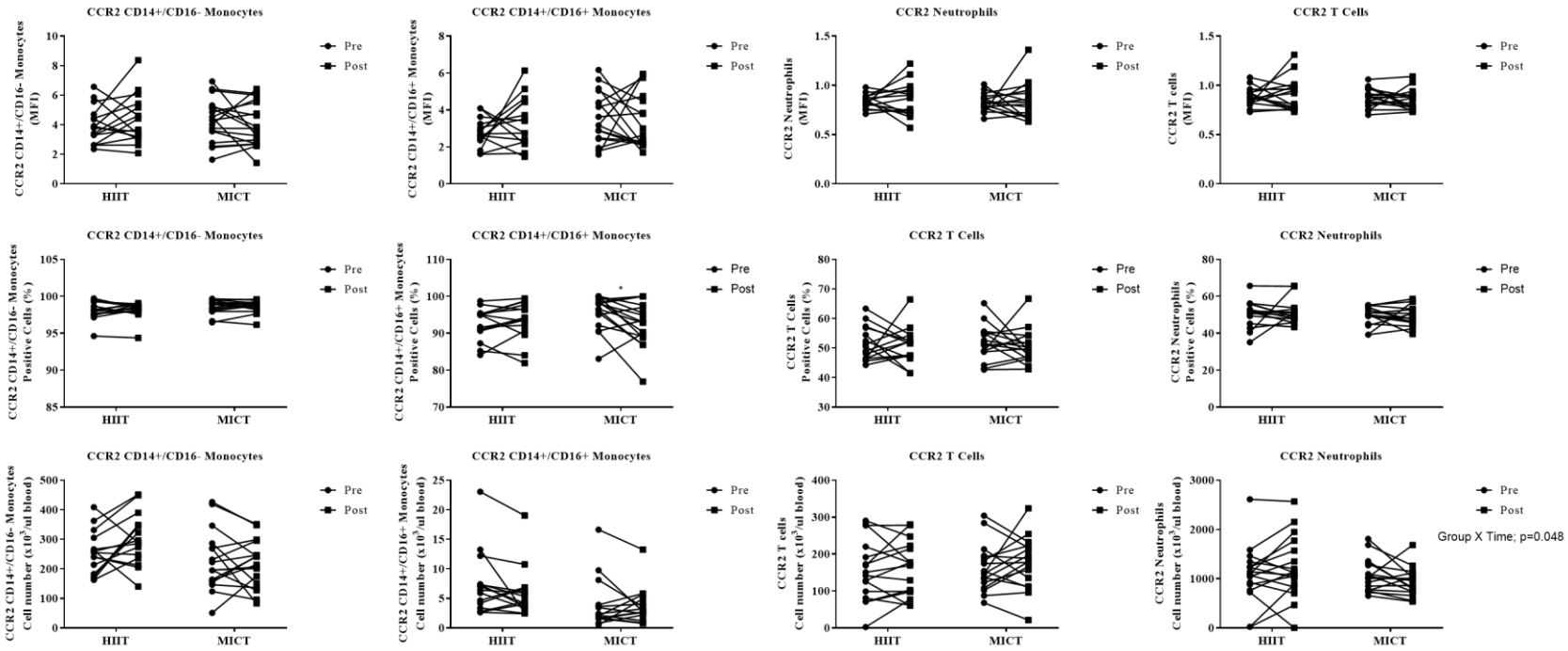

TNFα Production
TNFα Production (pg/ml)
1 ng/ml LPS
10 ng/ml LPS
IL10 (ng/ml)
TNFα Production (% of LPS alone)
LPS only = a
IL6 (ng/ml)
HIIT
MICT
Pre
Post
LPS only
1 ng/ml LPS
10 ng/ml LPS
Time X IL6 Ineraction; p=0.048
Time X Group Interaction; p=0.037
Time X IL6 Ineraction; p=0.049
1 LPS
10 LPS
IL10 Production (1 ng/ml LPS)
IL10 Production (10 ng/ml LPS)
IL6 Production (1 ng/ml LPS)
IL6 Production (10 ng/ml LPS)
IL10 Production (pg/ml)
IL6 Production (pg/ml)

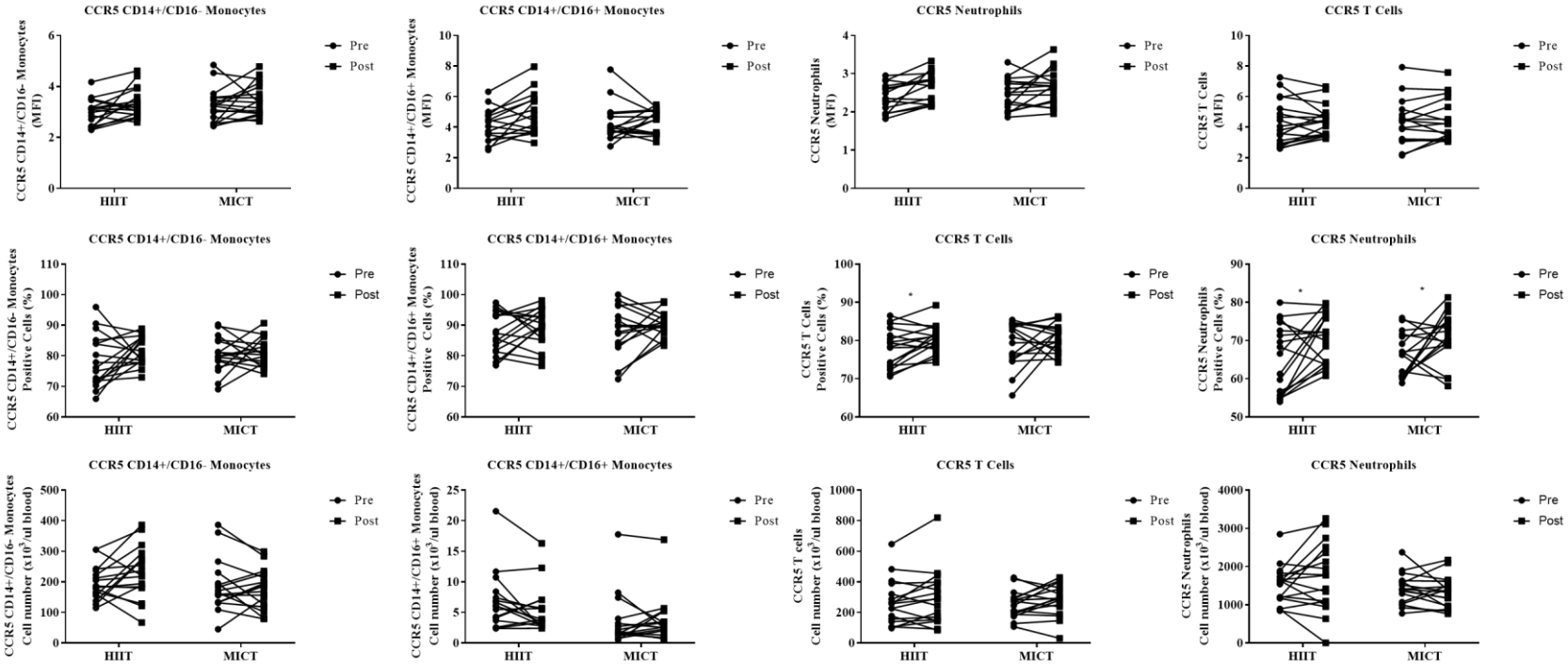

IL10R1
CD14+ Monocytes
IL10R1 CD14+ Monocytes (MFI)
IL10R1
CD16+ Granulocytes
IL10R1 CD16+ Granulocytes (MFI)
IL10R1
Lymphocytes
IL10R1 Lymphocytes (MFI)
IL6Ra
CD14+ Monocytes
IL6Ra CD14+ Monocytes (MFI)
IL6Ra
CD16+ Granulocytes
IL6Ra CD16+ Granulocytes (MFI)
IL6Ra
Lymphocytes
IL6Ra Lymphocytes (MFI)
White Blood Cell Count
White blood cell count
CD14+ Monocyte Count
CD14+ Monocyte count
Neutrophil Count
Neutrophil count
T Cell Count
T cell count
Pre
Post
HIIT
MICT

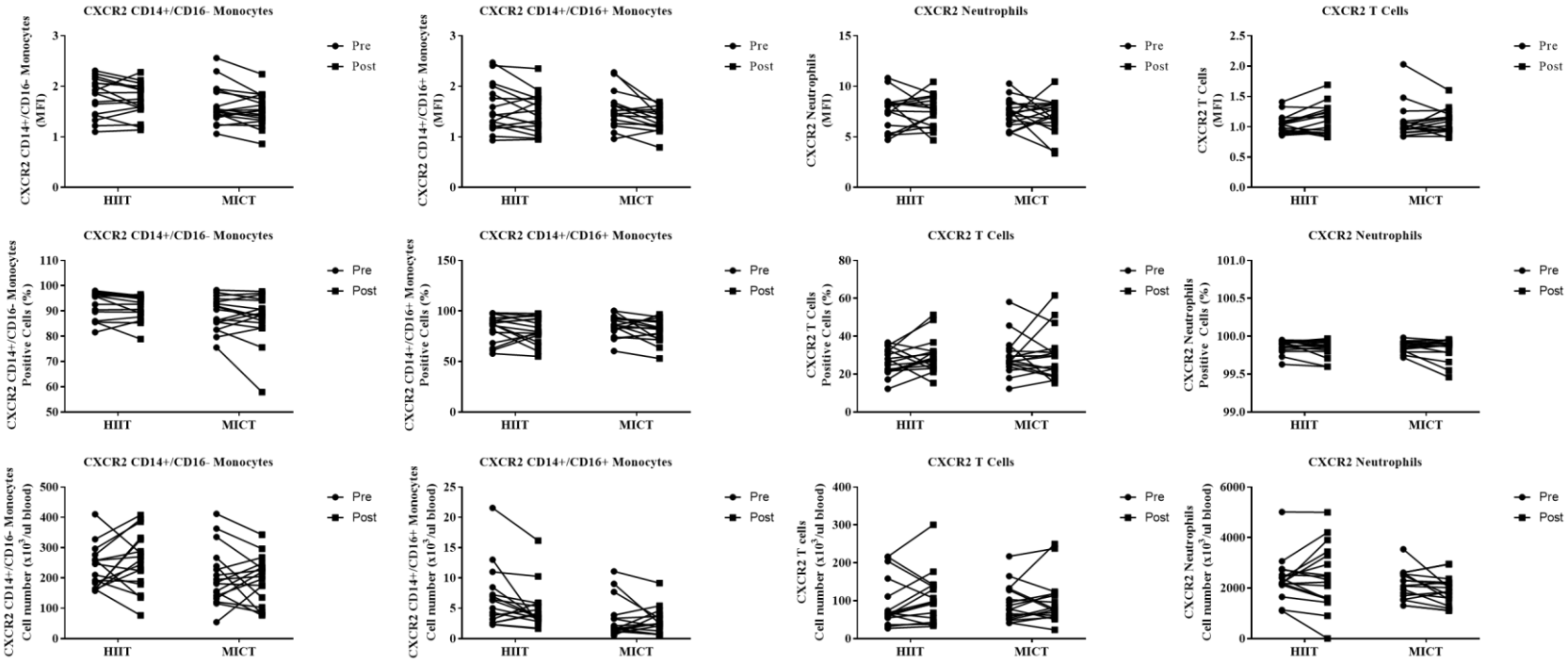
Appendix C: Individual data from Chapter 4




White Blood Cells
White blood cells (Hematology Count)
Lymphocytes
Lymphocytes (Hematology Count)
Monocytes
Monocytes (Hematology Count)
Granulocytes
Granulocytes (Hematology Count)
CCL2
CCL2 (pg/ml)
CCL3
CCL3 (pg/ml)
CXCL8
CXCL8 (pg/ml)
Leptin
Leptin (ng/ml)
HIIT
MICT
Pre
Post


CCR2 CD14+/CD16- Monocytes
CCR2 CD14+/CD16- Monocytes (MFI)
CCR2 CD14+/CD16+ Monocytes
CCR2 CD14+/CD16+ Monocytes (MFI)
CCR2 Neutrophils
CCR2 Neutrophils (MFI)
CCR2 T Cells
CCR2 T cells (MFI)
CCR2 CD14+/CD16- Monocytes
CCR2 CD14+/CD16- Monocytes Positive Cells (%)
CCR2 CD14+/CD16+ Monocytes
CCR2 CD14+/CD16+ Monocytes Positive Cells (%)
CCR2 T Cells
CCR2 T Cells Positive Cells (%)
CCR2 Neutrophils
CCR2 Neutrophils Positive Cells (%)
CCR2 CD14+/CD16- Monocytes
CCR2 CD14+/CD16- Monocytes Cell number (x10^5/ul blood)
CCR2 CD14+/CD16+ Monocytes
CCR2 CD14+/CD16+ Monocytes Cell number (x10^5/ul blood)
CCR2 T Cells
CCR2 T cells Cell number (x10^5/ul blood)
CCR2 Neutrophils
CCR2 Neutrophils Cell number (x10^5/ul blood)
Pre
Post
HIIT
MICT
Group X Time; p=0.048


CCR5 CD14+/CD16- Monocytes
CCR5 CD14+/CD16- Monocytes (MFI)
CCR5 CD14+/CD16+ Monocytes
CCR5 CD14+/CD16+ Monocytes (MFI)
CCR5 Neutrophils
CCR5 Neutrophils (MFI)
CCR5 T Cells
CCR5 T Cells (MFI)
CCR5 CD14+/CD16- Monocytes
CCR5 CD14+/CD16- Monocytes Positive Cells (%)
CCR5 CD14+/CD16+ Monocytes
CCR5 CD14+/CD16+ Monocytes Positive Cells (%)
CCR5 T Cells
CCR5 T Cells Positive Cells (%)
CCR5 Neutrophils
CCR5 Neutrophils Positive Cells (%)
CCR5 CD14+/CD16- Monocytes
CCR5 CD14+/CD16- Monocytes Cell number (x10^3/ul blood)
CCR5 CD14+/CD16+ Monocytes
CCR5 CD14+/CD16+ Monocytes Cell number (x10^3/ul blood)
CCR5 T Cells
CCR5 T cells Cell number (x10^3/ul blood)
CCR5 Neutrophils
CCR5 Neutrophils Cell number (x10^3/ul blood)
Pre
Post
HIIT
MICT


CXCR2 CD14+/CD16- Monocytes
CXCR2 CD14+/CD16- Monocytes (MFI)
CXCR2 CD14+/CD16+ Monocytes
CXCR2 CD14+/CD16+ Monocytes (MFI)
CXCR2 Neutrophils
CXCR2 Neutrophils (MFI)
CXCR2 T Cells
CXCR2 T Cells (MFI)
CXCR2 CD14+/CD16- Monocytes Positive Cells (%)
CXCR2 CD14+/CD16+ Monocytes Positive Cells (%)
CXCR2 T Cells Positive Cells (%)
CXCR2 Neutrophils Positive Cells (%)
CXCR2 CD14+/CD16- Monocytes Cell number (x10^3/ul blood)
CXCR2 CD14+/CD16+ Monocytes Cell number (x10^3/ul blood)
CXCR2 T cells Cell number (x10^3/ul blood)
CXCR2 Neutrophils Cell number (x10^3/ul blood)
HIIT
MICT
Pre
Post